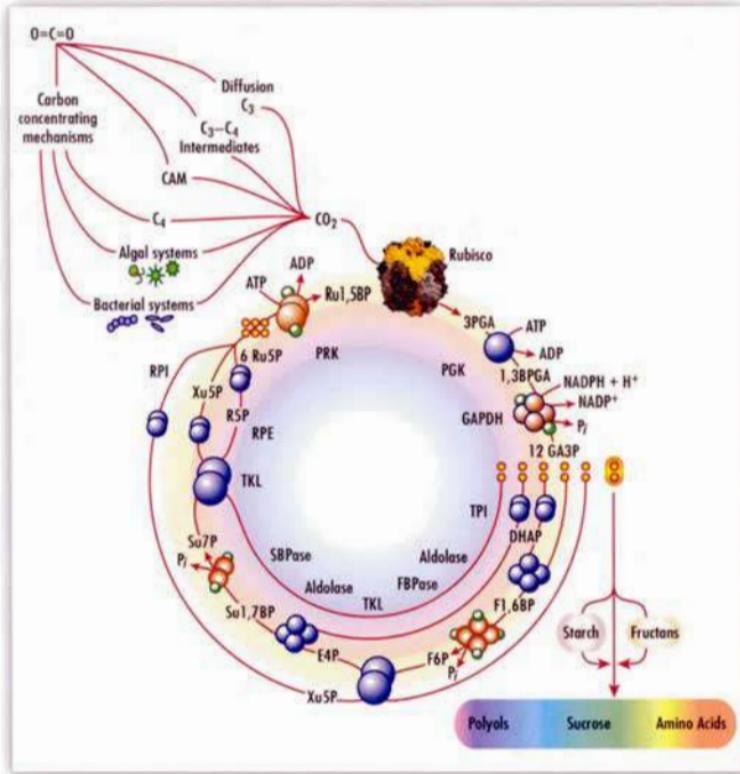


Photosynthesis: Physiology and Metabolism



Edited by

Richard C. Leegood, Thomas D. Sharkey
and Susanne von Caemmerer

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VOLUME 9

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Preface

Photosynthesis: Physiology and Metabolism is the ninth volume in the series *Advances in Photosynthesis* (Series Editor, Govindjee). Several volumes in this series have dealt with molecular and biophysical aspects of photosynthesis in the bacteria, algae and cyanobacteria, focussing largely on what have been traditionally, though inaccurately, termed the ‘light reactions’ (Volume 1, *The Molecular Biology of Cyanobacteria*; Volume 2, *Anoxygenic Photosynthetic Bacteria*, Volume 3, *Biophysical Techniques in Photosynthesis* and Volume 7, *The Molecular Biology of the Chloroplasts and Mitochondria in Chlamydomonas*). Volume 4 dealt with *Oxygenic Photosynthesis: The Light Reactions*, and volume 5 with *Photosynthesis and the Environment*, whereas the structure and function of lipids in photosynthesis was covered in Volume 6 of this series: *Lipids in Photosynthesis: Structure, Function and Genetics*, edited by Paul-André Siegenthaler and Norio Murata. Volume 8, edited by Harry A. Frank, Richard J. Cogdell, George Britton and Andrew J. Young, and published in 1999, deals with the *The Photochemistry of Carotenoids*.

Oxygenic photosynthesis (covered in Volume 4 of this series, edited by Don Ort and Charles Yocum) is normally viewed as the production of reducing power by photosynthetic electron transport and the subsequent metabolism and attendant physiology made possible by this reducing power. This current volume is the first one to deal with an in-depth discussion of some of this physiology and metabolism. Taken in its broadest sense, photosynthetic metabolism encompasses almost all that happens in leaves and other photosynthetic organs. In plants with Crassulacean Acid Metabolism, even some of the metabolism that occurs at night can legitimately be considered photosynthetic. However, photosynthetic metabolism is often viewed, especially in textbooks, solely as the means by which light energy is harnessed and CO_2 is fixed, and the importance of the light-dependent metabolism of nitrogen, sulphur, lipids etc., is largely unrecognized. If omission of these aspects is regarded as a crime (particularly by those who work in these fields!), then we must plead guilty. These other areas, though vitally important, would require a volume to themselves. In this book

we have concentrated on the acquisition and metabolism of carbon. However, a full understanding of reactions involved in the conversion of CO_2 to sugars requires an integrated view of metabolism. We have, therefore, commissioned international authorities to write chapters on, for example, interactions between carbon and nitrogen metabolism, on respiration in photosynthetic tissues and on the control of gene expression by metabolism. Photosynthetic carbon assimilation is also one of the most rapid metabolic processes that occurs in plant cells, and therefore has to be considered in relation to transport, whether it be the initial uptake of carbon, intracellular transport between organelles, intercellular transport, as occurs in C_4 plants, or transport of photosynthates through and out of the leaf. All these aspects of transport are also covered in the book.

The principal aim of the book is to provide final year undergraduates, graduate students and researchers with an up-to-date overview of photosynthetic carbon metabolism in plants, ranging from molecular to ecophysiological aspects. The book has been divided into 24 chapters in three subsections. The first section concentrates on the pathways and the regulation of CO_2 fixation, concentrating particularly on Rubisco, and on photorespiration, the consequence of the oxygenase activity of Rubisco. The second section deals with the fate of that carbon, on the cellular partitioning of carbon into products, such as sucrose, sugar alcohols, starch and soluble oligosaccharides such as fructans. An overview is presented of the cellular control of carbon fixation and partitioning, including the interactions of photosynthetic CO_2 assimilation with processes such as respiration, nitrogen metabolism, transport out of the leaf, and the feedback effects of accumulation of carbohydrates. In the third section the ways in which plants acquire CO_2 from their environment are discussed. These include the difficulties of obtaining an adequate supply of CO_2 from the aqueous environment, the optimization of leaf structure, and how some plants have developed C_4 photosynthesis (to overcome the carbon loss involved in photorespiration) and Crassulacean Acid Metabolism (primarily to conserve water, but also acting as a

CO_2 -concentrating mechanism). All these mechanisms result in advantages for these plants in certain environments.

The last time that these subjects have been comprehensively covered in a collected edition of reviews was in the early 1980s (although some aspects of the environmental regulation of photosynthesis were covered in Volume 5 of this series, edited by Neil Baker, and mentioned above). Since then there have been numerous developments, most dramatically in the molecular aspects of the subject, with knowledge proceeding apace on the structure, function and expression of the proteins involved in photosynthetic carbon acquisition and assimilation. The revolution has been such that it is now rare to find physiologists and biochemists who are not using molecular techniques or mutants and transgenic plants in their investigations. It is probably also true to say

that photosynthesis and carbon assimilation have, in the intervening years, gone through a period of being unfashionable, as sinks came to be considered more important than sources in limiting crop productivity. However, nearly all plant dry mass is derived from photosynthesis and so photosynthesis and crop productivity must be linked, but the co-ordinated regulation of growth and photosynthesis makes it difficult to demonstrate control of growth by photosynthesis.

The production of this volume has involved the efforts of a large number of people, but two in particular. Firstly, the authors, who have written chapters of a high standard and who have shown considerable patience during the protracted process of assembling and editing all the contributions. Secondly, we would like to thank Larry Orr for his skill and good humor in producing the book.

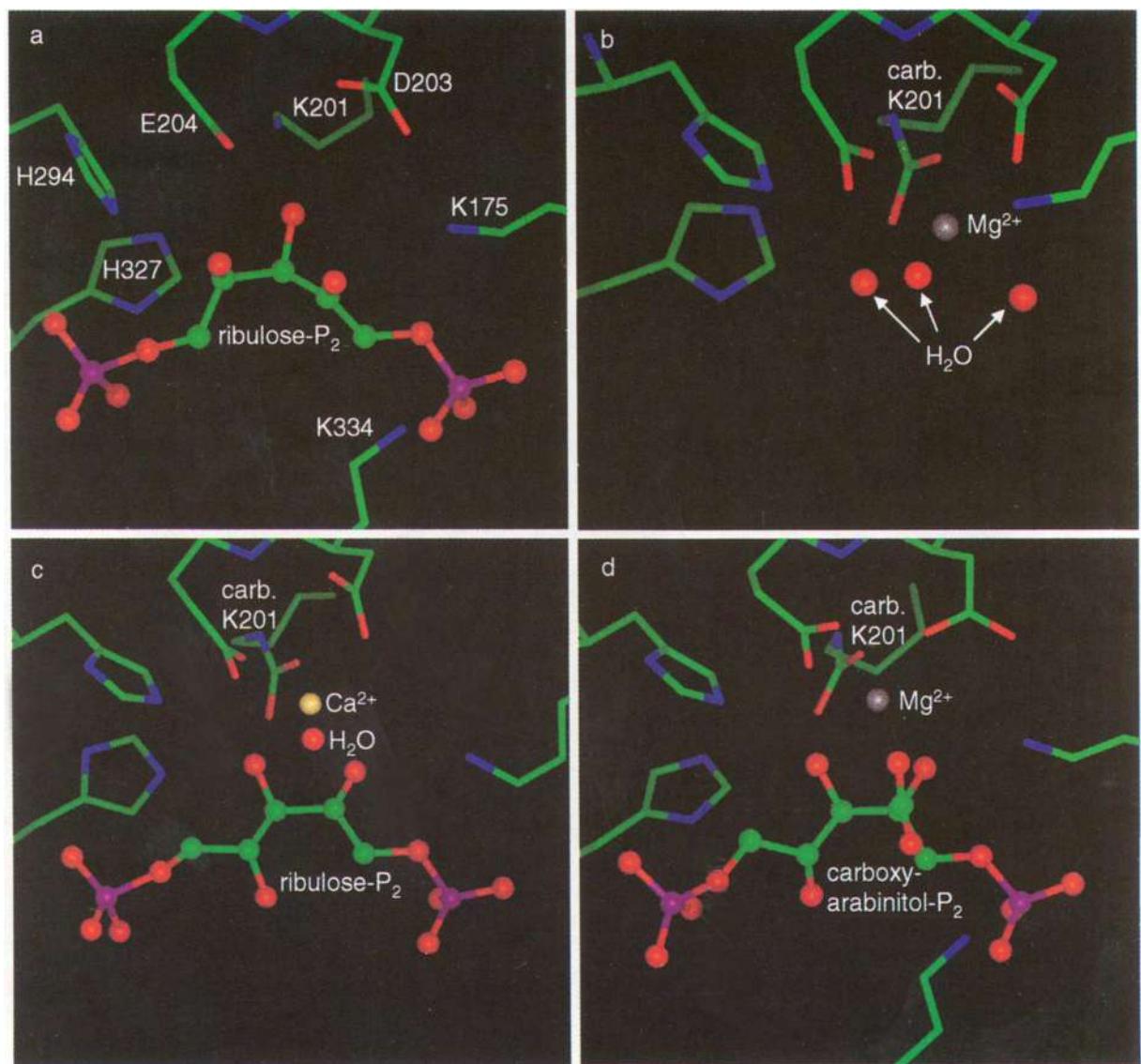
Richard C. Leegood

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Color Plates

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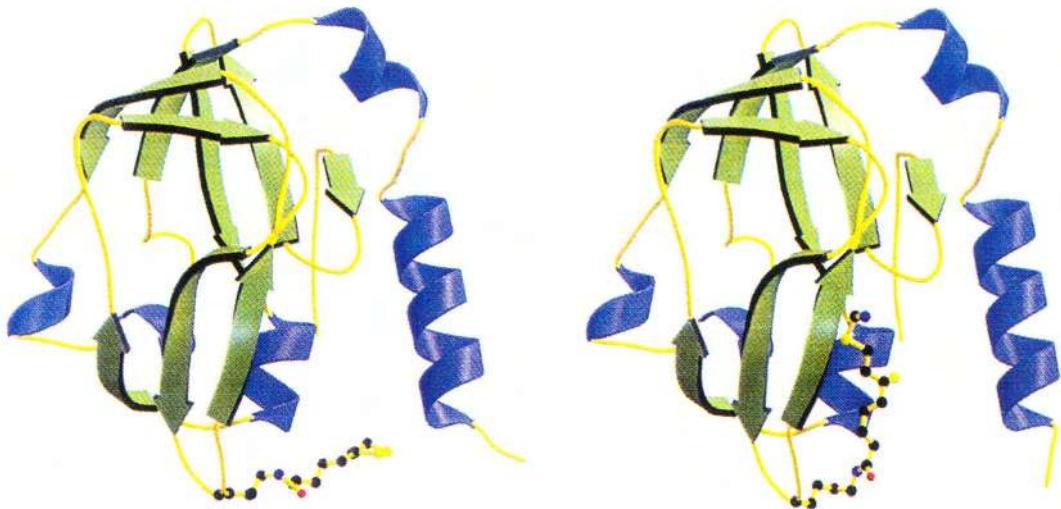


Color Plate 1. Views of the active site of spinach Rubisco showing residues involved in catalysis and binding of the metal ion (stick rendering). a, ribulose-P₂ bound to the decarbamylated (inactive), metal-free site (ER) (Taylor and Andersson, 1997); b, the carbamylated, Mg²⁺-activated site without substrate (ECM) (Taylor and Andersson, 1996); c, the carbamylated site with Ca²⁺ and ribulose-P₂ bound (ECMR) (Taylor and Andersson, 1997); d, the carbamylated, Mg²⁺-activated site with 2'-carboxyarabinitol-P₂ bound (ECM-analog) (Andersson, 1996). Heavy atoms of the phosphorylated ligands, metal ions and water molecules are rendered as balls. (See Chapter 3, p. 62, Fig. 4.)



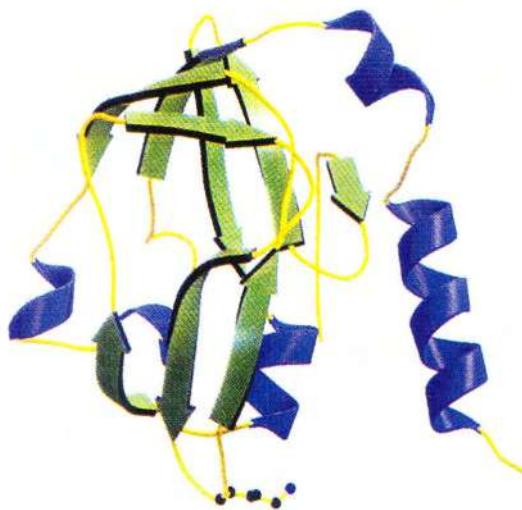
Color Plate 2. Stereo views of the structure of spinach Rubisco. Small subunits are colored in yellow and large subunits in two shades of grey. One dark grey and one light grey subunit together comprise the basic dimeric unit of large subunits. A, Space-filling rendering of the L₈S₈ hexadecamer showing the small subunits sandwiched between adjacent L₂ dimers and forming S₄ tetramers that cap each pole of the hexadecamer. B, Ribbon rendering of one L₂S₂ unit showing the elements of tertiary structure with bond ligands (Mg²⁺ and 2'-carboxyarabinitol-P₂) rendered in ball and stick and colored by atom. Reproduced from Andersson (1996) with permission. (See Chapter 3, p. 64, Fig. 6.)

STRUCTURE OF THE H-PROTEIN



Oxidized form

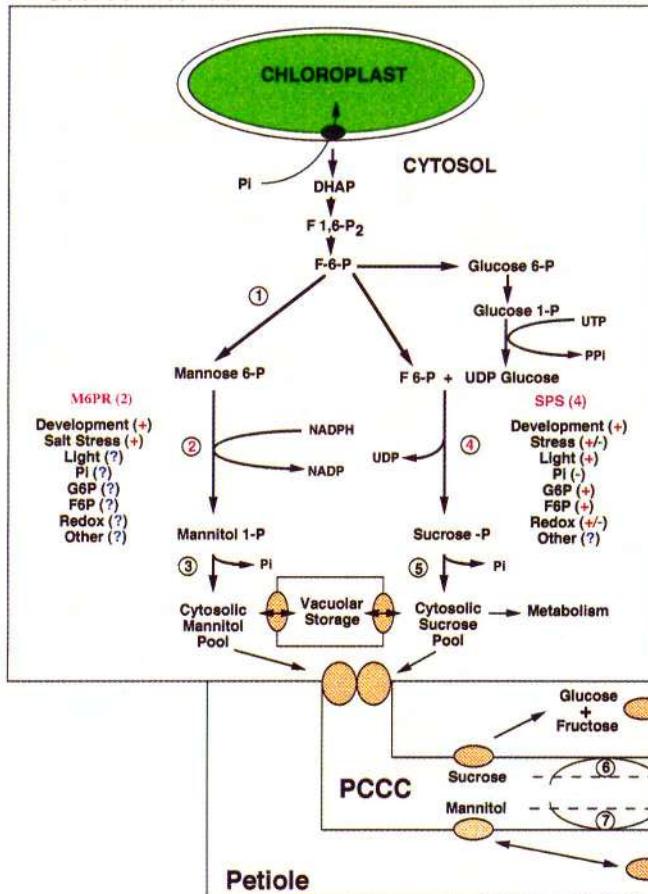
Methylamine-loaded form



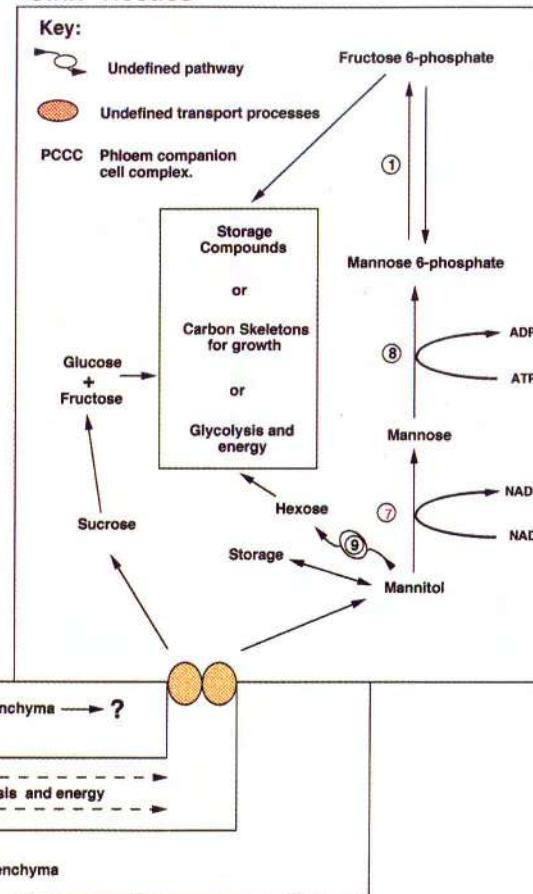
Apoprotein

Color Plate 3. Structure of the H-protein from the pea leaf glycine decarboxylase complex. Schematic ribbon representation of the overall folding of the proteins (oxidized form, methylamine-loaded form and apoprotein). The conformations of all these forms are virtually identical, consisting of seven β -strands in a sandwich structure made of two antiparallel β -sheets. Note that the lipoate cofactor attached to a specific lysine side chain is located in the loop of a hairpin configuration but following methylamine transfer it is pivoted to bind into a cleft at the surface of the H-protein. The apoprotein exhibits the same structure than the holoprotein indicating that the cofactor plays no determinant structural role. (See Chapter 5, p. 123, Fig. 3.)

Source Leaves



Sink Tissues



Color Plate 4. Schematic diagram showing important features involved in the metabolism and transport of the primary soluble products of photoassimilation (mannitol and sucrose). The information for this diagram comes primarily from work on celery (*Apium graveolens* var. dulce), but the synthetic pathway has been described in other higher plant species (see text). The diagram illustrates the parallel (and presumably competitive, in terms of substrates) mannitol and sucrose biosynthetic pathways. Various documented (+/-), and possible (?) modulators of the two key regulatory steps [M6PR (2) and SPS; (4)] in the mannitol and sucrose biosynthetic pathways, respectively, are listed. The diagram also illustrates the spatial separation of the anabolic and catabolic pathways for mannitol, and it is the dynamic balance between sink and source activity, translocation between them and sequestration in long and short term storage pools (involving as yet poorly described transport processes) that is ultimately responsible for regulating mannitol pools in the plant. Important enzymatic steps shown in the diagram are: 1) mannose 6P isomerase (note the roles in both anabolic and catabolic pathways); 2) mannose 6P reductase (M6PR); 3) mannitol-1-P-phosphatase; 4) sucrose-phosphate synthase (SPS); 5) sucrose-phosphate phosphatase; 6) sucrose synthase (SS); 7) mannitol-1-oxidoreductase (MTD); 8) hexokinase. Mannitol catabolism involves MTD (7), but alternative degradative enzymes exist in other species, e.g. mannitol 2-dehydrogenase (9) (see Table 1). (See Chapter 12, p. 277, Fig. 1.)

Chapter 1

Introduction

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Summary

An overview is presented of the principal mechanisms and regulatory processes involved in the acquisition of CO_2 by plants. Central to all these mechanisms is the Calvin-cycle and Rubisco, with its dual function as a carboxylase and as an oxygenase, catalyzing the first reaction in the phenomenon of photorespiration. The losses of carbon in photorespiration and the low levels of CO_2 in some habitats have led to the development of CO_2 -concentrating mechanisms, such as those found in aquatic organisms and in C_4 and CAM plants. Once CO_2 is fixed into triose-P, it can then be utilized to make carbohydrates for processes such as storage and export, or used to fuel respiration and biosynthesis in the plant. The use of genetic manipulation to further our understanding of photosynthetic carbon metabolism is discussed.

I. An Overview of Pathways and Mechanisms

A. Carbon Fixation

Fifty years ago, Melvin Calvin, Andrew Benson and their colleagues embarked on the painstaking task of elucidating the pathway of CO_2 fixation in plants. The formulation of the Calvin cycle was a spectacular achievement, for which Calvin received the Nobel prize, and an account of it in a small book called *The Path of Carbon in Photosynthesis* (Bassham and Calvin, 1957) is still eminently worth reading. The Calvin cycle (also termed the Benson-Calvin cycle, the Reductive Pentose Phosphate Pathway or Photosynthetic Carbon Reduction Cycle) is the only pathway in plants which can catalyze the net fixation of carbon dioxide and it lies at the heart of carbon metabolism in leaves. (Some bacteria can reverse the Krebs cycle but this carbon reduction pathway appears to be very limited.) The discovery of the Calvin cycle was followed by the characterization of its enzymes and their regulation. One of the key discoveries of Calvin's work was that RuBP was the primary CO_2 acceptor in plants, eventually leading to the discovery of Rubisco, which is now the most intensively studied enzyme in plant metabolism and one of the best studied proteins in plants, being one of relatively few plant proteins to have been crystallized. The remainder of Calvin cycle is increasingly studied at the molecular level, including investigations of the regulation of gene expression and its comparative biochemistry and evolution. It remains, therefore, at the forefront of photosynthesis research (Chapter 2, Martin et al.).

Rubisco has inevitably been the focus of attention in photosynthetic carbon metabolism not only because of its unique role, but also because of its inherent peculiarities and inefficiencies. Rubisco is peculiar because it contains two different subunits encoded by the nuclear and chloroplast genomes. These subunits have to be assembled within the chloroplast. The regulation and reaction mechanisms of Rubisco are also unique. These aspects are discussed in Chapter 3 (Roy and Andrews). Rubisco is an inefficient enzyme for two reasons. First, it has a low specific activity (about $3.6 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein). Plants therefore require large amounts of it. Rubisco

can account for as much as 50% of soluble protein in the leaf of a C_3 plant and is the major investment of nitrogen in leaves. Second, Rubisco is a bifunctional enzyme in that it catalyzes both the carboxylation and the oxygenation of RuBP. Oxygenation of RuBP leads to the production of one molecule of glycerate-3-P and one of glycinate-2-P. The latter is the substrate for the photorespiratory pathway (Chapter 5, Douce and Heldt) which results in the loss of one quarter of the carbon in glycinate-2-P as CO_2 . Consideration of the photorespiratory pathway provides a paradigm for the integration of photosynthetic carbon metabolism with the metabolic activities in the rest of the cell. The photorespiratory pathway involves other organelles (mitochondria and peroxisomes) besides the chloroplast, in photosynthetic carbon metabolism. Intracellular metabolite transport is, therefore, required at high rates during photosynthesis and has led to the specialized functions of organelles such as leaf mitochondria and peroxisomes. For example, glycine decarboxylase plays a dominant role in the mitochondria of leaf cells of C_3 plants, not only releasing CO_2 , but also NH_3 , and its sheer abundance has led to it being termed the 'Rubisco of plant mitochondria' by Douce. Photorespiration also has major implications for leaf nitrogen metabolism, because the rate of photorespiratory NH_3 release occurs at rates up to ten times the rate of primary NH_3 assimilation (i.e. NH_3 deriving from nitrate reduction). Since NH_3 is a far scarcer resource than CO_2 , NH_3 must be very efficiently re-fixed by glutamine synthetase and glutamate synthase in the chloroplast.

Plants which rely solely on diffusion for carbon acquisition are called C_3 plants. In these plants, the CO_2 concentration at Rubisco is lower than that in air because of resistances to diffusion primarily at the stomata and in the mesophyll. Recent advances using stable isotope and other methods have allowed an improved estimation of the concentration of CO_2 inside chloroplasts (Chapter 14, Evans and Loreto). In C_3 plants, losses of carbon due to photorespiration can be substantial (about a quarter of the rate of CO_2 fixation at moderate temperatures) and increase at higher temperatures. Due to the relatively low affinity of Rubisco for CO_2 and the occurrence of the competing oxygenase reaction, photosynthesis in C_3 plants is not saturated with CO_2 . The subtle optimization of leaf structure and stomatal function to facilitate the uptake of CO_2 by diffusion while keeping loss of water vapor to a minimum has been a major challenge to terrestrial plants and is dealt

Abbreviations: CAM – Crassulacean Acid Metabolism; CA1P – 2'-carboxybarbiton-1-P; PEP – phosphoenolpyruvate; Rubisco – ribulose 1,5-bisphosphate carboxylase-oxygenase; RuBP – ribulose 1,5-bisphosphate

with in Chapter 14 (Evans and Loreto).

Some photosynthetic organisms have developed mechanisms to enhance their acquisition of CO_2 from the environment. Many aquatic photosynthetic organisms have biophysical mechanisms for the direct uptake of inorganic carbon to overcome problems caused by diffusional limitations and the pH, which govern the species of inorganic carbon (CO_2 or HCO_3^-) available. Certain of these organisms, such as the cyanobacteria, have Rubisco with different kinetic properties and have developed a novel compartmentation of Rubisco and carbonic anhydrase in the carboxysome (Chapter 16, Badger and Spalding). Eukaryotic algae have pyrenoid bodies which may serve a similar function. This mechanism is paralleled in C_4 plants by the concentration of CO_2 in the bundle sheath where Rubisco is sequestered (Chapter 21, Sage and Pearcy).

In the case of C_4 and CAM plants, CO_2 is concentrated biochemically with resultant increases in carbon gain from the suppression of photorespiration, particularly at high temperatures, and increases in the efficiency with which resources such as nitrogen and water are utilized, which have important ecophysiological implications (Chapter 21, Sage and Pearcy). One of the principal means of studying the occurrence of C_3 , C_4 and CAM plants and their water-use efficiency has been the study of the fractionation of stable carbon isotopes, which have been employed extensively both in the laboratory and in the field (Chapter 17, Brugnoli and Farquhar). Crucial to all these photosynthetic adaptations is the presence (and in some cases absence) of carbonic anhydrase (Chapter 15, Coleman), which not only plays a role in C_3 photosynthesis, but is a key component of CO_2 -concentrating mechanisms in aquatic photosynthetic organisms and which can also be considered the first step of C_4 and CAM photosynthesis, since the substrate of PEP carboxylase is HCO_3^- , not CO_2 .

C_4 photosynthesis occurs mainly, though not exclusively, in the grasses. Although only about 5% of flowering plants are C_4 , C_4 plants are responsible for approximately 20% of global terrestrial productivity, largely because of their dominance in savanna grasslands (Lloyd and Farquhar, 1994). Over 30 years have elapsed since the elucidation of the C_4 pathway when the $^{14}\text{CO}_2$ pulse-chase techniques employed by Calvin were elegantly pressed into service again, first by Hugo Kortschak, then by Hal Hatch and Roger Slack (for a brief discussion of the

history of its discovery and some of the initial excitement engendered see Osmond, 1997 and Hatch, 1997). The C_4 pathway is an adjunct to the Benson-Calvin cycle and operates as a CO_2 concentrating mechanism that suppresses photorespiration (Chapter 18, Furbank et al.). What is particularly remarkable about C_4 photosynthesis is its integration of structure and function. C_4 photosynthesis involves the co-operation of two cell types, the mesophyll and bundle-sheath. As with photorespiration, C_4 photosynthesis requires not only intracellular transport of metabolites, between chloroplasts, cytosol, and mitochondria, but also involves rapid intercellular transport of metabolites between the mesophyll and bundle sheath (Chapter 19, Leegood). Understanding the factors that control the development of these two photosynthetic cell types and their different photosynthetic complements is a major challenge in developmental biology (Chapter 20, Dengler and Taylor), especially in view of attempts to engineer C_4 -type CO_2 concentrating mechanisms into C_3 crop plants (Mann, 1999).

The C_4 pathway represents a series of variations on a biochemical theme that has almost certainly evolved independently many times. This was possible because none of the enzymes or anatomical structures involved in C_4 photosynthesis is unique to these plants. There are at least three distinct biochemical subtypes of C_4 plants, classified in accordance with the enzyme which is employed to decarboxylate C_4 acids in the bundle-sheath (NADP-malic enzyme, NAD-malic enzyme and phosphoenolpyruvate carboxykinase). However, it is becoming increasingly apparent that this is an oversimplification and that there is very considerable biochemical and structural diversity among C_4 plants. C_4 photosynthesis is thought to have evolved within the last 30 million years in response to a period of low atmospheric CO_2 concentration and high oxygen concentration following the Cretaceous period (Chapter 21, Sage and Pearcy). Ehleringer et al. (1991) have proposed that the main driving force for the evolution of C_4 photosynthesis is the increased carbon gain that results from the suppression of photorespiration. A fascinating small group of plants, $\text{C}_3\text{-C}_4$ intermediates, which are, to varying degrees, intermediate in structure, biochemistry and gas-exchange between C_3 and C_4 plants can be viewed as models for the evolution of the C_4 syndrome (Chapter 22, Monson and Rawsthorne).

By comparison with the C_4 pathway, the pathway

of Crassulacean Acid Metabolism (CAM) was a less dramatic discovery (for example, observations of diurnal changes in acidity in leaves of *Bryophyllum calycinum* were recorded in 1813 by Heyne (Edwards and Walker, 1983)). Although CAM is usually considered to be a **CO₂-concentrating** mechanism, and an extremely effective one, especially under aquatic conditions (Keeley, 1996), the prime function of nocturnal stomatal opening is the conservation of water in environments in which the water supply is unpredictable. CAM is found not only in the succulents of arid regions, but more numerous among epiphytes, such as orchids and bromeliads. While CAM is only part of a suite of physiological and structural adaptations to an erratic water supply (Chapter 24, Borland et al.), it is also clear that, as in C₄ plants, CAM has evolved many times and that the mechanisms show considerable convergence. Both C₄ and CAM photosynthesis can be considered as variations on a C₃ biochemical theme with many common regulatory mechanisms (for example, the complex regulation of PEP carboxylase, which has largely been elucidated in first CAM, then C₄ plants). Unlike C₄ plants (although see Ueno, 1998), CAM plants exhibit an extraordinary degree of photosynthetic plasticity and in many plants CAM is induced either during development or in response to environmental stress. CAM plants such as *Mesembryanthemum crystallinum* have therefore become model systems for studying the environmental regulation of gene expression and the signal transduction pathways that are involved (Chapter 23, Cushman et al.).

B. Carbon Partitioning

The overall reaction of the Calvin cycle can be described as the fixation of three molecules of CO₂ into a three-carbon sugar phosphate, *triose-P*, with the incorporation of one molecule of Pi. The principal product of photosynthetic carbon assimilation in the chloroplast is triose-P. Triose-P can be exported to the cytosol where it can be utilized to make soluble sugars, sugar alcohols (Chapter 12 Loescher and Everard), soluble oligosaccharides such as fructans (Chapter 13, Cairns et al.) or used for respiration and amino acid biosynthesis (Chapter 7, Atkin et al.; Chapter 8, Foyer et al.). Alternatively, triose-P can be retained within the chloroplast, either to make starch (Chapter 9, Trehewey and Smith) or to regenerate the initial CO₂ acceptor, ribulose-5-bisphosphate. Thus large-scale transport across the chloroplast

envelope occurs during photosynthesis: the chloroplast is a Pi-importing, triose-P exporting organelle. These fluxes of carbon and Pi are mediated by the phosphate translocator, which catalyzes the obligatory counter-exchange of triose-P for Pi across the chloroplast envelope. The phosphate translocator is one of the best characterized of all the membrane transporters in plants (Chapter 6, Flügge). In addition, the chloroplast envelope also has transporters involved in the exchange of other metabolites such as dicarboxylates, adenylates, and hexoses, which are also involved in the turnover of carbohydrates, and the synthesis of compounds such as organic and amino acids.

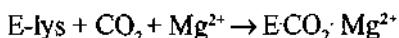
There are a range of regulatory mechanisms which balance the production of triose-P by the Calvin cycle with its utilization in the synthesis of sucrose and starch or other carbohydrates. The signals involved can act either within the cell or at a distance, i.e. from sink tissues. These include the regulation of enzymes such as sucrose-phosphate synthase by phosphorylation, of the cytosolic fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate, and short-term feedback, seen as fine control by phosphate (Chapter 8, Foyer et al.). However, while there is a considerable amount of evidence that changes in Pi can act as a message between carbohydrate synthesis and triose-P production in the short-term, acting via the phosphate translocator, there is little evidence that a fine control mechanism such as this acts in the long-term, when clearly what is needed is a modulation of relative capacities of the processes of triose-P and carbohydrate synthesis. Some of this may occur by post-translational modification of enzymes, but most is likely to occur by longer-term feedback on changes in gene expression, and hence the amounts of enzymes (coarse control). It is now evident that metabolically-induced changes in gene expression, particularly those mediated by sugars, play an important role in sink-source interaction in plants (Chapter 10, Graham and Martin). This has particular relevance to changes in photosynthetic capacity that are observed after growth of plants in elevated CO₂. It is clear that, if source capacity is increased or sink capacity is restricted, carbohydrates accumulate in the source leaves, both in the mesophyll and in the vasculature (Koroleva et al., 1998). Carbohydrate accumulation may be one of the mechanisms that brings about a reduction of photosynthetic capacity. The mechanisms mediating the export of carbohydrates and amino acids from leaves are, therefore, an essential part of our

understanding of the photosynthetic process (Chapter 11, Schobert et al.), as is an understanding of the compartmentation of metabolism between and within the different cell types of leaves.

C. Regulation and Control of Photosynthesis

In textbooks, photosynthesis has traditionally been demarcated into ‘light’ and ‘dark’ reactions, but it has been abundantly clear from the outset (i.e. Calvin’s experiments) that photosynthetic CO_2 fixation is wholly light dependent. Indeed, it is closely integrated with electron and proton transport in chloroplasts. For example, the pH of the darkened stroma is about 7, with a free Mg^{2+} concentration of 1–3 mM, whereas in the illuminated stroma the pH is about 8 and the free Mg^{2+} concentration 3–6 mM (Leegood et al., 1985). These electron-transport-driven changes in the stroma provide an environment which is close to the optimum for the operation of the enzymes of the Benson-Calvin cycle (see, for example, Baier and Latzko, 1975) or of enzymes of C_4 photosynthesis, such as NADP-malic enzyme (Chapter 18, Furbank et al.). The enzymes of photosynthetic carbon metabolism also exhibit a wide range of allosteric responses to metabolite effectors, which are discussed in many chapters in this volume. However, there are also unique mechanisms of regulation of some of these enzymes which occur by covalent modification.

In photosynthetic cells, at least three forms of covalent modulation occur, including changes in enzyme activity brought about by the thioredoxin system and by protein phosphorylation. Rubisco activity is modulated by two unique mechanisms. In vitro, Rubisco can be activated by preincubation with CO_2 and Mg^{2+} to form an active carbamylated enzyme. This carbamylation occurs on a lysine residue (lysine²⁰¹ of the spinach enzyme), but the CO_2 molecule involved is different from that involved in the reaction, although it plays a role in catalysis (Chapter 3, Roy and Andrews).



Carbamylation of Rubisco in vitro occurs only in the presence of millimolar concentrations of CO_2 , whereas *in vivo* the concentration of CO_2 in the leaf cell is only about 10 μM . Rubisco activase (Chapter 4, von Caemmerer and Quick) hydrolyzes ATP and allows carbamylation of Rubisco in the presence of physiological concentrations of CO_2 ($K_a(\text{CO}_2)$ = 4 μM). Binding of RuBP to the active site prevents

carbamylation and activase appears to be involved in removing bound RuBP and other ligands from the active site to allow carbamylation. Rubisco activase may also be involved in removing a tight-binding inhibitor, 2' carboxyarabinitol-1-P (CA1P; also known as hamamelonic acid 2'-phosphate; K_d , 32 nM) from Rubisco. CA1P is an analogue of the transition state intermediate, 3-keto-2-carboxy-arabinitol bisphosphate, which occurs during catalysis (Chapter 3, Roy and Andrews). Although a CA1P phosphatase has been purified (Charlet et al., 1997), little is understood about the synthesis of CA1P and its relationship to hamamelose phosphates that are present in chloroplasts during photosynthesis (Andralojc et al., 1996), or of the role and regulation of other tight-binding inhibitors of Rubisco (Parry et al., 1997).

The activities of a number of enzymes in the chloroplast are governed by the availability of the photosynthetically-generated reductant, thioredoxin (Chapter 2, Martin et al.), providing a direct link between electron transport and carbon assimilation. Ferredoxin-thioredoxin reductase (an iron-sulfur protein with a reducible disulphide bridge) catalyzes the reduction of thioredoxin by ferredoxin. Reduced thioredoxin is able to reduce disulfide bridges on the target enzymes, modulating their activities. There are also complex changes in the regulatory properties of these enzymes because light activation can change affinities for substrates or V_{max} . There are four target enzymes in the Benson-Calvin cycle: fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, ribulose-5-P kinase and glyceraldehyde-3-P dehydrogenase. Other enzymes of photosynthesis regulated by thioredoxin include NADP-malate dehydrogenase (in both C_3 and C_4 plants), the chloroplast ATPase and acetyl CoA carboxylase. This is not a blanket means of regulation since the response to thioredoxin can be modulated by other factors, such as substrate availability. The fact that enzymes have different midpoint redox potentials (E_m) also means that each enzyme will have a different sensitivity to light, since a difference in E_m of 30 mV will represent an equilibrium constant of 10 for a two-electron transfer. Thus the ATPase could remain substantially active while the activities of the bisphosphatases change markedly (Ort and Oxborough, 1992; Hirasawa et al., 1999).

In the last 15 years it has become clear that phosphorylation of plant enzymes is a major influence in the regulation of carbon metabolism in the cytoplasm of leaf cells (Chapter 8, Foyer et al.).

Sucrose-phosphate synthase, pyruvate, phosphate dikinase, phosphoenolpyruvate carboxykinase, PEP carboxylase, nitrate reductase (with its consequent impact on the demand for carbon skeletons) and, in the mitochondria, pyruvate dehydrogenase are all regulated by phosphorylation. PEP carboxylase, in particular, shows complex regulation by metabolites and by phosphorylation (Chapter 18, Fig. 3, Furbank et al.). There is a cascade of events involving changes in cytosolic conditions (and circadian rhythms in CAM plants) which trigger the synthesis and activation of PEP carboxylase kinase, which then leads to changes in the sensitivity to activators (glucose-6-P) and inhibitors (malate) in PEP carboxylase itself). This poses many questions about the significance of this complex regulation which occurs over very different time scales (turnover of the kinase on a diurnal basis, phosphorylation of PEP carboxylase in a matter of hours, and the almost immediate impact of allosteric regulation by metabolites). We remain largely ignorant as to why different mechanisms occur under different circumstances and, in the case of phosphorylation, the nature of the signal transduction events which activate and inactivate kinases. These mechanisms, particularly redox modulation, can be viewed simply as 'on-off' switches, but Fell (1997) has made the important point that multisite modulation (i.e. the simultaneous increase or decrease of enzyme activity, such as is achieved by redox regulation) acts as a dynamic means of tailoring enzyme activity to flux, so that no single enzyme dominates the control of flux. However, in view of the multiplicity of regulatory mechanisms which could modulate carbon assimilation, it is also likely that they operate in a very sophisticated manner, achieving the fine adjustment of enzyme activity to diurnal variations and transients in light, CO_2 , and temperature. Questions about the function of these different tiers of regulation will only be answered with a greater knowledge of the underlying biochemistry, and by the use of knock-out mutants and site-directed mutagenesis to create mutant enzymes for insertion into plants.

II. The Impact of Genetic Manipulation

A major advance in the last 10 years has been the increasing availability of mutants and transgenic plants. These are revolutionizing studies of the importance and role of individual proteins and of the

control of photosynthesis and other plant processes, many examples of which are discussed in the chapters of this book. As far as photosynthetic carbon metabolism is concerned, it has led to findings which have radically changed our thinking about the operation and regulation of plant metabolism. First, it has led to the view that there is considerable flexibility in many areas of plant metabolism and this, in turn, has led to a better appreciation of the metabolic diversity of plants. In many cases, enzyme contents have been decreased by antisense technology without any immediately apparent effects on overall fluxes, growth or phenotype. Examples include the lack of any appreciable influence of the complete loss of cytosolic pyruvate kinase in leaves, though not roots (Gottlob-McHugh et al., 1992; Knowles et al., 1998), and large decreases in citrate synthase (Kruse et al., 1998) or carbonic anhydrase (Price et al., 1994) in leaves. Very often, there must be alternative pathways which allow the plant to overcome a lack of these enzymes, although it must be stressed that antisense plants do not usually lack the enzyme entirely. A good example of flexibility in photosynthetic metabolism is modulation of the activity of the phosphate translocator (Chapter 6, Flügge). A 30% reduction in translocator capacity results in no change in photosynthetic capacity, but does result in decreased export of carbon during the day, enhanced starch accumulation and carbon export at night (partly as hexose), so that, over a diurnal cycle, carbon export from the chloroplast remains undiminished. An additional important point when considering the analysis of the control exercised by enzymes over the rate of photosynthesis is that marginal, essentially unmeasurable, changes during steady state photosynthesis may, over the growth period, be compounded to result in appreciable (and measurable) differences in growth. Recent studies of tobacco plants with carbonic anhydrase reduced to 2% of wild-type levels showed that this was accompanied by no measurable changes in photosynthetic CO_2 assimilation or morphology, but it was accompanied by a decrease in the CO_2 concentration (15 mbar) at the site of carboxylation (determined from carbon isotope composition). There would be a resultant marginal (4.4%), but unmeasurable, gain in the efficiency of photosynthesis in the wild-type (Price et al., 1994). This illustrates that the assessment of control by an enzyme may be limited not only by the ability to measure resultant changes but also to employ growth conditions in which any marginal

gain becomes evident. Similar advantages may accrue from the capacity for light-regulation of enzymes, for example, in response to fluctuating light environments, which would not be evident from plant material encountering the stable light regime maintained in growth cabinets.

Second, the availability of transgenic plants has changed our concepts about the regulation and control of plant metabolism. Control analysis involves asking how much a flux changes for a given change in enzyme activity (Kacser and Burns, 1973; Fell, 1997). Highly regulated enzymes, often catalyzing non-equilibrium reactions, have been traditionally predicted to be points of control (see Stitt, 1999, for discussion). However, it has become clear that considerable reductions in the amounts of many can occur without any deleterious effects, at least when measured under growth conditions, and that the enzyme in the wild-type may exert relatively weak control because regulation can compensate for the loss of catalytic capacity. For example, only when more than 85% of the activity of ribulose-5-P kinase had been removed in transgenic tobacco plants was photosynthesis inhibited, showing that the flux control coefficient in the wild-type was zero. Amounts of ribulose-5-P, ribose-5-P, ATP and fructose-6-P rose and amounts of RuBP, glyceraldehyde-3-P and ADP fell, all of which would tend to compensate for a decrease in total activity by activating ribulose-5-P kinase (Paul et al., 1995). In contrast, less highly regulated enzymes, such as the plastidic aldolase and transketolase, can show appreciable control because there is no means of compensating for loss of activity (Stitt 1999).

Rubisco illustrates how variable the control coefficient of a presumably important enzyme can be. A number of studies have employed transgenic tobacco plants expressing antisense against the small subunit of Rubisco to study how changing the amount of Rubisco influences photosynthetic fluxes and plant growth (Chapter 4, von Caemmerer and Quick; Stitt and Schulze, 1994). First, they show that regulation by increased carbamylated state can compensate for loss of activity, as discussed above. Second, they show that control by Rubisco depends strongly upon the environmental conditions. Third, they demonstrate complex interactions with nitrogen nutrition, such as effects on nitrate assimilation, because Rubisco represents such a large investment of nitrogen. Fourth, they show that changes in Rubisco are particularly pervasive and extend beyond carbon and nitrogen metabolism, water-use efficiency, dry matter

composition, biomass allocation and whole plant growth. They demonstrate that the control by an enzyme, while it can be viewed simply within a metabolic pathway, is also part of control of processes at the level of the whole plant and that control is shared with other non-enzymic processes, such as stomatal conductance. They also emphasize that photosynthesis cannot be considered as an isolated process within the plant. However, even given the complexity of interactions, the importance of Rubisco has been confirmed by analysis of transgenic plants when Rubisco has been substantially depleted or when the light intensity is high, requiring the full complement of Rubisco.

The use of molecular studies to understand photosynthetic physiology and metabolism is now pervasive and is found in nearly every chapter in this book. This has had and will continue to have a large impact on this field.

References

- Andralojc PJ, Keys AJ, Martindale W, Dawson GW and Parry MAJ (1996) Conversion of D-hamamelose into 2-carboxy-D-arabinitol and 2-carboxy-D-arabinitol 1-phosphate in leaves of *Phaseolus vulgaris* L. *J Biol Chem* 271: 26803–26809
- Baier D and Latzko E (1975) Properties and regulation of C-1-fructose-1,6-diphosphatase from spinach chloroplasts. *Biochim Biophys Acta* 396: 141–148
- Bassham and Calvin (1957) The path of carbon in photosynthesis. Prentice-Hall, Englewood Cliffs
- Charlet T, Moore BD and Seemann JR (1997) Carboxyarabinitol 1-phosphate phosphatase from leaves of *Phaseolus vulgaris* and other species. *Plant Cell Physiol* 38: 511–517
- Edwards GE and Walker DA (1983) C_3 ; C_4 mechanisms, and cellular and environmental regulation of photosynthesis. Blackwell, Oxford
- Ehleringer JR, Sage RF, Flanagan LB and Pearcy RW (1991) Climate change and the evolution of C_4 photosynthesis. *Trends Ecol Evol* 6: 95–99
- Fell D (1997) Understanding the Control of Metabolism. Portland Press, London
- Gottlob-McHugh SG, Sangwan RS, Blakely SD, Venleberghe GC, Ko K, Turpin DH, Plaxton WC, Miki BL and Dennis DT (1992) Normal growth of transgenic tobacco plants in the absence of cytosolic pyruvate kinase. *Plant Physiol* 100: 820–825
- Hatch MD (1997) Resolving C_4 photosynthesis: Trials, tribulations and other unpublished stories. *Aust J Plant Physiol* 24: 413–422
- Hirasawa M, Schürmann P, Jacquot J-P, Manieri W, Jacquot P, Keryer E, Hartman FC and Knaff DB (1999) Oxidation-reduction properties of chloroplast thioredoxins, ferredoxin-thioredoxin reductase, and thioredoxin-f-regulated enzymes. *Biochemistry* 38: 5200–5205

- Kacser H and Burns JA (1973) The control of flux. *Symp Soc Exp Biol* 27: 65–104
- Keeley JE (1996) Aquatic CAM photosynthesis. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, pp 281–295, Springer, Berlin
- Knowles VL, McHugh S, Hu Z, Dennis DT, Miki BL and Plaxton WC (1998) Altered growth of transgenic tobacco lacking leaf cytosolic pyruvate kinase. *Plant Physiol* 116: 45–51
- Koroleva OA, Farrar JF, Tomos AD and Pollock CJ (1998) Carbohydrates in individual cells of epidermis, mesophyll, and bundle sheath in barley leaves with changed export or photosynthetic rate. *Plant Physiol* 118: 1525–1532
- Kruse A, Fieuw S, Heineke D and Müller-Röber B (1998) Antisense inhibition of cytosolic NADP-dependent isocitrate dehydrogenase in transgenic tobacco plants. *Planta* 205: 82–91
- Leegood RC, Walker DA and Foyer CH (1985) Regulation of the Benson-Calvin cycle. In: Barber J and Baker NR (eds) *Photosynthetic Mechanisms and the Environment*, pp 189–258, Elsevier, Amsterdam
- Lloyd J and Farquhar GD (1994) ^{13}C discrimination during CO_2 assimilation by the terrestrial biosphere. *Oecologia* 99: 201–215
- Mann CC (1999) Genetic engineers aim to soup up crop photosynthesis. *Science* 283: 314–316
- Ort DR and Oxborough K (1992) In situ regulation of chloroplast coupling factor activity. *Ann Rev Plant Physiol Plant Mol Biol* 43: 269–291
- Osmond CB (1997) C_4 photosynthesis: Thirty or forty years on. *Aust J Plant Physiol* 24: 409–412
- Parry MAJ, Andralojc PJ, Parmar S, Keys AJ, Habash D, Paul MJ, Alred R, Quick WP and Servaites JC (1997) Regulation of Rubisco by inhibitors in the light. *Plant Cell and Environment* 20: 528–534
- Paul MJ, Knight JS, Habash D, Parry MAJ, Lawlor DW, Barnes SA, Loynes A and Gray JC (1995) Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: Effect on CO_2 assimilation and growth in low irradiance. *Plant J* 7: 535–542
- Price GD, von Caemmerer S, Evans JR, Yu J-W, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A and Badger MR (1994) Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation. *Planta* 193: 331–340
- Stitt M (1999) The first will be last and the last will be first: Non-regulated enzymes call the tune? In: Bryant JA, Burrell MM and Kruger NJ (eds) *Plant Carbohydrate Biochemistry*, pp 1–16, BIOS, Oxford
- Stitt M and Schulze D (1994) Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant Cell Environ* 17: 465–487
- Ueno O (1998) Induction of Kranz anatomy and C_4 -like biochemical characteristics in a submerged amphibious plant by abscisic acid. *Plant Cell* 10: 571–583

Chapter 2

The Calvin Cycle and Its Regulation

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Summary

The Calvin cycle is the starting point of carbon metabolism in higher plants. It is a typically eubacterial pathway, as comparative biochemistry of all of its enzymes from prokaryotes and eukaryotes has revealed. The structural basis of Calvin cycle function is reviewed with an attempt at a balanced consideration of biochemical and molecular findings. The structural diversity of prokaryotic enzymes is emphasized, since the genes encoding the pathway in eukaryotes have all been inherited by plants from prokaryotes through endosymbiosis. Curiously, the enzymes that constitute the pathway in different organisms are often structurally unrelated—what is conserved in evolution is merely the set of substrate conversions, not the enzymes that catalyze them. Some of the structural and regulatory properties of the enzymes were present in the antecedents of organelles, but others were newly acquired at the eukaryotic level. The expression of Calvin cycle genes is regulated by a wide spectrum of factors, though the molecular details of the regulation have yet to be unraveled. Findings that suggest the existence of multienzyme-like Calvin cycle complexes are summarized. The molecular basis of redox-modulated light regulation through the thioredoxin system and its importance for flexible control of the pathway under varying conditions is illustrated. Expression of Calvin cycle enzymes in response to external or internal stimuli is briefly reviewed, as are newer findings from the expression of antisense constructs of Calvin cycle enzymes in transgenic plants.

I. Introduction

The Calvin cycle is one of four known pathways of CO_2 fixation in nature, the three other pathways being the reverse (or reductive) citric acid cycle (Evans et al., 1966; Beh et al., 1993; Schönheit and Schäfer, 1995), the reductive acetyl-CoA (or Wood-Lungdahl) pathway (Fuchs and Stupperich, 1986; Ragsdale, 1991; Schönheit and Schäfer, 1995), and the recently discovered 3-hydroxypropionate pathway (Strauss and Fuchs, 1993; Ishii et al., 1996). However, the Calvin cycle is the only pathway of CO_2 fixation known to occur in plants (Fig. 1). It therefore figures prominently in plant biochemistry, albeit under various acronyms, among them the reductive pentose

phosphate pathway (RPPP), the photosynthetic carbon reduction (PCR) cycle, the Calvin-Benson-Bassham (CBB) pathway, the Benson-Calvin cycle, the C₃ cycle, and so on. The enzymes of the Calvin cycle have been previously reviewed by Latzko and Kelly (1979), Robinson and Walker (1981) and Leegood (1990). Regulation of the Calvin cycle has been reviewed by Buchanan (1980), Macdonald and Buchanan (1990), Geiger and Servaites (1994) and, in cyanobacteria, by Tabita (1994). Historical developments surrounding the elucidation of the pathway have been briefly summarized elsewhere (Schnarrenberger and Martin, 1997).

In the Calvin cycle, ATP and NADPH from the light reactions of the photosynthetic membrane are expended to reduce CO_2 to carbohydrate. From the standpoint of ATP investment per mole of CO_2 fixed, the Calvin cycle is the most costly of the four CO_2 fixation pathways known (Strauss and Fuchs, 1993). The basics of the pathway were clarified through ¹⁴C tracer studies in eukaryotic algae over 40 years ago (Calvin, 1956). The net reaction can be summarized as



Mutants defective in CO_2 fixation in the facultatively anaerobic, chemoautotrophic proteobacteria *Rhodobacter sphaeroides* (Gibson and Tabita, 1996) and *Ralstonia eutropha* (previously *Alcaligenes eutrophus*) (Kusian and Bowien, 1997) have been a

Abbreviations: 1,3BPGA - 1,3 bisphosphoglycerate; 3PGA - 3-phosphoglycerate; Aldolase - fructose-1,6-bisphosphate aldolase; CBB - Calvin-Benson-Bassham; cpDNA - chloroplast DNA; CTE - C-terminal extension; DHAP - dihydroxyacetone phosphate; DTT - dithiothreitol; E4P - erythrose 4-phosphate; F1,6BP - fructose 1,6-bisphosphate; F6P - fructose 6-phosphate; FBPase - fructose-1,6-bisphosphatase; FTR - ferredoxin/thioredoxin reductase; GA3P - glyceraldehyde 3-phosphate; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; MDH - malate dehydrogenase; PGK - phosphoglycerate kinase; P_i - inorganic phosphate; PRK - phosphoribulose kinase; R5P - ribose-5-phosphate; RPE - ribulose-5-phosphate 3-epimerase; RPI - ribose 5-phosphate isomerase; Ru1,5BP - ribulose 1,5-bisphosphate; Ru5P - ribulose-5-phosphate; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; SBPase - sedoheptulose-1,7-bisphosphatase; Su1,7BP - sedoheptulose 1,7-bisphosphate; Su7P - sedoheptulose 7-phosphate; Td - thioredoxin; TKL - transketolase; TPI - triosephosphate isomerase; Xu5P - xylulose 5-phosphate

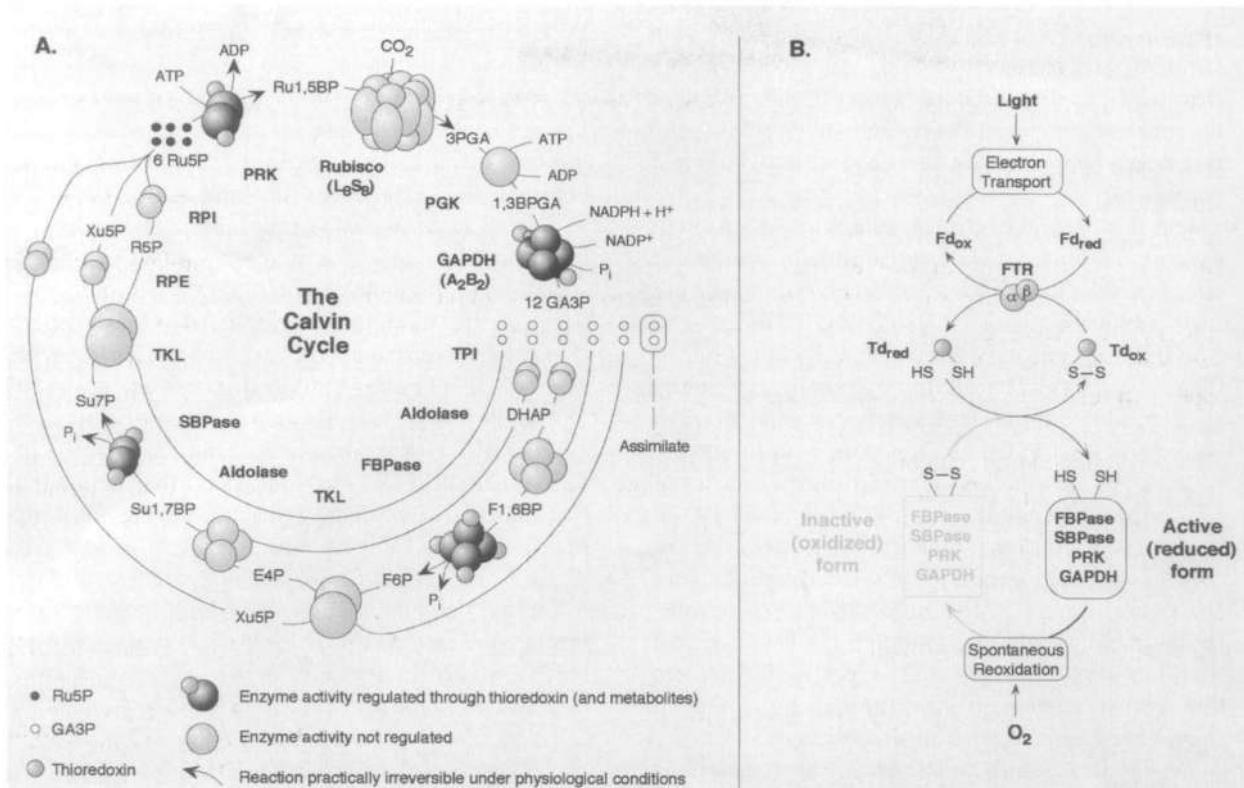


Fig. 1. The Calvin cycle and its regulation in spinach chloroplasts. A. Enzymes and substrates. Relative subunit sizes are drawn roughly to scale. The four regulated enzymes are indicated by darker shading. Lines without arrowheads indicate freely reversible reactions. B. The ferredoxin-thioredoxin system. Fd_{red}: reduced ferredoxin. Fd_{ox}: oxidized ferredoxin. FTR: ferredoxin thioredoxin reductase. Td_{red}: reduced thioredoxin. Td_{ox}: oxidized thioredoxin.

powerful tool for understanding the molecular biology and the genetic regulation of the pathway in these organisms. Molecular sequences are known for all of the enzymes of the pathway from spinach chloroplasts (Martin and Schnarrenberger, 1997) and from the genome sequence of the cyanobacterium *Synechocystis* PCC6803 (Kaneko et al., 1996) and, with a few exceptions, from *Rhodobacter sphaeroides* (Gibson and Tabita, 1997) and *Ralstonia eutropha* (Bommer et al., 1996). Several lines of reasoning support the view that in order to understand the Calvin cycle of higher plant chloroplasts in a broader context, it is useful to consider regulation and structural diversity within the pathway among eubacteria.

First, the pathway did not evolve *de novo* in plants, but rather was inherited from eubacteria via the endosymbiotic origins of organelles. As a consequence, many—but not all—of the regulatory properties that are observed among the enzymes of the higher plant pathway arose at the prokaryotic level and were simply maintained within the plant

lineage, having been genetically transmitted from the cyanobacterial antecedents of plastids. The enzymes of the pathway in chloroplasts are not all acquisitions from cyanobacteria, some are acquisitions from mitochondria (Martin and Schnarrenberger, 1997; Martin and Müller, 1998) that were rerouted during evolution to a new target organelle (Martin and Herrmann, 1998).

Second, in most plastids, at least one enzyme of the pathway (one or both subunits of Rubisco) is still encoded in chloroplast DNA (cpDNA), establishing a requirement for coordination of gene expression between plastids and the nucleus in order to properly express the pathway. Indeed, the plastids of some algae even still possess the *cbbR* gene (Stoebe et al., 1998) which encodes a homologue of the transcriptional regulator of Calvin cycle gene expression in eubacteria.

Third, the quantitatively most important mechanism governing the activity of higher plant Calvin cycle enzymes—light activation via the thioredoxin

system—is present and active in cyanobacteria. This regulatory mechanism was also inherited by plants from the eubacterial antecedents of plastids, although the molecular basis for the covalent transitions in the target enzymes can differ between cyanobacteria and plants.

Fourth, although the mechanisms of Calvin cycle gene regulation in eubacteria are probably much less complex than those in eukaryotes, by no means are they irrelevant to our understanding of eukaryotic Calvin cycle gene regulation. On the contrary, due to their simplicity and tractability, mechanisms of Calvin cycle gene regulation are much better understood in eubacteria than in eukaryotes. And with the recent discovery of a cyanobacterial homologue of phytochrome (Hughes et al., 1997; Yeh et al., 1997), it appears that at least some of the basic machinery for Calvin cycle gene regulation through light in eukaryotes were simply inherited from prokaryotes through endosymbiosis, although the actual signal transduction pathways in prokaryotes and eukaryotes that lead to gene regulation through light will, in many cases, turn out to be quite different.

Finally, although the series of substrate conversions that constitute the Calvin cycle are strictly conserved across eubacteria and eukaryotes, the same degree of conservation does not apply to the enzymes that catalyze those reactions. In fact, in this chapter we will see that the pathway in proteobacteria, cyanobacteria and higher plant chloroplasts consists of enzymes that catalyze identical reactions, but, in some cases, that are altogether unrelated at the level of sequence, structure and reaction mechanism (see Martin and Schnarrenberger, 1997).

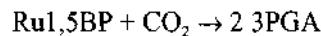
Many of the subsequent chapters in this volume deal, in one way or another, with various aspects of the Calvin cycle, including Rubisco itself (Chapters 3, (Roy and Andrews) and 4 (von Caemmerer and Quick)), metabolite transport (Chapter 6, Flügge), C4 metabolism (Chapters 18 (Furbank et al.) and 19 (Leegood)) and chloroplast-cytosol interactions (Chapters 7 (Aiken et al.) and 8 (Foyer et al.)). In this chapter, we will focus on structural, functional and regulatory aspects of the enzymes that constitute the pathway, emphasizing insights provided by molecular approaches, but considering classical biochemical aspects as well.

II. The Enzymes of the Calvin Cycle

A schematic comparison of Calvin cycle enzymes in

the α -proteobacterium *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Bowien et al., 1993) and those encoded in the genome of the cyanobacterium *Synechocystis* PCC6803 (Kaneko et al., 1996a, 1996b) reveals that pathways in these bacteria comprise the same sets of substrate conversions, but in several cases with the help of enzymes that are non-homologous—or very nearly so (Fig. 2). Such structurally distinct but functionally homologous enzymes are traditionally designated as class I/class II enzymes, a term that will be used here. Differences also exist between the pathways in spinach chloroplasts and *Synechocystis* (e.g. use of class I vs. class II aldolase, respectively), but as depicted in Fig. 2, these differences are less grave than across the two eubacteria compared. The following sections provide a synopsis of structural and functional diversity for each Calvin cycle enzyme. Regulation of individual enzymes by covalent modification through the ferredoxin/thioredoxin system (recently reviewed by Jacquot et al., 1997b) will be considered later in this chapter.

A. Ribulose-1,5-bisphosphate Carboxylase/oxygenase



Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco) catalyzes the initial CO_2 fixation step. The mechanism involves an activating carbamylation reaction between CO_2 and the ϵ -amino group of an active site lysine residue in the large subunit (Lorimer and Miziorko, 1980). Carbamylation is promoted by Rubisco activase (Portis, 1992). For details of Rubisco kinetics, catalytic mechanism and regulation, see Chapters 3 (Roy and Andrews) and 4 (von Caemmerer and Quick). The crystal structure of Rubisco from spinach chloroplasts is known at great resolution, it is a stout cylindrical tetramer of L₂ dimers that are ‘glued’ together by four small subunits at each end (Shibata et al., 1996; Andersson, 1996). Two structurally distinct Rubisco enzymes are known. Class I (or form I) Rubisco has a native M_r of about 560 kDa and consists of eight large subunits (LSU, M_r ~55 kDa each) and eight small subunits (SSU, M_r ~15 kDa each) comprising the L₂S₈ holoenzyme. Assembly of the heterohexadecamer requires the aid of chaperonins in both chloroplasts and eubacteria (Goloubinoff et al., 1989; Gatenby and Viitanen, 1994; Gutteridge and Gatenby, 1995).

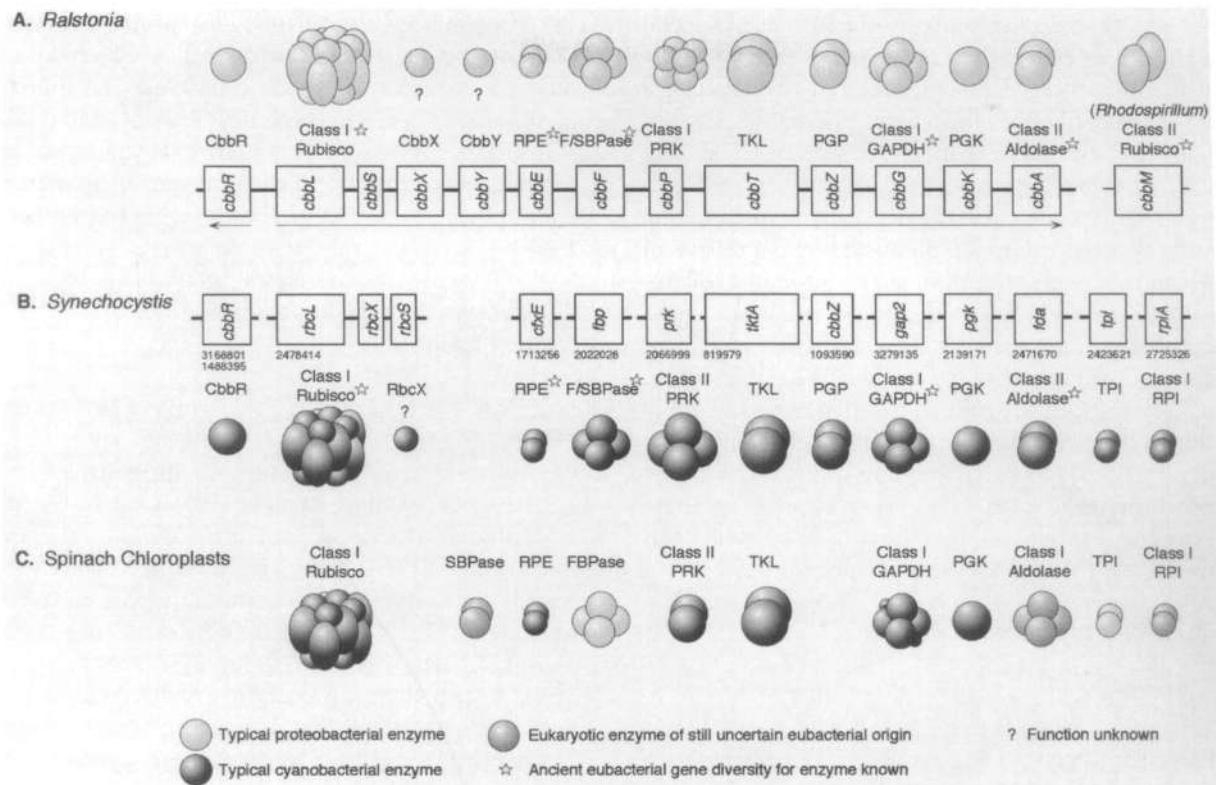


Fig. 2. Comparison of gene organization and subunit structure of Calvin cycle enzymes in bacteria and higher plants. A. The *cbb* operon of *Ralstonia eutropha* as summarized from data in Bowien et al. (1993), from which the gene designations were taken. Direction of transcription is indicated with arrows. Note that TPI and RPI are not encoded in the operon. PGP: phosphoglycolate phosphatase. B. Positions of *cbb* genes in *Synechocystis* PCC 6803 as summarized from data in Kaneko et al. (1996a, 1996b), from which the gene designations were taken. Numbers indicate the genome coordinate of the first nucleotide of the gene. RbcX was recently shown to possess a chaperonin function for folding of cyanobacterial Rubisco (Li and Tabita, 1997). C. The enzymes from spinach chloroplasts, all of which except the large subunit of Rubisco are nuclear encoded (summarized from data in Martin and Schnarrenberger (1997)). The cyanobacterial (plastid) or proteobacterial (mitochondrion) origins of the spinach genes are indicated with shading.

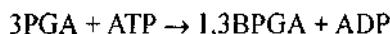
Class II Rubisco consists only of large subunits of $M_r \sim 55$ each (L_{2-6} holoenzyme) (Gibson and Tabita, 1996; Kusian and Bowien, 1997). The class I and class II large subunits share about 30% amino acid identity, indicating that they share a common ancestor. Rubisco gene diversity is a complicated matter and has been discussed in detail elsewhere (Watson and Tabita, 1996; Martin and Schnarrenberger, 1997). At least two very ancient gene duplications (or lateral transfers) have occurred in Rubisco evolution, one that gave rise to the class I and class II enzymes and a second that gave rise to the two distinct families of class I Rubisco found in chlorophytic ('green' or G-type Rubisco) and rhodophytic plastids ('red' or R-type Rubisco), respectively (Martin and Schnarrenberger, 1997). Cyanobacteria and many γ - and β -proteobacteria studied to date possess G-type Rubisco (Watson and Tabita, 1996), whereas the proteobacteria *Ralstonia*, *Rhodobacter* and *Xanthoflavus* encode R-

type Rubisco in their *cbb* (Calvin-Benson-Bassham) operons. But *Rhodobacter*, like *Rhodospirillum*, *Hydrogenovibrio* and several other eubacteria, also encode and express the class II enzyme (Falcone and Tabita, 1991; Stoner and Shively, 1993; Gibson and Tabita, 1996). Notably, the curious Rubisco gene in the *Methanococcus* genome encodes an active, O₂-sensitive enzyme (Watson et al., 1999).

Higher plants, like cyanobacteria, possess G-type class I Rubisco, whereby in all species studied, the large subunit is encoded as a single copy in the cpDNA and the small subunit is encoded in the nucleus, usually as a gene family (see below). The primitive photosynthetic protist *Cyanophora paradoxa* is an exception, however, in that both the large and the small subunits of its G-type Rubisco are encoded in the cpDNA (Lambert et al., 1985). Rhodophytes and photosynthetic protists that have obtained their plastids from rhodophytes via

secondary endosymbiosis (McFadden et al., 1996; Van de Peer et al., 1996) encode both subunits of R-type Rubisco in their cpDNA, and also encode a homologue of *cbbR*, the transcriptional regulator of Calvin cycle operons in proteobacteria. The only examples in which eukaryotes have been shown to possess class II Rubisco have been described for the very diverse group of photosynthetic protists of secondary symbiotic origin known as dinoflagellates (Morse et al., 1995; Rowan et al., 1996), where, quite surprisingly, the gene for the class II Rubisco large subunit is encoded in the nucleus. The diversity of eukaryotic Rubisco genes is, in all likelihood, simply the result of sampling from ancient eubacterial gene diversity present in the common ancestor of endosymbiotic organelles, very similar to allele sampling in population genetics, but on a geological time scale (Martin and Schnarrenberger, 1997).

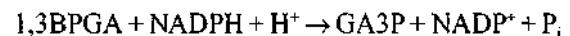
B. Phosphoglycerate Kinase



Phosphoglycerate kinase (EC 2.7.2.3, PGK) catalyzes the reversible transfer of the γ -phosphate of ATP to the carboxyl group of 3-phosphoglycerate (3PGA), forming 1,3-bisphosphoglycerate (1,3BPGA) for the subsequent reduction step. In all prokaryotic and eukaryotic sources studied to date, the active enzyme is a monomer with an M_r of ~44 kDa (Fothergill-Gilmore and Michels, 1993). The crystal structure of the enzyme from several sources is known. PGK is unusual in that substrate binding induces a dramatic conformational change: the two 'wings' of the butterfly structure are bent upon 3PGA and ATP binding by over 30 degrees, displacing distal regions of the domains by some 27 Å (Bernstein et al., 1997). The chloroplast and cytosolic isoenzymes can be separated with conventional techniques, roughly 90% of the PGK activity is localized in higher plant chloroplasts (Pacold and Anderson, 1975; Köpke-Secundo et al., 1990; McMorrow and Bradbeer, 1990). In *Chlamydomonas reinhardtii*, a cytosolic isoenzyme seems to be lacking (Schnarrenberger et al., 1990; Kitayama and Togasaki, 1995). Chloroplast PGK from various sources shows biphasic kinetics with $K_m(3\text{PGA})$ of ~400 μM and $K_m(\text{ATP})$ of ~500 μM at low substrate concentrations, with a pH optimum around 7.5 (Köpke-Secundo et al., 1990). The enzyme has not been found to be strongly regulated by allosteric effectors or by light (Leegood, 1990). The

enzyme has been cloned from several higher plants (Longstaff et al., 1989; Bertsch et al. 1993) and was mapped in wheat (Chao et al., 1989). The higher plant nuclear genes for both the chloroplast and the cytosolic enzymes were obtained from cyanobacteria through endosymbiotic gene transfer (Brinkmann and Martin, 1996; Martin and Schnarrenberger, 1997).

C. Glyceraldehyde-3-phosphate Dehydrogenase



Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13, NADP⁺-dependent GAPDH) catalyzes the reversible reductive step of the Calvin cycle. In catalysis, 1,3BPGA forms a highly reactive thioester bond with the thiol moiety of the active site cysteine residue (Cys^{149}) under elimination of the acyl phosphate. The carbonyl group of the covalently bound intermediate is reduced to a hemithioacetal by hydride transfer from NADPH and glyceraldehyde 3-phosphate (GA3P) is released from the enzyme through cleavage of the hemithioacetal bond (Bränden and Eklund, 1980). Chloroplast NADP⁺-dependent GAPDH has activity with both NAD(H) and NADP(H). Although the $K_m(\text{NADH})$ of the enzyme is too low to be relevant during (anabolic) CO₂ fixation, NADPH being strongly preferred by the enzyme (Cerff, 1978a), recent studies indicate that the NAD⁺-activity may play an important role during (catabolic) ATP-synthesis in the dark (Backhausen et al., 1998). The kinetic properties are complex and depend upon the activation state of the enzyme (Cerff, 1978a; Wolosiuk and Buchanan, 1978). In the forward reaction the fully active purified enzyme has a $K_m(1,3\text{BPGA})$ of roughly 30 μM and a $K_m(\text{NADPH})$ of 40 μM (Baalmann et al., 1995). For the reaction in intact chloroplasts, a $K_m(1,3\text{BPGA})$ of 1 μM has been estimated (Fridlyand et al., 1997).

Class I and class II GAPDH enzymes are known that share only 15–20% sequence identity, both are tetramers of M_r ~150 kDa, consisting of ~37 kDa subunits. Eukaryotes, eubacteria, and one halophilic archaeabacterium possess class I GAPDH (Prüß et al., 1994; Brinkmann and Martin, 1996). The tertiary structure of class I GAPDH is known for numerous sources (Biesecker et al., 1977; Michels et al., 1996). Class II GAPDH has been found only in archaeabacteria (Hensel et al., 1987; Fabry and Hensel,

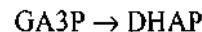
1988; Zwickl et al., 1990). No crystal structures have been published for the class II enzyme.

Higher plant Calvin cycle GAPDH differs from all other known GAPDH enzymes in that it is an A_2B_2 heterotetramer rather than a homotetramer (Cerff and Chambers, 1979; Ferri et al., 1990; Scagliarini et al., 1993). The tetrameric A_2B_2 enzyme can reversibly aggregate to a multimeric form of 600 to 800 kDa, being less active than the dissociated form (Cerff, 1978a, 1978b; Pupillo and Faggiani, 1979; Waraswapat et al., 1980; Trost et al., 1993). This multimeric form was probably the form of the enzyme first purified from plants (Yonuschot et al., 1970). Cytosolic GAPDH (EC 1.2.1.12) and non-phosphorylating GAPDH (EC 1.2.1.9), although tetramers, do not show this reversible oligomer formation (Pupillo and Faggiani, 1979). No strong allosteric effectors are known for higher plant GAPDH, but in the cyanobacterium *Synechocystis* PCC6803 a low MW fraction has been described that reduces the activity of Calvin cycle GAPDH in the reverse (oxidative) direction (Koksharova et al., 1998).

A novel, **NAD⁺-dependent** plastid-specific GAPDH (GapCp) was recently described from *Pinus* chloroplasts that coexists with A_2B_2 GAPDH of the Calvin cycle. It shows no detectable activity with **NADP⁺** and has a $K_{m(NAD)}$ of 62 μM and $K_{m(GA3P)}$ of 344 μM (Meyer-Gauen et al., 1994; Meyer-Gauen et al., 1998). GapCp from *Pinus* is possibly similar to the **NAD⁺-GAPDH** reported from isolated, non-photosynthetic plastids of developing cauliflower buds (Neuhaus et al., 1993). There is also biochemical evidence for a similar **NAD⁺-specific** plastid GAPDH in ripening sweet pepper fruits where, in cooperation with an **NAD⁺-dependent** MDH, it appears to be important in the distribution of reducing equivalents between plastid and cytosol (Backhausen et al., 1998). In some photosynthetic tissues, for example in pine seedlings, GapCp (an **NAD⁺-specific** enzyme) appears to coexist with A_2B_2 Calvin cycle GAPDH (**NADP⁺-specific**) (Meyer-Gauen et al., 1994; Schnarrenberger, unpublished). In some non-photosynthetic tissues, GapCp may functionally replace the A_2B_2 enzyme. By analogy, in some photosynthetic protists, an **NADP⁺-specific** GAPDH enzyme has been recruited from an **NAD⁺-specific** ancestral enzyme (Liaud et al., 1997; Fagan et al., 1998). The nuclear gene for higher plant Calvin cycle GAPDH was obtained by plants from cyanobacteria, the cytosolic enzyme appears to have been obtained from the mitochondrial symbiont

genome (Martin et al., 1993; Henze et al., 1995). The GapA and GapB subunits of the A_2B_2 enzyme arose through gene duplication during chlorophyte evolution (Meyer-Gauen et al., 1994). The B subunit is implicated in regulatory properties of the enzyme (Scagliarini et al., 1998) and possesses a CTE of roughly 30 amino acids relative to the A subunit that is involved in thioredoxin-dependent regulation (see Section VI).

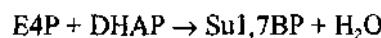
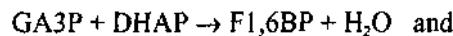
D. Triosephosphate Isomerase



Triosephosphate isomerase (EC 5.3.1.1, TPI) catalyzes the rapid and reversible ketose-aldoose isomerization of dihydroxyacetone phosphate (DHAP) and GA3P. The native enzyme in eubacteria and eukaryotes is a homodimer of ~27 kDa subunits (Fothergill-Gilmour and Michels, 1993), in hyperthermophilic archaebacteria TPI is a homotetramer of 25 kDa subunits (Kohlhoff et al., 1996). The Calvin cycle enzyme of higher plant chloroplasts is a homodimer of ~27 kDa subunits (Kurzok and Feierabend, 1984; Henze et al., 1994; Schmidt et al., 1995). For both the chloroplast and cytosolic enzymes separated from leaves $K_{m(DHAP)}$ is ~2 mM and $K_{m(GA3P)}$ is ~700 μM (Kurzok and Feierabend, 1984). The crystal structure of the enzyme from many sources is known (Velanker et al., 1997).

As for PGK, class I/class II forms of TPI have not been described. Calvin cycle TPI of higher plant chloroplasts arose through a duplication of the pre-existing eukaryotic nuclear gene for cytosolic TPI, accompanied by the acquisition of a transit peptide (Henze et al., 1994; Schmidt et al., 1995). But since the preexisting nuclear gene was itself acquired via endosymbiotic gene transfer from ancestors of mitochondria (Keeling and Doolittle, 1997), the Calvin cycle of higher plant chloroplasts functions with TPI enzyme of mitochondrial origin that was rerouted to the plastid during evolution (Martin and Schnarrenberger, 1997).

E. Fructose-1,6-bisphosphate/Sedoheptulose-1,7-bisphosphate Aldolase

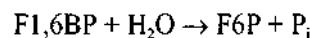


Fructose-1,6-bisphosphate aldolase (EC 4.12.1.13, aldolase) catalyzes the reversible aldol condensation of dihydroxyacetone phosphate and either GA3P or erythrose-4-phosphate to yield fructose-1,6-bisphosphate or sedoheptulose-1,7-bisphosphate, respectively. Both activities are part of the Calvin cycle. Two very distinct types of aldolase enzymes occur in nature that differ in their catalytic mechanism (Rutner, 1964; Marsh and Lebherz, 1992). Class I aldolase enzymes form a Schiff-base with the substrate during catalysis via condensation of the *ε*-amino group of an active-center lysine residue with the carbonyl group of the substrate. Class II aldolase enzymes require divalent cations such as Zn²⁺, Fe²⁺ or Ca²⁺ as cofactors which stabilize the carbanion intermediate formed during the reaction. The dual specificity for F1,6BP and Su1,7BP formation by aldolase applies to the chloroplast enzyme and to the cytosolic enzyme, both of the class I type in higher plants (Brooks and Criddle, 1966; Moorehead and Plaxton, 1990) and of the class II type in *Cyanophora paradoxa* (Flechner et al., 1999). Class I aldolases are homotetramers whereas class II aldolases are homodimers. The subunit size of both classes of aldolase enzymes is ~40 kDa, but class I and class II aldolase monomers share no detectable sequence similarity. This, in addition to the different catalytic mechanisms and unrelated crystal structures for class I (Blom and Sygusch, 1997) and class II (Cooper et al., 1996) aldolase, clearly indicates that these two classes of aldolase enzymes are the result of evolutionary functional convergence. For separated spinach chloroplast and cytosolic class I aldolase, K_{m(F1,6BP)} is 20 μM and 1 μM, respectively, whereas K_{m(Su1,7BP)} is 6 μM and 4 μM, respectively. The corresponding values for chloroplast and cytosolic class II aldolase from *Cyanophora paradoxa* are K_{m(F1,6BP)} 1 mM and 660 μM, respectively, whereas K_{m(Su1,7BP)} is 200 μM and 230 μM, respectively (Flechner et al., 1999).

Class I and class II aldolases have a very complex phylogenetic distribution across prokaryotes and eukaryotes (Henze et al., 1998). Most eubacteria, including all cyanobacteria studied to date, typically possess class II aldolase (Rutter, 1964; Antia, 1967), although class I aldolase is known in eubacteria (Witke and Götz, 1993). Halophilic archaeabacteria can possess either class I or class II aldolase (Dhar and Altekar, 1986). Interestingly, the *Methanococcus* genome does not encode a recognizable homologue of either class I or class II aldolase (Bult et al., 1996),

although methanogens are known to possess aldolase activity (Yu et al., 1994; Schönheit and Schäfer, 1995), raising the possibility that a class III aldolase will eventually be found. A possible candidate for such a new class of aldolase has been described from a halophilic archaeabacterium (Krishnan and Altekar, 1991) that possesses a (mechanistically) class I aldolase consisting of 27 kDa (rather than 40 kDa) subunits with novel properties. Among higher eukaryotes, fungi typically possess class II aldolase whereas metazoa and higher plants possess class I aldolase (Schnarrenberger et al., 1990; Marsh and Lebherz, 1992; Tsutsumi et al., 1994). *Euglena gracilis* is exceptional among eukaryotes in that it possesses both class I aldolase (in the chloroplast) and class II aldolase (in the cytosol) (Pelzer-Reith et al., 1994b). In addition to class I and class II aldolase, ancient eubacterial gene duplications are known in class II aldolase evolution that have given rise to aldolase-related enzymes specialized for substrates other than sugar phosphates (Plaumann et al., 1997). The Calvin cycle of both proteobacteria and cyanobacteria operates with class II aldolase, while aldolase of higher plant chloroplasts is a class I enzyme, and the paucity of sequences for class I aldolase from prokaryotes makes it currently impossible to tell whence the class I gene for the chloroplast enzyme arose (Plaumann et al., 1997). To add to this conundrum of diversity, the Calvin cycle in cyanelles of *Cyanophora paradoxa* operates with a class II aldolase (Gross et al., 1994). Thus, Calvin cycle aldolase of plastids has arisen at least twice in evolution, and the data for class I aldolase of *Euglena*'s chloroplasts suggest that a third independent origin of Calvin cycle aldolase in plastids is likely (Plaumann et al., 1997).

F. Fructose-1,6-bisphosphatase



Fructose-1,6-bisphosphatase (EC 3.1.3.11, FBPase) catalyzes the cleavage of the phosphoester bond on C1 to yield fructose-6-bisphosphate (F6P). In most proteobacteria and cyanobacteria, the FBPase and SBPase reactions of the Calvin cycle are catalyzed by a single enzyme (F/SBPase) with dual specificity for both substrates (Gerbling et al., 1986; Gibson and Tabita, 1988; Yoo and Bowien, 1995; Paoli et al., 1995). *Xanthobacter flavus* is an exception (see below). F/SBPase from cyanobacteria (Gerbling et

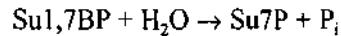
al., 1985) is a tetramer of ~40 kDa subunits, as is FBPase from spinach (Marcus and Harrsch, 1990). The crystal structure of spinach chloroplast FBPase has been determined (Villeret et al., 1995).

FBPase catalyzes a highly exergonic reaction that is virtually irreversible under physiological conditions, and it is one of the key targets for regulation of the Calvin cycle. Activity of the enzyme is undetectable in the dark (oxidized state), but increases to maximum activities within a few minutes of illumination due to thiol reduction via the thioredoxin system (Buchanan, 1980). FBPase is specifically activated by thioredoxin *f* (hence the designation *f*) (Buchanan, 1980; Lopez-Jaramillo et al., 1997). The mechanism of chloroplast FBPase regulation was revealed by altered kinetics observed in the presence of Mg²⁺, pH, and thiols (Zimmermann et al., 1976). Activation of chloroplast FBPase by reduced thiols affects a dramatic increase of substrate affinity of >20-fold (Charles and Halliwell, 1980), $K_m(Mg\text{F1,6BP})$ of the fully activated enzyme is 6 μM as compared to 130 μM for the oxidized enzyme (Cadet and Meunier, 1988b). The sensitivity of the chloroplast enzyme to low concentrations of mercuric ions has been studied in several species (Ashton, 1998a).

Both chloroplast and cytosolic FBPase of higher plants are highly regulated (see Section VI), but by quite different mechanisms (Latzko et al., 1974; Zimmermann et al. 1976). The cytosolic enzyme is a control point for regulating flux through gluconeogenesis. Like its homologues from the cytosol of non-photosynthetic eukaryotes, it is subject to strong allosteric inhibition by AMP and regulation through F2,6BP (Stitt, 1990a, 1990b), whereas thioredoxin has no effect. The chloroplast enzyme on the other hand, is insensitive to both AMP and F2,6BP. Curiously, these distinct regulatory properties seem to have evolved specifically in the plant lineage. This is because higher chloroplast FBPase arose through gene duplication of the preexisting nuclear gene for cytosolic FBPase, that itself appears to have been acquired from mitochondria (Martin et al., 1996a; Schnarrenberger and Martin, 1997), indicating that—as in the case of TPI—the higher plant Calvin cycle functions with an FBPase enzyme of mitochondrial origin. The archaeabacteria *Methanococcus maripaludis* and *Haloarcula vallismortis* possess high FBPase activity (Altekar and Rangaswamy, 1992; Yu et al., 1994) but the enzyme has not been purified from any archaeon and the *Methanococcus* genome does not encode a recognizable gene for FBPase

(Bult et al., 1996), raising the possibility that structurally unrelated class I and class II FBPase enzymes may exist.

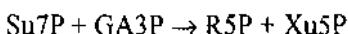
G. Sedoheptulose-1,7-bisphosphatase



A highly specific sedoheptulose-1,7-bisphosphatase (EC 3.1.3.37, SPB) is not known from prokaryotes, although *Xanthobacter flavus* differentially expresses two distinct F/SBPase isoenzymes that both accept F1,6BP and Su1,7BP as substrates. The isoenzyme expressed during autotrophic growth (CbbF) has nearly equal activities with F1,6BP and Su1,7BP (3:1, respectively) as substrates, with a $K_m(\text{F1,6BP})$ of 3 μM, the other isoenzyme possesses much lower activity with Su1,7BP (van den Bergh et al., 1995). Similarly, *Synechococcus* PCC 7942 possesses two immunologically distinct tetrameric FBPase isoenzymes, one of which is specific for F1,6BP, the other of which efficiently cleaves both F1,6BP and Su1,7BP (Tamoi et al., 1996). The cyanobacterial F/SBPase isoenzymes thus have slightly more similar properties to those found in higher plants, where chloroplast FBPase and SBPase in plants are separate enzymes encoded by distinct but distantly related nuclear genes (Raines et al., 1988,1992; Martin et al., 1996a). In contrast to FBPase and bacterial F/SBPase, which are tetramers, SBPase from higherplant chloroplasts is a dimer of ~35 kDa subunits (Nishizawa and Buchanan, 1981; Cadet et al., 1987). Also in contrast to many bacterial F/SBPase enzymes, higher plant chloroplast FBPase and SBPase show a high specificity for their respective substrates, whereby chloroplast SBPase is highly, but not completely specific for Su1,7BP (Zimmermann et al., 1976; Breazeale et al., 1978; Cadet and Meunier, 1988b). Chloroplast SBPase, as FBPase, is redox-modulated by thioredoxin *f* (Breazeale et al., 1978; Nishizawa and Buchanan, 1981). The reduced (activated) enzyme has a $K_m(\text{Su1,7BP})$ of 50 μM and a $K_m(\text{F1,6BP})$ of 380 μM (Cadet and Meunier, 1988b). But since the $K_m(\text{F1,6BP})$ of reduced (activated) chloroplast FBPase is 6 μM, the F1,6BP activity of SPB is probably of little or no physiological relevance. Similar reasoning applies to the SBPase activity of chloroplast FBPase (Ashton, 1998b). SBPase from wheat has been expressed in *E. coli* (Dunford et al., 1998). F2,6BP, a potent allosteric regulator of cytosolic FBPase, has no allosteric effect on chloroplast SBPase, but can

act as a competitive inhibitor (Cadet and Meunier, 1988b). The evolutionary relationship between chloroplast SBPase and eukaryotic FBPase and eubacterial F/SBPase enzymes is unclear (Martin et al., 1996a), but it appears that the specialization of SBPase from a bifunctional F/SBPase ancestor occurred at the prokaryotic level.

H. Transketolase

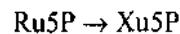


Transketolase (EC 2.2.1.1, TKL) catalyzes the reversible, thiamine diphosphate-dependent transfer of a two carbon ketol group from either fructose-6-phosphate or sedoheptulose-7-phosphate (Su7P) to glyceraldehyde-3-phosphate to yield xylulose-5-phosphate (Xu5P) and either erythrose-4-phosphate or ribose-5-phosphate (R5P), respectively. TKL from various sources is a homodimer of 74 kDa subunits (Feierabend and Gringel, 1983). The crystal structure of the yeast enzyme is known (Nikkola et al., 1994; Nilsson et al., 1997). The catalytic mechanism involves nucleophilic attack of the substrate carbonyl group via the C2 carbanion of thiamine diphosphate (ThDP): the rate-limiting C2 deprotonation step requires interaction of N1' in the ThDP pyrimidine ring with Glu⁴¹⁸ (Kern et al., 1997).

Beyond the studies of Murphy and Walker (1982), who purified the enzyme 400-fold, and Feierabend and Gringel (1983), who found only a single chloroplast species, little attention has been given to the biochemistry of this Calvin cycle enzyme. Substrate affinities for the plant enzyme have been reported as 100-130 μM for Xu5P, E4P and R5P (Murphy and Walker, 1982), for human erythrocytes the values $K_{m(\text{Xu5P})}$, 20 μM , $K_{m(\text{R5P})}$, 30 μM and $K_{m(\text{F6P})}$, 2 mM were found (Himmo et al., 1989). For the purified enzyme from spinach chloroplasts $K_{m(\text{Xu5P})}$, 77 μM and $K_{m(\text{R5P})}$, 330 μM were found (Teige et al., 1998). TKL has been cloned from *Craterostigma* (Bernaccia et al., 1995), spinach (Flechner et al., 1996) and potato (Teige et al., 1996). The enzyme from spinach chloroplasts has been expressed in highly active form in *E. coli* (Flechner et al., 1996). Spinach leaves appear to possess only a single TKL enzyme, localized exclusively in the chloroplast (Feierabend and Gringel, 1983; Schnarrenberger et al., 1995). TKL shows structural similarity to several other enzymes

involved in ThDP-dependent C2 metabolism: pyruvate decarboxylase and the E1 subunit of pyruvate dehydrogenase (Robinson and Chun, 1993). The nuclear gene for the Calvin cycle enzyme of higher plants was acquired from cyanobacteria (Martin and Schnarrenberger, 1997).

I. Ribulose-5-phosphate 3-epimerase



Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1, RPE) catalyzes the reversible interconversion of ribulose-5-phosphate and xylulose-5-phosphate. RPE is a homodimer of ~23 kDa subunits in animals (Karmali et al., 1983), *Ralstonia* (Kusian et al., 1992) and spinach (Nowitzki et al., 1995). The spinach enzyme has been purified to homogeneity and N-terminally sequenced (Teige et al., 1998). The purified spinach enzyme migrates as an octamer, the $K_{m(\text{Ru5P})}$ was determined as 250 μM (Teige et al., 1998). RPE from the red alga *Galdieria sulphuraria* has a $K_{m(\text{Ru5P})}$ of ~830 μM (J. Girnus, W. Gross and C. Schnarrenberger, unpublished). Spinach leaves appear to possess only a single RPE enzyme, localized in chloroplasts (Schnarrenberger et al., 1995). RPE has been cloned from sorghum and spinach (Nowitzki et al., 1995) and potato (Teige et al., 1995), the enzyme from spinach chloroplasts has been expressed in active form in *E. coli* (Nowitzki et al., 1995). More recently, the enzyme from spinach chloroplasts was cloned again, and was expressed in *E. coli* again (Chen et al., 1998). Neither the mechanism of catalysis nor the tertiary structure have been reported from any source. Class I / class II RPE enzymes have not been described, but three very distantly *rpe*-related genes exist in the *E. coli* genome, indicating the presence of relatively ancient eubacterial gene families (Nowitzki et al., 1995). The nuclear gene for higher plant Calvin cycle RPE was acquired from cyanobacteria (Martin and Schnarrenberger, 1997).

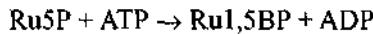
J. Ribose-5-phosphate Isomerase



Ribose-5-phosphate isomerase (EC 5.3.1.6, RPI) catalyzes the reversible isomerization of ribose-5-phosphate and ribulose-5-phosphate. RPI has not been identified in the *cbb* operons of photosynthetic proteobacteria (Gibson and Tabita, 1996). No crystal

structures have been reported for this enzyme. Rutner (1970) purified RPI from spinach 2800-fold. Only a single enzyme was found, a homodimer of 23 kDa subunits, as later shown for *Arabidopsis* (Babadzhanova and Bakaeva, 1987), that had a $K_m(RPI)$ of 460 μM . Chloroplast RPI from spinach (Martin et al., 1996b) has been cloned, it has sequence similarity to RpiA from *E. coli* (Hove-Jensen and Maigaard, 1993). But *E. coli* also possesses a gene for a second functional RPI enzyme, RpiB, that is a homodimer of 16 kDa subunits. It shows no sequence similarity to RpiA, but very high similarity to galactose-6-phosphate isomerases (Sørensen and Hove-Jensen, 1996). Thus for RPI, class I (e.g. spinach RPI or RpiA of *E. coli*) and class II (RpiB of *E. coli*) enzymes should be distinguished. No cytosolic isoenzyme of RPI was found in spinach leaves (Schnarrenberger et al., 1995). Calvin cycle (class I) RPI from spinach has identifiable homologues encoded in the *Synechocystis* and *Methanococcus* genomes, but due to paucity of reference sequences, the evolutionary origin of the plant nuclear gene is still unclear.

K. Phosphoribulokinase



Phosphoribulokinase (EC 2.7.1.19, PRK) transfers the γ -phosphate of ATP to the C1 hydroxyl group of ribulose-5-phosphate, regenerating the primary CO_2 acceptor. Class I and class II PRK enzymes are known (Tabita, 1994; Brandes et al., 1996a; Martin and Schnarrenberger, 1997). Class I PRK is encoded in proteobacterial *cbb* operons. It is an octamer of ~30 kDa subunits with allosteric inhibition through AMP and allosteric activation through NADH (Runquist et al., 1995). Crystal structure data has been reported for class I PRK (Roberts et al., 1995; DHT Harrison et al., 1998). Class II PRK is found in cyanobacteria and higher plants. It is a dimer of ~44 kDa subunits in chloroplasts (Porter et al., 1986; Clasper et al., 1994). The enzyme can associate to tetramers in *Synechocystis* (Wadano et al., 1995). The 300-fold purified enzyme from the chromophytic protist *Heterosigma carterae* is a tetramer of 53 kDa subunits with a $K_m(\text{Mg-ATP})$ of 208 μM and $K_m(\text{Ru5P})$ of 226 μM (Hariharan et al., 1998). Crystal structures have not been reported for the class II enzyme. The catalytic properties of class I and class II PRK differ markedly (Tabita, 1988). The nuclear gene for the

higher plant Calvin cycle enzyme was acquired from cyanobacteria (Martin and Schnarrenberger, 1997).

PRK catalyzes a highly exergonic reaction and is strongly regulated by the thioredoxin system (Buchanan, 1980). The oxidized (dark) enzyme possesses only about 2% of the activity of the fully active (reduced) form (Surek et al., 1985). Kinetic values of $K_m(\text{Mg-ATP})$ of 60 μM and $K_m(\text{Ru5P})$ of 110 μM were reported for the spinach enzyme expressed in the yeast *Pichia pastoris* (Brandes et al., 1996a), similar to values determined for the purified native activated wheat enzyme (Surek et al., 1985). In contrast to GAPDH, FBPase, and SBPase, thioredoxin activation of PRK does not involve lowering of K_m values, but affects the V_{max} (Porter et al., 1986).

III. Calvin Cycle Gene Organization, Expression, and Regulation in Eubacteria

Several excellent reviews on this topic have appeared recently (Tabita, 1994; Gibson and Tabita, 1996; Bommer et al., 1996; Gibson and Tabita, 1997; Kusian and Bowien, 1997; Shively et al., 1998). Mutant strains of *Rhodospirillum rubrum* (Falcone and Tabita, 1993), *Rhodobacter sphaeroides* (Gibson et al., 1991), and *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Bowien et al., 1993) defective for autotrophic growth continue to uncover new genes involved in Calvin cycle function and regulation. The structure and regulation of Calvin cycle operons and gene clusters has been investigated in several eubacteria. Among eubacteria, the most complete picture of gene organization exists for the β -proteobacterium *Ralstonia eutropha* and the cyanobacterium *Synechocystis* PCC6803. The structural organization of Calvin cycle genes in these organisms could not possibly differ more.

Ralstonia eutropha possesses the largest *cbb* operon characterized to date (Bowien et al., 1993; Bommer, 1996). With two exceptions (ribose-5-phosphate isomerase and triosephosphate isomerase) it encodes the entire pathway, and is transcribed as one polycistronic mRNA under the regulation of CbbR (Windhövel and Bowien, 1991), a member of the LysR family of transcriptional regulators (Tabita, 1994). The opposite extreme is realized in *Synechocystis*, where no two genes for Calvin cycle enzymes occur as neighbors in the genome (Kaneko et al., 1996a, 1996b). The *Synechocystis* genes are not separated by just a few hundred or a few thousand

bases, they are strewn around the 3.6 Mb genome with no recognizable pattern whatsoever. Even the *rbcL/rbcS* operon is disrupted, the genes for the two subunits being separated by an ORF of still unknown function, *rbcX*. *Synechocystis* possesses two genes homologous to *cbbR*, but neither the function of their products are known, nor whether Calvin cycle genes in *Synechocystis* form a regulon. Comparatively little is known about regulation, coordinated or otherwise, of cyanobacterial Calvin cycle genes (Beuf et al., 1994; Li and Tabita, 1994; Gibson and Tabita, 1996; Xu and Tabita, 1996).

In *Xanthobacter flavus*, *cbb* genes are distributed across at least two operons, the *gap-pgk* cluster is not contiguous with the *cbb* operon, but it is part of a *cbb* regulon under CbbR control (Meijer et al., 1996). A second *cbb* operon is present on a large plasmid in *Ralstonia* that is nearly identical to the chromosomal operon (Bowien et al., 1993). The *cbb* operons studied from *Ralstonia* (**β -proteobacteria**), *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Xanthobacter flavus*, and *Nitrobacter vulgaris* (all **α -proteobacteria**) show very little conservation of gene order across species, other than the fact that *cbbR* is usually transcribed, as in *Ralstonia*, on the opposite strand from a divergent promoter (Gibson and Tabita, 1996). Given the dispersed nature of the *Synechocystis* genes, it is conceivable that either these operons were assembled in independent lineages from an ancestrally dispersed state, or that fragmentation of an ancestral operon has occurred in *Synechocystis*, accompanied by rearrangements in proteobacteria. Visible rearrangement of *cbb* genes in **α -proteobacteria** suggests that considerable structural reorganization of *cbb* operons has occurred in these genomes during evolution. There have been reports of Calvin cycle specific activities in halophilic archaeabacteria (Rawal et al., 1988; Altekar and Rajagopalan, 1990; Rajagopalan and Altekar, 1994) but the enzymes have not been characterized in detail.

A general picture of higher level control of signal transduction and gene regulation for the Calvin cycle and its integration into the general metabolism of photosynthetic eubacteria is beginning to emerge. The presumably top level of hierarchy involves the RegA/RegB (PrrA/PrrB) two-component sensor-kinase system (Joshi and Tabita, 1996). This system appears to integrate the control, expression and feedback of regulons for photosystem biosynthesis (Sganga and Bauer, 1992; Eraso and Caplan, 1994;

Mosley et al., 1994; Allen et al., 1995), nitrogen metabolism (*nif*-system), and CO_2 fixation (Qian and Tabita, 1996; Joshi and Tabita, 1996). The regulatory cascade is apparently influenced by the redox state of the cells, the level of oxygen, and the presence of various carbon and nitrogen sources (Joshi and Tabita, 1996). Since precisely these factors (redox state, oxygen, carbon and nitrogen) are known to have dramatic and, in some cases, interdependent effects on plant metabolism and nuclear gene expression (Turpin and Weger, 1990; Chen et al., 1993; Escoubas et al., 1995; Kozaki and Takeba, 1996; Wingsle and Karpinski, 1996; Karpinski et al., 1997), these findings from bacterial systems may have a degree of model character for understanding related phenomena in eukaryotic systems, where the molecular basis of regulation is less thoroughly understood. Although it seems unlikely at first sight that these same prokaryotic molecular components will be found to be involved in plant signal transduction, the principles of regulatory response implemented by eukaryotic signaling/regulation machinery may ultimately prove to be very similar. A remarkable study recently provided strong evidence that some components for rapidly transducing redox signals in higher plants perceive the redox state of the plastoquinone pool directly in the thylakoid membrane (Pfannschmidt et al., 1999). Whether or not nuclear encoded bacterial two-component systems, which are still encoded in some chloroplast genomes (Stoebe et al., 1998; Martin et al., 1998), might be involved in such processes, is an attractive question.

IV. Calvin Cycle Expression in Plants

A. Quantification of Activities

Regulation of the Calvin cycle enzyme expression has often been monitored using Rubisco as a marker for the pathway. PRK, TKL RPI and RPE may fulfill the same purpose, because they appear to be localized in chloroplasts exclusively as well (Schnarrenberger et al., 1995). The other enzymes may fulfill functions in other pathways. For example PGK, GAPDH, TPI, aldolase and FBPase are involved in the gluconeogenetic and partially in the glycolytic reaction sequence, and the oxidative pentose phosphate pathway relies on many enzymes of the Calvin cycle (Schnarrenberger et al., 1995). Expression studies of

Calvin cycle enzymes that possess cytosolic homologues require not only measurement of total activities in crude extracts but also quantification of the amount of activities attributable to chloroplast and cytosolic isoenzymes. This is particularly necessary for the gluconeogenetic enzyme activities PGK, GAPDH, TIM, aldolase and FBPase isoenzymes, which may be separated by ion-exchange chromatography but not by gel filtration. In special cases, it is also possible to distinguish cytosol- and chloroplast-specific activities by virtue of their different substrate specificity for NADH and NADPH, as in the case of GAPDH, or by their differential response to pH, sulphydryl reagents, and Mg^{2+} , as for FBPase. But compartmentation of plant carbohydrate metabolism is not an evolutionarily conserved property across species (Schnarrenberger et al., 1990), and within a given plant it varies across developmental stages and tissues. Worse yet, across species, completely different enzymes are involved that must be assayed by different means, for example class I and class II Calvin cycle aldolase (Gross et al., 1994; Plaumann et al., 1997; Flechner et al., 1999). Thus, Rubisco is a valuable marker for regulation of Calvin cycle expression, but other enzymes may show very different regulation patterns, and sweeping generalizations to the effect that, beyond the Calvin cycle, 'plants' have this, that or the other pathway of sugar phosphate metabolism in this or that compartment are not possible.

The chloroplast activities of PGK, GAPDH, aldolase and FBPase in green leaves usually account for about 90% of the total activity (Heber et al., 1963; Latzko et al., 1974; Krüger and Schnarrenberger, 1983; Schnarrenberger and Krüger, 1986; Lebherz et al., 1984; Köpke-Secundo et al., 1990; McMorrow and Bradbeer, 1990), chloroplast TPI accounts for only about 50% of the total activity (Kurzok and Feierabend, 1984). For other isoenzyme activities of starch metabolism and the oxidative pentose phosphate pathway in green leaves, the cytosolic isoenzyme appears to account for most of the prevalent activity (Schnarrenberger, 1987). It appears that the activities of the regenerative part of the Calvin cycle (RPI, RPE, TKL) may not require isoenzyme separation in most cases, since they are probably located exclusively in the chloroplasts (Schnarrenberger et al., 1995), except in some specialized tissues and species (e.g. TKL in *Craterostigma*: Bernacchia et al., 1995). For these three enzymes no chloroplast/cytosol isoenzymes

can be separated in spinach leaves (Schnarrenberger et al., 1995). It is well known that various Calvin cycle enzymes vary considerably in their maximal activities among higher plants, various algae and eubacteria (Smillie, 1963; Heber et al., 1967; Latzko and Gibbs, 1968; Latzko and Gibbs, 1969; Kelly and Latzko, 1979), as do activities in other pathways.

B. Expression Studies of Enzyme Activities and Transcription

The literature on expression of Calvin cycle enzymes is vast. One of the most widely studied aspects is the increase of enzyme activity and mRNA levels in response to light. The influence of light on expression of genes involved in photosynthesis has been reviewed (Chory et al., 1996; Kloppstech, 1997). Phytochromes (Schopfer, 1977; Pratt, 1995), blue light, and UV-receptors play important roles in this regulation, that ultimately reaches genes for many Calvin cycle enzymes. It is also well-known that the glycolytic, cytosolic isoenzymes of several Calvin cycle activities are, as a rule, not responsive to light and are induced under anaerobic conditions (Sachs, 1994; Kennedy et al., 1992).

Complete cDNAs have been characterized for several Calvin cycle enzymes from several sources, and all of the Calvin cycle enzymes from spinach chloroplasts have been cloned (Flechner et al., 1996; Martin et al., 1996a). Rubisco gene expression has been studied in by far the greatest detail of all of the Calvin cycle enzymes. Transcription factors involved specifically in *rbcS* gene expression, e.g. GT-1 (Lam and Chua, 1990; Sarokin and Chua, 1992) and GT-2 (Gilmartin et al., 1992) have been characterized. In general, expression of Calvin cycle genes in plants, particularly in etiolated seedlings, is stimulated by light. This can occur through elevated transcription, or, as recent studies of the *Cen* gene in *Chlamydomonas* mutants have shown, post-transcriptionally at the level of mRNA stability (Hahn et al., 1996). Various cis elements have been described for Calvin cycle genes from different sources, including the WF-1 element upstream of the genes for SBPase and FBPase of wheat (Miles et al., 1993), the Gap and AE boxes upstream of the *Arabidopsis GapA* and *GapB* genes (Conley et al., 1994; Kwon et al., 1994; Park et al., 1996), and an octameric motif in the first intron of the maize *GapA1* gene (Donath et al., 1995; Köhler et al., 1996). The FBPase promoter also contains a DNA binding site for the GT-1 factor

which mediates light activation of expression through phytochrome in promoters of oat and rice (Lloyd et al., 1991b). In addition to the small subunit of Rubisco, for which numerous gene structures are known (Wolter et al., 1988; DeRocher et al., 1993; Fritz et al., 1993), several higher plant Calvin cycle gene structures have been characterized. These include *GapA* from maize (Quigley et al., 1988), *Arabidopsis* (Shih et al., 1992), and several other sources (Kersanach et al., 1994), SBPase and FBPase from wheat (Raines et al., 1988, 1992), aldolase from rice (Tsutsumi et al., 1994) and *Chlamydomonas* (Pelzer-Reith et al., 1995), and PRK from wheat (Lloyd et al., 1991a).

In studies of the transcript levels of all Calvin cycle enzymes in various tissues during spinach development (Henze, 1997), it was observed that most mRNAs were present in all green leaf tissue in roughly the same relative quantities, with the exception of *rbcS* mRNA, that was present at roughly 10-fold higher steady-state levels. In etiolated cotyledons, all mRNA levels were reduced at least 10- to 20-fold relative to green leaves. Upon illumination, mRNAs for *rbcS*, aldolase, SBPase and PRK increased within 2 h after illumination, followed by the other mRNAs. After 24 h of illumination, mRNA levels were indistinguishable from those in green leaf tissue. It is still too early to tell whether genes of the Calvin cycle in higher plants are regulated as a unit, or whether their activity is simply modulated as part of a general greening response of the gene regulatory machinery to light and redox state. In the red alga *Galdieria sulphuraria*, that can grow heterotrophically or autotrophically (Gross et al., 1999), isoenzymes of many Calvin cycle activities (aldolase, RPE, PGK, FBPase, and GAPDH) are specifically induced during the transition from heterotrophic to autotrophic growth (J. Girnus, C. Schnarrenberger, W. Gross, unpublished)

There are far too many reports involving expression studies of Calvin cycle genes and enzyme activities to permit thorough review. In Table 1 we have tried to provide access to some of that literature, including many studies that cannot be found by computer-searching (and omitting many studies that can). Rubisco is well known for its inducibility by phytochrome. The effects of phytochrome and other light receptors on the remaining Calvin cycle enzymes have been studied in less detail and in many of the early studies, the quantification of chloroplast vs.

cytosol enzyme activities was not considered. Table 1 is by no means complete, but we hope that readers find parts of it useful.

C. Gene Regulation Through High CO₂, Sugar Sensing, and Redox State

A sugar-sensing system has been discussed in plants that may be able to significantly influence gene expression (Sheen, 1990, 1994; Koch, 1996; van Oosten and Besford, 1996; Jang and Sheen, 1997; Chapter 10, Graham and Martin). Sugars like glucose, fructose and sucrose cause strong repression of genes for photosynthetic functions, resulting in e.g. reduction of photosynthetic pigments and Calvin cycle enzymes while other sugars are largely ineffective. Glucose feeding can reduce the steady-state mRNA levels of several Calvin cycle genes in wheat, including FBPase, SBPase, PGK and *rbcS* (Jones et al., 1996). Among the Calvin cycle enzymes, activity and protein of Rubisco decline steadily within several days. This was demonstrated in cell suspension cultures of *Chenopodium rubrum* and in intact tobacco and potato leaves which were cold-girdled for 12 h to reduce assimilate export (Krapp et al., 1993), detached spinach leaves fed with glucose through the petioles (Krapp et al., 1991), mesophyll cells of tobacco (Criqui et al., 1992) and by comparing green and bleached leaves of transgenic tobacco plants expressing a yeast-derived invertase in the apoplast (Stitt et al., 1990). In some of these systems it was shown that glucose treatment resulted in the repression of these and other photosynthesis-related genes. In cell suspension cultures of *Chenopodium rubrum*, *rbcS* mRNA levels are reduced within several hours and run-on experiments with isolated nuclei indicated that also the synthesis of *rbcS* is reduced, as is ³⁵S-methionine incorporation into the Rubisco protein, indicating an inhibition of de-novo synthesis (Krapp et al. 1993). In glucose-fed tobacco protoplasts and leaf discs, *rbcS* transcript levels are reduced within hours upon glucose addition, but *rbcL* transcript levels are reduced at a much slower rate (Criqui et al., 1992). Thus, regulation takes place primarily at a transcriptional level. Information on glucose repression of other Calvin cycle enzymes is limited to FBPase and GAPDH, both of which, like Rubisco, show similarly declining activities in the presence of glucose (Stitt et al., 1990; Krapp et al. 1991, 1993).

Elevated CO₂ (about 1000 ppm) can also influence

gene expression (reviewed by von Oosten and Besford, 1996), including those for some Calvin cycle enzymes. For example, whole tomato plants grown at elevated CO_2 , relative to ambient CO_2 -grown plants, for 10 days showed reduced Rubisco activity in the second half of this period, probably accounting for the long-term decline in photosynthetic efficiency under high CO_2 (Yelle et al., 1989). *rbcS* transcript levels are greatly reduced in tomato plants within 4 days, while *rbcL* transcript levels decline less pronounced. This effect was enhanced in detached leaves, indicating repression by elevated internal glucose levels (van Oosten et al., 1994). On the contrary, low levels of CO_2 resulted in an overexpression of *rbcS* (Krapp et al., 1993). Besides Rubisco, also the activities of PGK and GAPDH were reduced under elevated CO_2 condition, though only in fully developed leaves (Besford, 1990). However, Krapp et al. (1991) observed less inhibition at high CO_2 than under ambient CO_2 at saturating irradiation and even less under low irradiation. It should be noted that any inhibition seen under these conditions might also be attributable to nitrogen limitation that can become apparent at increased growth rates (Kozaki and Takeba, 1996).

Van Oosten and Besford (1995) showed that transcript levels of plastid-encoded *rbcL* and other genes involved in photosynthesis (*psbA*, *psaAB*) were reduced in mature leaves by elevated CO_2 . Expression of nuclear genes associated with the Calvin cycle such as Rubisco activase are also reduced by elevated CO_2 (van Oosten et al., 1994). Nuclear encoded *rbcS* transcript levels were reduced in tomato plants exposed to high CO_2 , as were plastid-encoded *rbcL* transcript levels, though less markedly, and these effects could be simulated by sugar feeding (van Oosten and Besford, 1994). In terms of Rubisco content, tomato plants responded to high CO_2 in a manner similar to plants grown with low nitrogen supply (van Oosten et al., 1995). In bird-cherry trees grown under conditions where nutrients were not limiting, Rubisco activity decreased in response to high CO_2 (Wilkins et al., 1994). Clearly, there is an interdependence between CO_2 availability, nitrogen, redox state, sugar levels and light levels that influence gene expression. An *Arabidopsis* mutant defective in a gene that might be involved in integrating or transducing sugar-related signals was recently described (van Oosten et al., 1997). Furthermore, the redox state of the thylakoid membrane itself has been recently shown to regulate the transcription of plastid

genes involved in maintaining redox balance (Pfannschmidt et al., 1999), a process that certainly entails the Calvin cycle as the primary means for regenerating NADP^+ . The question of which and how many signaling pathways are involved in maintaining redox balance in a manner that affects the Calvin cycle is still open.

D. Regulation in Specific Systems

A system involving preferential breakdown of 70S chloroplast ribosomes on Calvin cycle enzymes was used in early studies, because it permitted the site of enzyme synthesis to be determined long before the coding capacity of chloroplast genomes had been determined (Feierabend and Schrader-Reichhardt, 1967). Nuclear-encoded enzymes are still synthesized on 80S ribosomes and imported to chloroplasts under permissive low (22 °C) temperatures or non-permissive high (32 °C) temperatures. Among the enzymes of sugar phosphate metabolism assayed, GAPDH, PGK, TPI, TKL, FBPase, RPI, PRK and aldolase were recovered in chloroplasts at non-permissive conditions (Feierabend and Brassel, 1977; Feierabend, 1979, 1986; Feierabend and Gringel, 1983; Kurzok and Feierabend, 1983, 1986; Otto and Feierabend, 1989), however, Rubisco was absent (Feierabend, 1979), transcripts of Rubisco were repressed as well (Winter and Feierabend, 1990).

Another well studied system of Calvin cycle expression is green and white leaf tissue of the ‘albostrians’ mutant of barley. This mutant shows a variegated pattern of white and green striped leaves with non-Mendelian inheritance (Hagemann and Scholz, 1962). Rubisco, GAPDH, aldolase, and FBPase were strongly reduced in white leaf tissue (Börner et al., 1976; Bradbeer and Börner, 1978; Boldt et al., 1992). In contrast, the cytosolic counterparts of the Calvin cycle enzymes, the enzymes of starch metabolism and the key enzymes of the oxidative pentose phosphate pathway were virtually unchanged (Boldt et al., 1992). Transcripts of Rubisco were totally repressed in white tissue but were enhanced in green tissue through phytochrome (Hess et al., 1991). The transcripts of chloroplast PRK, GAPDH, PGK, aldolase, and FBPase were repressed in white tissue, while those of cytosolic GAPDH and PGK were slightly enhanced (Hess et al., 1993, 1994; Boldt et al., 1994). The phenomenon is interpreted as the action of a plastid derived factor or signal which represses many (but not all) nuclear-

Table 1. Expression studies of Calvin cycle enzymes in various systems

Organism	Tissue	Factor	References
Phosphoglycerate kinase			
Wheat	Leaves	Light	Raines et al., 1988, 1989; Longstaff et al., 1989
	Leaves	Sugar	Jones et al., 1996
Tobacco	Leaves	Light	Bringloe et al., 1996
	Plants	CO ₂	Besford, 1990
Rye	Leaves	Heat bleaching	Otto and Feierabend, 1989; Feierabend, 1979; Kurzok and Feierabend, 1986
Barley	Leaves	Albostrians mutant	Boldt et al., 1992; Hess et al., 1994
<i>Euglena</i>	Cells	Light, auto/ heterotrophy	Smillie, 1963, 1968; Latzko and Gibbs 1996
Glyceraldehyde-3-phosphate dehydrogenase			
Mustard	Seedlings	Light	Cerff, 1973; Cerff and Quail, 1974
Radish	Cotyledons	Light	Schnarrenberger et al., 1975; Schmidt et al., 1987
<i>Arabidopsis</i>	Plants	Light	Conley et al., 1994; Kwon et al., 1994; Park et al., 1996; Conley and Shih, 1995; Dewdney et al., 1993
		Heat, anoxia, sugar	Yang et al., 1993
	Mutants	Light	Conley and Shih, 1995
		Norflurazon	Conley and Shih, 1995
Maize	Callus	Light	Donath et al., 1995; Köhler et al., 1995
		Anoxia	Russel and Sachs, 1989; Köhler et al., 1996a,b
many Tobacco, <i>Arabidopsis</i>	Leaves	Light	Cerff and Kloppstech, 1982
	Callus	Light	Shih and Goodman, 1988
Rice	Seedlings	Anoxia	Ricard et al., 1989
Spinach	Leaves	Glucose	Krapp et al., 1991, 1993
Sorghum	Leaves	Light	Oelmüller and Mohr, 1985
Tobacco	Plants	Invertase transgenics	Stitt et al., 1990
Tomato	Plants	CO ₂	Besford, 1990
Rye	Leaves	Heat bleaching	Otto and Feierabend, 1989; Feierabend, 1979
Barley	Leaves	Albostrians mutant	Bradbeer et al., 1979; Bradbeer and Börner, 1978; Boldt et al., 1992; Hess et al., 1994
Ice plant	Leaves	Salt stress	Oestrem et al., 1990; Vernon and Bohnert, 1992; Vernon et al., 1993
Tobacco	Plants	Antisense	Price et al., 1995
	Cells	Light, autotrophy	Smillie, 1963, 1968; Dockerty and Merrett, 1979; Latzko and Gibbs, 1969
<i>Euglena</i>	Cells	Light, mutants	Schmidt and Lyman, 1974
Triosephosphate isomerase			
Rye	Leaves	Light	Kurzok and Feierabend, 1986
	Leaves	Heat bleaching	Feierabend, 1979, 1986; Kurzok and Feierabend, 1986
Barley	Leaves	Albostrians mutant	Boldt et al., 1992
	Cells	Autotrophy	Mo et al., 1973
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963, 1968; Latzko and Gibbs, 1969
Fructose-1,6-bisphosphate aldolase			
Spinach	Cotyledons	Light	Krüger and Schnarrenberger, 1985
Maize	Cotyledons	Anoxia	Kelley and Freeling, 1984; Kelley and Tolan, 1986; Dennis et al., 1988
Pea	Leaves, stem, root	Light, Anoxia	Pelzer-Reith and Schnarrenberger, unpublished
Rice/tobacco	Leaves	Light	Kagaya et al., 1995; Nakamura et al. 1997
Rice	Seedlings	Calcium	Nakamura et al., 1996
Rye	Leaves	Heat bleaching	Feierabend, 1979
Barley	Leaves	Albostrians mutant	Boldt et al., 1992, 1994; Hess et al., 1994
Castor bean	Endosperm	Development	Nishimura and Beevers, 1981

Table 1. (Continued)

Organism	Tissue	Factor	References
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963, 1968; Latzko and Gibbs, 1969; Mo et al., 1973;
	Cells	Endogenous rhythms	Karlan and Russel, 1976; Dockerty and Merrett, 1979
	Cells	Light, mutants	Pelzer-Reith et al., 1994a; Malik, 1997
Fructose-1,6-bisphosphatase			
Barley, pea	Seedlings	Light	Huffacker et al., 1966; Keller and Huffacker, 1967; Graham et al., 1966
Rye, pea	Leaves	Light	Feierabend, 1966; Feierabend and Pirson 1966; Graham et al., 1968
Wheat	Leaves	Light	Raines et al., 1988; Miles et al., 1993; Lloyd et al., 1991b
	Leaves	Sugar	Jones et al., 1996
Potato	Leaves, stem, tuber, root	Overexpression	Kossmann et al., 1992
	Plants	Antisense	Kossmann et al., 1994
Maize	Leaves	Chilling	Kingston-Smith et al., 1997
Tobacco	Plants	Antisense	Bilger et al., 1995; Fisahn et al., 1995
Spinach	Leaves	Glucose	Krapp et al., 1991, 1993
Tobacco	Plants	Invertase transgenics	Stitt et al., 1990
Tomato	Leaves	Chilling	Brüggemann et al., 1994
Tomato	Fruits	Ripening	Büker et al., 1998
Rye	Leaves	Heat bleaching	Feierabend, 1979, 1986
	Leaves	Albostrians mutant	Boldt et al., 1992; Hess et al., 1993
Castor bean	Endosperm	Development	Nishimura and Beevers, 1981
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963; Latzko and Gibbs, 1969
Ribose-5-phosphate isomerase			
Rye, pea	Leaves	Light	Feierabend, 1966; Feierabend and Pirson 1966; Graham et al., 1968
Barley, pea	Seedlings	Light	Huffacker et al., 1966; Keller and Huffacker, 1967; Graham et al., 1966
Rye	Leaves	Heat bleaching	Feierabend, 1979, 1986
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963; Latzko and Gibbs, 1969
Horsegram	Leaves	Salinity	Reddy et al., 1998
Ribulose-5-phosphate 3-epimerase			
Potato	Leaves	Light	Teige et al., 1995
<i>C. reinhardtii</i>	Cells	Mutant	Hahn et al., 1996
<i>Euglena</i>	Cells	Light, autotrophy	Latzko and Gibbs, 1969
Transketolase			
Rye, pea	Leaves	Light	Feierabend, 1966; Feierabend and Pirson 1966; Graham et al., 1968
Potato	Leaves	Light	Teige et al., 1996
Craterostigma	Leaves	Dessication	Bernacchia et al., 1995
Rye	Leaves	Heat bleaching	Feierabend, 1979; Feierabend and Gringel, 1983
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963; Latzko and Gibbs, 1969
Sedoheptulose-1,7-bisphosphatase			
Wheat	Leaves	Light	Miles et al., 1993
	Leaves	Sugar	Jones et al., 1996
<i>Euglena</i>	Cells	Light, autotrophy	Latzko and Gibbs, 1969
<i>Arabidopsis</i>	Leaves	Light	Willingham et al., 1994
<i>C. reinhardtii</i>	Cells	Light	Hahn et al., 1998
Tobacco	Leaves	Antisense	Harrison et al., 1998a

Table 1. (Continued)

Organism	Tissue	Factor	References
Phosphoribulokinase			
Barley, pea	Seedlings	Light	Huffacker et al., 1966; Keller and Huffacker, 1967; Graham et al., 1966
Wheat	Leaves	Light	Raines et al., 1988, 1989; Longstaff et al., 1989
	Leaves	Senescence	Kamber and Feller, 1998
Rye	Leaves	Heat bleaching	Feierabend, 1979, 1986
	Leaves	Albostrians mutant	Bradbeer and Börner, 1978; Hess et al., 1994
Ice plant	Leaves	Salt stress	Michalowski et al., 1992
Tobacco	Plants	Antisense	Gray et al., 1995; Paul et al., 1995; Banks et al., 1999
	Leaves	Hydroxyl radicals	Shen et al., 1997
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963; Latzko and Gibbs, 1996
	Cells	Light, mutants	Schmidt and Lyman, 1974
Rubisco			
Bean	Leaves	Chloramphenicol	Ireland and Bradbeer, 1971
Spinach	Leaves	Glucose	Krapp et al., 1991
Radish	Cotyledons	Phytochrome	Fourcroy, 1986
Rye, pea	Leaves	Light	Feierabend, 1966; Feierabend and Pirson 1966; Graham et al., 1968
	Leaves	Light	Schmidt et al., 1987
Rye	Leaves	Sugar	Jones et al., 1996
Pea	various	Heat bleaching	Feierabend, 1979, Winter and Feierabend, 1990
<i>Chenopodium</i>	Cell culture	Blue light	Fluhr et al., 1986
Potato	Cell culture	Cold girdling	Krapp et al., 1993
Maize	Bundle sheath	Cold girdling	Krapp et al., 1993
<i>Lycopersicon</i>	Plants	Light	Purcell et al., 1995
Tobacco	Protoplasts, leaf discs	CO ₂	Yelle et al., 1989; van Oosten et al., 1994; Krapp et al., 1993
		Glucose	Criqui et al., 1992
Tobacco	Plants	Invertase transgenic	Stitt et al., 1990
	Leaves	Rubisco activase transformant	Mate et al., 1993; Jiang et al., 1994
Tobacco	Plants	Antisense	Rodermel et al., 1988; Quick et al., 1993, 1993; Hudson et al., 1992; Fichtner et al., 1993; Gunasekera and Berkowitz, 1993; Lauerer et al., 1993; Masle et al., 1993; Krapp et al., 1994; Evans et al., 1994; Sicher et al., 1994; Stitt and Schulze 1994; von Caemmerer et al., 1994; Andrews et al., 1995; Jiang and Rodermel, 1995; Furbank et al., 1994
<i>Arabidopsis</i>	Plants	Rubisco activase transgenics	Eckardt et al., 1997
	Cell cultures	Light	Kaldenhoff et al., 1994
Barley	Leaves	Albostrians mutant	Börner et al., 1976; Hess et al., 1991
Castor bean	Endosperm	Development	Nishimura and Beevers, 1981
<i>Euglena</i>	Cells	Light, autotrophy	Smillie, 1963; Latzko and Gibbs, 1969
	Cells	Light, mutants	Schmidt and Lyman, 1974

encoded plastid proteins in the nucleus (Boldt et al., 1990; Hess et al., 1994; Hess et al., 1997). This factor/signal also represses most genes of the glycolate pathway in plastids and peroxisomes, indicating functional unity of repression of photosynthetic functions (Boldt et al., 1997). Hahn et al. (1996) recently described a nuclear gene in *Chlamydomonas reinhardtii* that posttranscriptionally

affects mRNA levels for several chloroplast proteins, including RPE.

Expression of Calvin cycle genes has been studied in the facultative CAM plant *Mesembryanthemum crystallinum* (ice plant). GAPDH mRNA accumulates in response to salt stress (Vernon and Bohnert, 1992; Vernon et al., 1993), whereby PRK expression is reduced by salt stress (Michalowski et al., 1992).

Transcript levels for cytosolic GAPDH increase during the transition from C3 to CAM metabolism (Ostrem et al., 1990). In C4 plants, only the bundle sheath cells contain a complete set of Calvin cycle enzymes (see also Chapter 18, Furbank et al.).

Enzymes of the Calvin cycle can also be found in plastids of non-green tissues. An extreme example is the endosperm tissue of developing and germinating castor bean which never greens (Plaxton, 1996). Many of the plastidic isoenzymes of the Calvin cycle are present in this tissue and show an expression pattern following the typical fat-to-sugar conversion with maximum activities 5 days after germination (Nishimura and Beevers, 1981). The question is whether these enzymes really function in CO_2 fixation or whether they are due to a leaky expression. Alternatively, they could function in the oxidative pentose phosphate cycle which can provide NADPH for nitrate reduction. This has been implied by work of Emes and Fowler (1978) on TKL and transaldolase. A cytosolic class I aldolase with specificity for both F1,6BP and Su1,7BP was found in carrot storage roots but the plastidic homolog was missing (Moorhead and Plaxton, 1990). A general consideration of metabolism in chromoplasts was summarized by Camara et al (1995).

E. Calvin Cycle Enzymes and Expression in Euglena gracilis

Euglena gracilis can grow autotrophically and heterotrophically on various substrates (Kitaoka et al., 1989; Brandt and Wilhelm, 1990). A first screen for cytosolic and chloroplast enzyme activities of sugar phosphate metabolism was presented by Smillie (1963). Enzyme levels related to photosynthesis and degradative reactions like glycolysis seem to be antagonistically regulated under autotrophic and heterotrophic growth conditions, respectively. Most enzyme activities of the Calvin cycle increase during greening though to various degrees and decrease after transfer to heterotrophic conditions (Latzko and Gibbs, 1969; Kitaoka et al., 1989).

Enzyme activities in *Euglena gracilis* may also be regulated by light. This phenomenon was dissected into a blue- and a red-light reaction by the use of mutants (Schmidt and Lyman, 1974): In wild-type cells blue light increased the activities of Rubisco, PRK, chloroplast GAPDH, and chloroplast aldolase twice as effectively as red light. Mutant Y9ZNa1L had no chlorophyll and showed a blue-light but no

red-light effect. Mutant Y11P22DL had small amounts of chlorophyll and showed the same activity in red and blue light. Mutant W14ZNa1L had no chloroplasts and no Rubisco but the same activity of GAPDH, PRK and aldolase as dark-grown wild-type cells, but this activity was not increased by light. In other mutants (W3BLU and W8BHL) with no plastid DNA small amounts of cytosolic class I aldolase were recorded (Karlan and Russell, 1976). Chloroplast development in *Euglena gracilis* is sensitive to glucose repression. When heterotrophic cells are transferred to autotrophic conditions, chloroplast development starts from proplastids. The presence of glucose inhibits greening and synthesis of Rubisco (Reinbothe et al., 1991a). During dedifferentiation from chloroplasts to proplastids, on the other hand, Rubisco synthesis ceased immediately upon transfer to the dark in the presence of glucose (Reinbothe et al., 1991b).

Chloroplast and cytosolic GAPDH are both present in *Euglena gracilis* cells, have been purified and show no immunochemical cross-reaction (Grisson and Kahn, 1974; Theiss-Seuberling, 1984). Chloroplast GAPDH is activated by dithiothreitol and/or thioredoxin (Theiss-Seuberling, 1981). During chloroplast development of *Euglena gracilis* in the light NADP-GAPDH increases in activity (Hovenkamp-Obbema and Stegwee, 1974). Both enzymes are encoded by nuclear genes and possess several unusual sequence attributes (Henze et al., 1995).

The aldolase isoenzymes of *Euglena gracilis* belong to the class I and class II type and are compartmented in the chloroplasts and in the cytosol, respectively (Rutter, 1964; Mo et al., 1973; Pelzer-Reith et al., 1994b; Plaumann et al., 1997). Under autotrophic conditions, the chloroplast enzyme is more active than the cytosolic enzyme (Mo et al., 1973; Karlan and Russel, 1976). This pattern is reversed during growth under heterotrophic conditions (Mo et al., 1973). While chloroplast and cytosolic aldolase of higher plants differ little in their biochemical parameters (Anderson and Pacold, 1972; Buckowiecki and Anderson, 1974; Krüger and Schnarrenberger, 1983; Lebherz et al., 1984), the cytosolic class II aldolase of *Euglena* has a much higher $K_m(\text{F}1,6\text{BP})$ value and a broader pH optimum than class I aldolase (Pelzer-Reith et al., 1994b). Both aldolases of *Euglena gracilis* show endogenous rhythmicity in the light and in the dark (Pelzer-Reith et al., 1994a; Malik, 1997). The expression of transcripts and the enzymes appears to be regulated

posttranscriptionally (Malik, 1997). Chloroplast and cytosolic isoenzymes of TPI were separated from *Euglena gracilis* (Mo et al., 1973). The chloroplast type A enzyme is high in autotrophic and low in heterotrophic cells while the cytosolic type B isomerase predominates under heterotrophic growth conditions. An antagonistic regulation under autotrophic and heterotrophic growth conditions is also implied for the chloroplast and cytosolic FBPase when measured at pH 8.5 and 6.9, respectively (Latzko and Gibbs, 1969).

Structure and expression of nuclear genes for chloroplast proteins in *Euglena* are unusual in several respects. Many encode polyprotein precursors of chloroplast proteins (Houlné and Schantz, 1988, 1993). An example of such a polyprotein is *rbcS* in *Euglena*. The nuclear gene is transcribed as an mRNA encoding eight nearly identical concatenate small subunits that are translated as a 140 kDa cytosolic polyprotein, all eight subunits are imported into chloroplasts with the aid of a single transit peptide, and then proteolytically processed from the polyprotein into individual subunits for Rubisco assembly (Chan et al., 1990). This unusual polyprotein organization appears to be restricted to some nuclear-encoded genes in protists of secondary symbiotic origin, i.e. protists that acquired their plastids by engulfing photosynthetic eukaryotes rather than prokaryotes. [This notion was first suggested for *Euglena* (Gibbs, 1978) and subsequently demonstrated to be the case for several photosynthetic protists (Maier, 1992; McFadden et al., 1994; Melkonian, 1996; Van de Peer et al., 1996; McFadden et al., 1997)]. Such plastids are surrounded by three or more membranes instead of two, and precursor import is therefore more complex, involving ER-processing of a signal peptide prior to chloroplast uptake in the case of *Euglena* (Kishore et al., 1993; Sulli and Schwartzbach, 1996). It is likely that a number of *Euglena*'s nuclear genes for chloroplast proteins stem from the secondary symbiont and were therefore transferred twice in evolution: once from cyanobacteria to the chlorophyte nucleus, and once more from the chlorophyte nucleus to the nucleus of the *Trypanosoma*-like host (Henze et al., 1995; Plaumann et al., 1997). *Euglena*'s nuclear genes for Calvin cycle GAPDH (Henze et al., 1995), aldolase (Plaumann et al., 1997), TKL and TPI (W. Martin, unpublished) are not encoded as polyproteins, indicating that this unusual organization is restricted to certain transcripts. *Euglena*'s nuclear genes for

rbcS and cytosolic GAPDH contain a novel class of highly structured introns that have not been described from any other eukaryotes (Henze et al., 1995; Tessier et al., 1995). Also, spliced leader sequences are found at the 5' end of many of *Euglena*'s nuclear transcribed mRNAs (Tessier et al., 1991). Such spliced leaders have been implicated in the RNA-processing of polycistronically transcribed eukaryotic operons found in the euglenozoan lineage and in *Caenorhabditis* (Hirsch, 1994).

In other photosynthetic protists, little is known at the molecular level about Calvin cycle enzymes and gene structure, but this can be expected to change in the future since these organisms are turning up quite a number of surprising findings. For example the dinoflagellates *Gonyaulax* (Morse et al., 1995) and *Symbiodinium* (Rowan et al., 1996) use class II Rubisco in their Calvin cycle. Moreover, those class II Rubisco genes are nuclear encoded—and in *Symbiodinium* as a polyprotein, as in the case of *Euglena*'s *rbcS*. Other photosynthetic protists seem to lack chloroplast- and cytosol-specific isoenzymes of sugar phosphate metabolism. *Chlamydomonas reinhardtii* is an extreme example, since this alga has no cytosolic isoenzymes of sugar phosphate metabolism for at least eight enzyme activities, among them aldolase (Schnarrenberger et al., 1994), suggesting that the general compartmentation of carbohydrate metabolism may be surprisingly variable across protists.

V. Enzyme Interactions and Multienzyme-like Complexes

In 1970, Rutner noted that ‘...there are now several well-documented cases of multi-enzyme complexes (e.g. fatty acid synthase, pyruvic dehydrogenase [...]'), there is a tendency to implicate them in other sequential biochemical reactions’ (Rutner, 1970) and delineated some straightforward mass-activity stoichiometric difficulties encountered when such complexes are considered in the context of the Calvin cycle. Since that time, there have been many reports that some enzymes of the Calvin cycle may form multienzyme-like complexes, findings that have often been discussed in the context of metabolic channeling of intermediates. In enzymological studies prior to 1980, these complexes were rarely observed. The majority of reports deal with complexes isolated from pea and spinach chloroplasts and from green

algae, similar associations between Calvin cycle enzymes have not been observed in any cyanobacteria or photosynthetic proteobacteria. The reports differ substantially with respect to the number and nature of protein-protein interactions observed. There are still many open questions in this area, and there is currently no consensus concerning the nature, function or significance of such complexes. Various complexes have been isolated by ultracentrifugation in sucrose gradients, by exclusion chromatography in the presence of stabilizing compounds such as glycerol, and by ion-exchange chromatography. The multienzyme-like complexes should be distinguished from multimeric forms of individual enzymes which themselves can aggregate in purified form, for example GAPDH (Baalmann et al., 1994), FBPase (Grotjohann, 1997) or RPE (Teige et al., 1998).

Several reports concern complexes consisting of two or three enzymes. A complex containing Rubisco, RPI (90 kDa) and PRK (54 kDa) was reported from pea leaves (Sainis and Harris, 1986) that catalyzed R5P-dependent CO_2 fixation in the presence of ATP, and contained about 4–5% of RPI and PRK activities in the complexed form. In a similar complex from spinach, 75% of PRK and 7% of RPI were found to associate and copurify with Rubisco (Sainis et al., 1989). The ratio of PRK to Rubisco was estimated to be 1:1 to 1:3. In another report, a complex of PRK with GAPDH was isolated from *Scenedesmus obliquus* (Nicholson et al., 1987). The stoichiometry was estimated to be $\text{GAPDH}_8\text{PRK}_6$ with a (too low) molecular mass of 560 kDa. Also, PRK and GAPDH were found to form a complex coexisting with the free enzyme forms in spinach (Clasper et al., 1991). A novel, 12 kDa chloroplast protein (CP12) has recently been described from higher plants that shares high sequence similarity with the CTE of the GapB subunit (Pohlmeyer et al., 1996). CP12 interacts with GAPDH in affinity chromatography and with PRK in the yeast two-hybrid system. Under oxidizing conditions, CP12 interacts with both proteins to form ~600 kDa complexes and has been suggested to be involved in regulation (Wedel et al., 1997; Wedel and Soll, 1998).

A complex was isolated from *Chlamydomonas reinhardtii* consisting of $\text{PRK}_2\text{GAPDH}_2$ with an average molecular mass of 460 kDa (Avilan et al., 1997; Lebreton et al., 1997), equal to the sum of individual masses of the free enzymes. The dissociation of the complex can be achieved by reducing agents like DTT, NAD(P)H, reduced

ferredoxin or reduced thioredoxin, accompanied by an increase particularly in PRK activity. PRK is inactive in the oxidized form (see Section VI) and gained some activity during complex formation with GAPDH in a manner similar but not identical to chaperonin action (Lebreton et al., 1997). However, this increase corresponds to only a few percent of the activity of the reduced form present in the light. The subsequent dissociation of the complex by reducing agents causes a conformation change in PRK, another 20-fold increase in PRK activity with a 4-fold decrease in $K_{m(\text{R5P})}$ and a 2-fold decrease in $K_{m(\text{ATP})}$. During complex dissociation, GAPDH showed a relative increase in favor of NADP^+ over NAD^+ activity (Avilan et al., 1997). On the other hand, the complex could form spontaneously, upon addition of NAD^+ or oxidized glutathione (Avilan et al., 1997; Lebreton et al., 1997). The association between GAPDH and PRK in *Chlamydomonas* involves two enzymes that catalyze non-consecutive steps in the pathway and the complex is present under dark conditions where there should be no Calvin cycle activity. Thus, the complex is unlikely to be involved in channeling in the classical sense (Gontero et al., 1994; Ricard et al., 1994).

There have been reports of larger Calvin cycle multienzyme-like complexes involving several additional enzymes. The first such larger complex contained PRK, Rubisco, PGK, and GAPDH and was reported by Müller (1972), who recognized that the fragile complex is dissociated by NADPH or ATP and that the enzymes involved are activated during dissociation. The complex had a molecular mass of 400 kDa, less than the value of 700 to 800 kDa expected. Sasajima and Yoneda (1974) found that RPI, TKL and RPE copurify. More recent reports have detected complexes with an M_n in the range of 500 to 1000 kDa (Gontero et al., 1988; Gontero et al., 1993; Rault et al., 1993; Sainis and Srinivasan, 1993; Süß et al., 1993, 1995). The enzymes involved in these complexes and their stoichiometry differ in individual laboratories, the function is generally interpreted as metabolic channeling.

Gontero et al. (1988) found a complex consisting of Rubisco, RPI, PRK, PGK and GAPDH. The complex was fairly stable and homogeneous during ultracentrifugation. DTT increased the activity of the individual enzymes. The complex catalyzed CO_2 fixation with R5P, ATP, NADPH and CO_2 . The molecular mass of the complex was estimated as 520 kDa and the ratio of the individual enzymes inferred

to be 2PRK:2GapA:2GapB:2RbcS:4RbcL, in addition to some RPI and PGK (Rault et al., 1993). Because the molecular mass of the individual enzymes is anticipated to be much larger than that of the complex, it was suggested that Rubisco might exist in an L₄S₂ form (Rault et al., 1993), differing from that of the crystallized enzyme (Shibita et al., 1996). If the complex is subjected to SDS-PAGE, several protein bands are observed corresponding to the bands of the individual enzymes.

A complex isolated from spinach contained Rubisco, PRK, GAPDH, SBPase, ferredoxin-NADP reductase (FNR) and chaperonin 60 (Süss et al., 1993) and fixed CO₂ from R5P. The complex was stable at low salt conditions (<200 mM KCl) and dissociated under high salt conditions (>250 mM KCl). Ammonium sulfate (1 M) or pH 4.5 completely dissociated the complex. All enzymes of the complex were found almost exclusively attached to the outer surface of thylakoid membranes during gold immunolabeling except Rubisco, which showed also stromal localization (Süss et al., 1993a, 1993b; Adler et al., 1993). Similar association with thylakoids had previously been reported for Rubisco (Grissom and Kahn, 1974; McNeil and Walker, 1981), PRK (Fischer and Latzko, 1979) and GAPDH (Grissom and Kahn, 1974). A newly described ~600 kDa complex from tobacco contained Rubisco, PRK, RPI and carbonic anhydrase (Jebanathirajah and Coleman, 1998).

In other reports, complexes consisting of PGK-GAPDH (Malhotra et al., 1987; Macioszek and Anderson, 1987; Macioszek et al., 1990), GAPDH-TPI, aldolase-TPI, GAPDH-aldolase (Anderson et al., 1995), and PRI and PRK (Anderson, 1987; Skrukrud et al., 1991) have been found. Cytosolic PGK and GAPDH were also found to form a bienzyme complex (Weber and Berhard, 1982; Malhorta et al., 1987). Complexes of aldolase-GAPDH, PGK-GAPDH and aldolase-TPI from chloroplasts were isolated and characterized in pea (Anderson et al., 1995). It has been suggested that interaction among GAPDH, TKL and aldolase around SBPase may lead to a direct transfer of GA3P among these enzymes (Marques et al., 1987). Finally, PRI and PRK were shown to have kinetics in a complex state that differed from those anticipated for substrates used by non-complexed enzymes (Anderson, 1987). The theoretical kinetics of Calvin cycle multienzyme complexes have been modeled (Gontero et al., 1994; Ricard et al., 1994). In multienzyme complexes, the kinetics become increasingly complicated because

of the combined action of several enzymes.

Note that the sum of molecular weights of the native enzymes from spinach chloroplasts shown in Fig. 1 is about 1500 kDa, Rubisco alone contributing a third of that. Since Rubisco is far more abundant than any of the other Calvin cycle enzymes in plastids, it is clear that not all active Calvin cycle enzymes can exist in a complexed state (Rutner, 1970).

Summing up these findings on Calvin cycle multienzyme complexes, it appears clear that interactions between various enzymes do exist, but there is no consensus on which or how many enzymes interact and whether the same enzymes interact in different species. The metabolic relevance of these complexes is still unclear. Pressing problems concerning these associations have yet to be solved.

First, the isolated enzyme complexes are usually described to *dissociate* in the presence of reducing thiols (light), but the key regulatory factor of overall Calvin cycle activity is light-mediated (redox) *activation* through reduced thiols (see below). This discrepancy is difficult to reconcile with metabolic channeling, since the kinetic data indicate higher enzyme activities for associated enzymes via transfer of substrates in a consecutive reaction sequence, but flux through the pathway in the dark is basically nil (associated state) due to severe down-regulation of FBPase, PRK and SBPase (and moderate down-regulation of GAPDH) in the dark (i.e. in absence of reduced thioredoxins). But if the overall activities of the complexed enzymes are higher, as many such studies indicate, we are left with the question of the physiological relevance of improved kinetics for (dark-) associated enzyme complexes, since the active forms are dissociated in the light (see Section VI).

Second, if associations between enzymes are as critical to Calvin cycle function as the interpretations of many multienzyme studies would suggest, then problems in understanding the pathway ensue when the results from antisense inhibition of Calvin cycle enzymes are considered (see section VII). This is because antisense studies have shown that most (but not all) Calvin cycle activities must be reduced on the order of five- to ten-fold to affect a significant reduction in assimilation rate under normal growth conditions. If the brunt of assimilation occurs in complexes, limiting any one component should be expected to have a more drastic effect. Further work is needed to clarify the general significance of these enzyme association phenomena.

VI. Biochemical Regulation in Chloroplasts

Light governs not only Calvin cycle gene expression in higher plants, it is also the foremost determinant of enzyme activity, and hence flux through the pathway (Buchanan, 1980; Wolosiuk et al., 1993). Light regulation of the Calvin cycle is achieved by modulation of enzyme activity of four enzymes through the ferredoxin/thioredoxin system: FBPase, SBPase, PRK and GAPDH (Buchanan, 1991). The first three enzymes are obvious targets for regulation, since they catalyze reactions that are irreversible under physiological conditions, their regulatory principle is the same: reduced activity as a result of oxidation of regulatory cysteines by O_2 in the dark, full activity through reduction of regulatory cysteines by reduced thioredoxin in the light. Activity is continuously adjusted in the light through continued reoxidation (O_2) and reduction (thioredoxin). The steady-state between these interconvertible enzyme forms is individually influenced by metabolites (Scheibe, 1990). The fourth reaction, catalyzed by GAPDH, is reversible. Its activity is dependent upon its state of aggregation, redox modulation being the prerequisite enabling this metabolite-induced interconversion under physiological conditions.

A. The Ferredoxin/Thioredoxin System

Ferredoxin (Fd) reduced by photosynthetic electron flow provides electrons for NADP⁺ reduction via Fd/NADP reductase, for nitrite reduction via nitrite reductase, for sulfite reduction via sulfite reductase, for the reductive generation of glutamate from oxoglutarate via glutamate synthase (GOGAT), and for the reduction of the thioredoxin (Td) via ferredoxin-thioredoxin reductase (FTR) (reviewed by Buchanan, 1980; Woodrow and Berry, 1988; Scheibe, 1990; Buchanan, 1991; Knaff and Hirasawa, 1991; Wolosiuk et al., 1993; Jacquot et al., 1997b). FTR is composed of two different subunits, subunit A is rather variable between organisms, subunit B is more highly conserved and contains an Fe-S cluster in addition to conserved cysteines involved in redox transfer, but it is not a flavoprotein (Tsugita et al., 1991; Falkenstein et al., 1994). Thioredoxins are small heat-stable proteins that occur in all organisms and in many compartments. In the chloroplast various isoforms occur that differ in their primary structures and specificities: Tdm, Tdf_A , and Tdf_B (reviewed by

Eklund et al., 1991). Invitro, Tdm primarily activates NADP-dependent malate dehydrogenase (NADP-MDH), and inactivates chloroplast glucose-6-phosphate dehydrogenase (G6PDH), while Tdf preferentially activates chloroplast FBPase, SBPase, PRK, GAPDH in addition to the chloroplast coupling factor CF1 (reviewed by Buchanan, 1991). Whether this pattern of specificities also holds in stroma, where the protein concentration is very high, remains to be established.

Traditionally, light/dark modulation of chloroplast enzymes was considered as an all-or-nothing ‘on/off’-switch, but more recently it has become apparent that it is also a means to fine-tune enzyme activities in the light (Scheibe, 1990, 1991, 1995). This is because O_2 present at high concentrations in the chloroplast (Steiger et al., 1977) continuously reoxidizes the cysteines generated by thioredoxin-mediated electron flow to the target enzymes. Light-modulated enzymes thus exist as two interconvertible enzyme forms that are subject to covalent modification (reduction and reoxidation of cystine/cysteine residues), comparable to those enzymes that are subject to protein phosphorylation/dephosphorylation (Scheibe, 1990). In both cases, energy is consumed to drive the cycle between the two forms, but in the light, energy in the form of reducing equivalents is abundant and poses no significant drain on the photosynthetic membrane.

Target enzymes as well as the chloroplast thioredoxins are characterized by the very negative midpoint redox potentials of their regulatory cysteines (Faske et al., 1995). For NADP-MDH, FBPase and PRK these are around -380 mV, similar to that of the nonphysiological reagent dithiothreitol (DTT), and even more negative than the value of -350 mV for Tdm and Tdf (Gilbert, 1984). These redox potentials are all more negative than those of NADP(H) (-320 mV), and of glutathione (-260 mV), indicating that these protein thiols cannot be reduced by cellular reductants other than reduced ferredoxin. In some cases, mixed disulfides can be formed with low molecular weight thiols such as glutathione (Ocheretina and Scheibe, 1994). That certain chloroplast proteins occur in an oxidized form is a rather special attribute, since usually only extracellular proteins tend to exhibit this property (Fahey et al., 1977). For chloroplast enzymes it is this specific property which is the basis for a very flexible regulatory system.

B. Target Enzymes

The light/dark-modulated chloroplast enzymes are characterized by their unusually negative redox potentials. As a result of this, they are only in the reduced state when electrons of very negative redox potential from ferredoxin are available in the light; otherwise they relax to their oxidized state. The redox potentials themselves, however, are subject to change by specific metabolites, mostly the substrate or the product of the respective enzyme reaction. These metabolites are also known to act as effectors of the reductive and/or the oxidative part of the redox cycle. At equilibrium (with the redox buffer, *in vitro*) or at steady-state (*in vivo*) changes in the relative effector concentrations result in a more or less pronounced shift of the ratio between oxidized and reduced enzyme form (in a concentration-dependent manner) (Faske et al., 1995). Redox-modulated chloroplast enzymes generally exhibit high similarities with their non-redox-modulated homologues from other sources, but also tend to possess unique, cysteine-bearing sequence motifs that are responsible for their regulatory properties (Scheibe, 1990).

Chloroplast FBPase is the classical target for light regulation via thioredoxin (Buchanan, 1980). There is a strong dependence of FBPase activity upon the F1,6BP concentration, i.e. FBPase cannot easily be activated by DTT (or in the light) in the absence of F1,6BP. The resulting regulatory pattern is a strict feedforward mechanism of FBPase activation due to increasing F1,6BP levels (Scheibe, 1991). Several studies have investigated the mechanism of activation using FBPase overexpressed in *E. coli* (Jacquot et al., 1995; Hermoso et al., 1996; Jacquot et al., 1997a; Lopez-Jaramillo et al., 1997; Sahrawy et al., 1997). Chloroplast FBPase possesses a conspicuous insertion of 12–15 amino acids in the central region of the primary structure with two conserved cysteine residues separated by five amino acids (C₁₇₃ and C₁₇₈) preceded by a third conserved cysteine (C₁₅₃) further N-terminal (Marcus et al., 1988; Raines et al., 1988). In *vitro* mutagenesis of C₁₇₃ and C₁₇₈ results in enzymatically active FBPase enzymes that can no longer be regulated by thioredoxin, indicating that these may be specific targets of thioredoxin regulation (Jacquot et al., 1995). But in a more recent study, C₁₅₃ was also found to be responsible for redox dependence (Jacquot et al., 1997a). Replacing these three cysteines with serine residues in rapeseed FBPase also resulted in enzymes that were active in a manner largely

independent of redox modulation (Rodríguez-Suarez et al., 1997). It is not yet clear how redox modification of these three target cysteines modulates FBPase activity at the mechanistic level. For spinach FBPase, it appears that only a single disulfide bridge is formed, that between C₁₅₃ and C₁₇₄, as shown by mutant protein studies (A. Reichert and R. Scheibe, unpublished). Binding of thioredoxin to FBPase may be mediated by electrostatic interactions (Mora et al., 1998).

Pea Tdm overexpressed in *E. coli* can activate spinach FBPase, in contrast to previous findings, and K₇₀E mutagenesis of pea Tdm leads to a 50% decrease in FBPase activation (Lopez Jaramillo et al., 1997). A poorly conserved region of the FBPase alignment is found immediately preceding the redox regulatory cysteines. Deletion of this region yields active FBPase that can no longer be redox-regulated (Sahrawy et al., 1997). The same deleted region was expressed in *E. coli* as a 19 amino acid fragment (P₁₄₉-G₁₆₇), and shown to strongly interact with pea Tdm and, surprisingly, to increase the efficiency of Tdm in FBPase activation (Hermoso et al., 1996). These findings suggest that this may be the Td docking site that causes a conformational change upon binding.

Chloroplast GAPDH is among the first enzymes for which light regulation was known (Marcus 1960; Ziegler and Ziegler, 1965) and where a posttranslational modification was thought to modulate activity changes (Müller et al., 1969). Light/dark modulation of the enzyme correlates with changes in its aggregation state in isolated intact spinach chloroplasts. In the dark, only the less active, ~600 kDa form of chloroplast GAPDH appears to be present. In the light, the 150 kDa (tetrameric) form with a specific activity on the order of 120 U·mg⁻¹ (Trost et al., 1993; Scagliarini et al., 1993; Baalmann et al., 1994, 1995) predominates. Conversion to the activated 150 kDa form under reducing conditions (reduced thioredoxin) is achieved in the presence of low 1,3-bisphosphoglycerate (1,3BPGA) concentrations (K_a = 1–2 μM) and is accompanied by a 20-fold increase of the affinity for the substrate 1,3BPGA (Baalmann et al., 1994, 1995). In the presence of reduced thioredoxin, this transition is modulated by low concentrations of 1,3BPGA and is accompanied by a 20-fold increase of the affinity for 1,3BPGA as a substrate. The oxidized form can only be activated at unphysiologically high concentrations of the effector 1,3BPGA (Baalmann et al., 1994; 1995). Aggregation of active chloroplast GAPDH to ~600

kDa forms can also be induced in vitro by addition of $140 \mu\text{M NAD}^+$ (Pupillo and Giuliani-Piccarri, 1975; Cerff, 1982). The mature A and B subunits of spinach chloroplast GAPDH expressed in *E. coli* associate to active \mathbf{A}_4 and \mathbf{B}_4 forms, as does the derivative of the B subunit lacking the CTE, which is four-fold more active than the \mathbf{B}_4 form (Baalmann et al., 1996). These findings indicate that aggregation-mediated activation is dependent upon the 30 amino acid long carboxyterminal extension (CTE) of the B subunit, consistent with independent findings from partial proteolysis studies (Scheibe et al., 1995). The two conserved cysteines in the CTE may be the targets for thioredoxin reduction, but other models have been proposed (Pacold et al., 1995).

PRK is activated by incubation with reduced thioredoxin and inactivated by oxidation. The target cysteine residues involved in Td regulation of spinach chloroplast PRK were identified through chemical modification as \mathbf{C}_{16} and \mathbf{C}_{55} , the latter being close to the ATP-binding site (Porter et al., 1988), findings which were substantiated through in vitro mutagenesis and expression of the active enzyme in the yeast *Pichia pastoris* (Brandes et al., 1996a, 1996b). Although the cosubstrate and effector ATP decreases the rates of both activation and inactivation, it does not significantly influence the ratio between oxidized and reduced PRK at equilibrium (Faske et al., 1995). Therefore, redox-activation of PRK in the light occurs in a metabolite-independent manner, whereby the activated enzyme is then subject to non-covalent regulation of its catalytic activity by various metabolites (Gardemann et al., 1983).

Chloroplast SBPase, like FBPase, is enzymatically inactive in the oxidized state, but activity is restored within minutes by reduced sulphydryls, thioredoxins f_s , f_b and m can activate the enzyme (Cadet and Meunier, 1988a). Although SBPase and FBPase are related enzymes, the central insertion containing the redox-responsive cysteine residues of FBPase is, surprisingly, lacking in SBPase (Raines et al., 1992). Results from molecular modeling have suggested that interdomain disulfides might be involved in chloroplast SBPase redox regulation (Anderson et al., 1996). The target cysteines for SBPase regulation via thioredoxin have been identified through mutagenesis studies (Raines et al., 1998). It has also been suggested that flux through SBPase might be limiting for flux through the pathway (Pettersson and Ryde-Pettersson, 1989).

Oxidation of cysteines is a well-known reaction

occurring upon folding and secretion of proteins, often assisted by the action of thioredoxin-like proteins such as protein-disulfide isomerase (for review see: Loferer and Hennecke, 1994). However, these reactions are essentially irreversible and confer stability to the proteins in an oxidizing extracellular environment. The fact that intracellular proteins are oxidized in a reversible manner is unique to photosynthetic systems. Oxidation is accompanied by drastic changes of particular enzymic parameters as V_{max} , K_m and K_a , thus fulfilling important criteria of a covalent modification likely to be of relevance in vivo (Ziegler, 1985). For PRK, oxidation results in a decrease of its V_{max} to essentially zero. For FBPase and SBPase, oxidation decreases affinity for sugar bisphosphate and Mg^{2+} and shifts the pH optimum to higher values. Oxidation of Calvin cycle GAPDH results in the requirement for increased activator (1,3BPGA) concentrations (Trost et al., 1993; Baalmann et al., 1994), whereby 1,3BPGA is responsible for the dissociation of the aggregated low-affinity form to generate the high-affinity $\mathbf{A}_2\mathbf{B}_2$ heterotetramer.

The electron pressure required for the reduction of GAPDH and of PRK is rather low when compared to FBPase and SBPase, so that electron flow through thioredoxin will not be limiting even at low light, thus enabling the reductive step of the Calvin cycle and the regeneration of the CO_2 acceptor to occur under all conditions. Flux through these steps is thus determined by the availability of substrates. The chloroplast isoforms of glucose-6-phosphate dehydrogenase (G6PDH), ATPase and NADP-MDH are also subject to covalent redox-modification, but will not be considered here.

C. Physiological Consequences

Actual enzyme activities in isolated chloroplasts achieved at various light intensities will strongly depend on the metabolic status. Chloroplast GAPDH activity is at 100% even at low light intensities when ATP and 3PGA are present, since they readily generate 1,3BPGA concentrations required for GAPDH activation through the (unregulated) PGK reaction (Baalmann et al., 1994). The actual activity, however, will be lower due to the lack of 1,3BPGA as a substrate (Fridlyand et al., 1997). Thus the flux at this step is always adjusted to the overall flux through the Cycle. Flux through the PRK reaction in the light is also determined by substrate levels, since the enzyme

is in the fully active form at all light intensities (Scheibe, 1995). In contrast, the activation state of FBPase is strongly dependent upon the presence of F1,6BP which promotes reductive activation and inhibits oxidative inactivation (Scheibe, 1995). In intact chloroplasts, any decrease of electron pressure induced by the addition of electron acceptors decreases FBPase activation and leads to increased F1,6BP levels that apparently are not sufficient to support activation under these conditions (Holtgrefe et al., 1997), indicating that a strict control of CO_2 fixation is exerted by redox state and energy charge.

Correlations of light intensity and photosynthetic electron transport on the one hand, and stromal enzyme activities on the other, have been analyzed (Harbinson et al., 1990; Sassenrath-Coleetal., 1994). In some cases, a more or less positive correlation was found. But in others, as at low O_2 or CO_2 concentration, this did not appear to be the case (Harbinson and Foyer, 1991; Sassenrath-Cole et al., 1994). Under some conditions, the metabolite levels will also be altered, in turn shifting the activation states of the enzymes at a given thylakoid redox state. Therefore, it is important to include both electron pressure, metabolite levels and enzyme concentrations *in vivo* (Harris and Koniger, 1997) into models of photosynthetic regulation, since the redox potential of the isolated enzyme alone does not reflect the dynamic situation *in vivo* (Kramer et al., 1990).

Taken together, the differential light- and metabolite-dependent modulation of redox-modulated enzymes provides the basis for an extremely flexible fine-tuning. In isolated chloroplasts, this mechanism has been shown to establish a strict hierarchy between all reactions consuming light-generated electrons, so that no competition between the various pathways will occur (Backhausen et al., 1994). Due to the differential affinity of the various electron acceptors for electrons from reduced ferredoxin, the essential reactions as NADP^+ reduction, nitrite reduction and thioredoxin-independent enzyme activation will preferentially occur under physiological conditions. The required ATP/NADPH ratio is adjusted by the action of the malate valve that is controlled by the light/dark-modulated $\text{NADP}^+ \text{-malate}$ dehydrogenase present in all chloroplasts studied to date (Backhausen et al., 1994). Only an excess of electrons will flow into the Mehler/ascorbate reaction and into cyclic electron flow (Steiger and Beck, 1981; Heber and Walker, 1992).

In contrast, at higher concentrations the unphysiological electron acceptors H_2O_2 and nitrite can drain electrons from the Calvin cycle (Robinson et al., 1980; Backhausen et al., 1994) and thus inhibit photosynthesis.

VII. Studies of Calvin Cycle Enzymes with Antisense RNA

The effects of antisense RNA on carbohydrate partitioning in plants (Sonnewald et al., 1994; Frommer and Sonnewald, 1995; Furbank and Taylor, 1995; Stitt and Sonnewald, 1995) and on general aspects of regulation (Furbank and Taylor, 1995) can also be found throughout this volume. Antisense RNA rarely results in a complete inhibition of gene expression, residual gene expression can often supply sufficient enzyme activity to maintain a wild-type phenotype (Furbank and Taylor, 1995), and altered phenotypes of plants with reduced levels of Calvin cycle enzyme are sometimes only visible under conditions where factors such as light, CO_2 or mineral nutrition are limiting. Antisense technology has opened up new avenues of investigation and gene knockouts through insertional mutagenesis are increasingly becoming available in 'higher' plants, which were previously only available in 'lower' plants, notably the moss *Physcomitrella patens* (Schaefer and Zryd, 1997). Antisense is obviously an important tool for identifying controlling steps in photosynthetic carbon assimilation, and thus for identifying new enzymes as targets for plant protection strategies (Hoefgen et al., 1995), making it an essential tool for applied research. For this reason it is possible that many antisense studies will only become public knowledge after patent issues have been settled.

The first study using antisense technology on Calvin cycle enzymes was that of Rodermel et al. (1988), who were able to reduce RbcS protein and mRNA levels in transgenic tobacco, and surprisingly found that this influenced the total amount of RbcL protein synthesized, but not the *rbcL* mRNA level. In a later study, they were able to show that this is because RbcS protein exerts a positive effect on the translation efficiency of *rbcL* mRNA in plastids, but not on the translation efficiency of other plastid mRNAs (Rodermel et al., 1996), providing insights into a longstanding problem of how nuclear and plastid gene expression might be coordinated. Many papers have appeared on antisense expression of

rbcS focussing on the numerous effects that reduced Rubisco amounts have on tobacco metabolism under a plethora of light, temperature, CO_2 , and nitrogen availability conditions (Quick et al., 1991, 1992; Hudson et al., 1992; Fichtner et al., 1993; Gunasekera and Berkowitz, 1993; Lauerer et al., 1993; Masle et al., 1993; Krapp et al., 1994; Evans et al., 1994; Sicher et al., 1994; Stitt and Schulze, 1994; Von Caemmerer et al., 1994; Andrews et al., 1995; Jiang and Rodermel, 1995; Eckhardt et al., 1997). Varying degrees of reduced photosynthesis rates and various manifestations of altered redox state are usually observed in these plants. Furbank et al. (1994) studied the effect of *rbcS* antisense in a C_4 plant and also found that, as in C_3 plants, Rubisco activity was a major determinant of photosynthetic flux under high light intensities at elevated CO_2 .

Rubisco activase has also been inhibited by antisense. Plants required elevated CO_2 to grow reasonably, Rubisco carbamylation was reduced and twofold increases in Rubisco levels were observed (Mate et al., 1993). In a separate study, reduction of Rubisco activase was not found to influence *rbcS* or *rbcL* mRNA or protein levels (Jiang et al., 1994). Reduction of *Arabidopsis* Rubisco activase to about 40% of the wildtype levels also produced plants that grow poorly, suggesting that *Arabidopsis* does not produce Rubisco activase in vast excess of its needs (Eckardt et al., 1997).

Reduction of chloroplast FBPase in potato to 15% of normal levels decreases tuber yield, but reduction to 36% did not effect yield, although photosynthesis was impaired (Kossmann et al., 1994). Chloroplast FBPase was reduced in tobacco and potato to roughly 20% of normal levels. These antisense mutants had impaired electron transport capacity, and plants kept at low temperatures and low light to reduce photoinhibition showed increased levels of deepoxidized xanthophylls in a manner that depended upon light and temperature (Bilger et al., 1995; Fisahn et al., 1995). Reduction of cytosolic FBPase in potato lowered sucrose biosynthesis and increased leaf starch levels, but did not have negative effects on tuber yield under greenhouse conditions (Zrenner et al., 1996). Transgenic potato plants expressing antisense chloroplast FBPase showed reduced sizes and numbers of starch grains (Muschak et al., 1997).

Price et al. (1995) measured metabolite pools in antisense tobacco with chloroplast GAPDH reduced to as little as 7% of normal activity, and found that the 3PGA pool remained quite stable, whereas the

Ru1,5BP pool decreased as soon as GAPDH was below wildtype level. Reduction of PRK in tobacco to below 15% of normal levels resulted in up to five-fold increased levels of R5P, Ru5P, F6P and ATP, and up to 4-fold decreases in levels of Ru1,5BP, 3PGA and ADP. These metabolite levels largely compensated for PRK activity reduced down to 5% of the wildtype level, suggesting that it does not control flux through the pathway (Gray et al., 1995; Paul et al., 1995). Importantly, the extent of these effects depends in a crucial manner upon growth conditions, and most studies to date have been performed in controlled climate chambers under moderate light, rather than under variable ambient conditions including strong transients. Transgenic tobacco expressing antisense Rubisco activase showed reduced CO_2 fixation rates and increased Rubisco levels (He et al., 1997). Antisense chloroplast aldolase effectively inhibited photosynthesis in potato (Haake et al., 1998). Reduction of PRK activity by 94% through antisense in tobacco had little effect on photosynthetic rate, except in the presence of low nitrogen (Banks et al., 1999).

It can be expected that antisense studies of the remaining Calvin cycle enzymes, clones for which are available from higher plants (Martin and Schnarrenberger, 1997) will eventually be reported. It is already becoming apparent that reducing the activity of TPI, PGK, TKL, RPE, RPI, which are not allosterically regulated in any particular manner, will more dramatically impair photosynthesis and growth (Stitt, 1999). However, it is also possible that no single enzyme limits total flux through the pathway, and that control may be distributed across various steps at which compensation can be exerted. Yet notably, very severe inhibition of photosynthesis was reported in tobacco expressing antisense SPBase, suggesting that this enzyme activity might exert strong control over the photosynthetic rate in this tissue (EP Harrison et al., 1998).

VIII. Concluding Remarks

The structural diversity known among Calvin cycle enzymes is great, but it is doubtful that its full breadth has been discovered. The numerous examples of class I and class II enzymes known from the pathway should prompt the question of just how difficult or unlikely it is for functionally (substrate-product) identical, but structurally (binding-catalysis)

distinct enzymes to evolve in nature *de novo*. Recent experiments suggest that it may not be as difficult as one might think: Wagner et al. (1995) produced *antibodies* that catalyze the class I aldolase reaction with a variety of ketones and aldehydes—some of which yield a k_{cat} of $7 \times 10^{-3} \text{ min}^{-1}$ and a K_m of 17 μM —these antibodies are true synthetic enzymes and employ the identical, Schiff-base mechanism as known from natural class I aldolase. If new enzymes can be generated in the lab in spans of years, then cells, given billions of years, should certainly be able to vastly surpass that result. Conversely, functional diversity can also emanate from one and the same structure, as shown by the biochemical data of Zhao et al. (1995), who found that erythrose-4-phosphate dehydrogenase of *E. coli* is encoded by a gene that was previously thought to code for a GAPDH. The latter principle may also be extended to the regulatory level, since the redox-regulatory motifs of one and the same enzymes can differ dramatically across species (Jacquot et al., 1997b). Uncovering the breadth of structural and functional diversity thus requires a balance of biochemical and molecular studies. Further investigation of the pathway in prokaryotes and photosynthetic protists should reveal new and unexpected examples of how sampling from ancient prokaryotic gene diversity can bring forth functionally equivalent, yet structurally divergent assemblies of Calvin cycle enzymes, pathways which, despite their origins from a maze of ancient diversity, bear—rightly so—a common unifying name.

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References

- Adler K, Arkona C, Manteuffel R and Süss K-H (1993) Electron-microscopical localization of chloroplast proteins by immunogold labelling on cryo-embedded spinach leaves. *Cell Biol Intern* 17: 213–220
- Allen JF, Alexiev K and Hakansson G (1995) Photosynthesis: Regulation by redox signaling. *Curr Biol* 5: 869–872
- Altekar W and Rajagopalan R (1990) Ribulose bisphosphate carboxylase activity in halophilic archaeabacteria. *Arch Microbiol* 153: 169–174
- Altekar W and Rangaswamy V (1992) Degradation of endogenous fructose during catabolism of sucrose and mannitol in halophilic archaeabacteria. *Arch Microbiol* 158: 356–363
- Anderson LE (1987) Ribose-5-phosphate isomerase and ribulose-5-phosphate kinase show apparent specificity for a specific ribulose 5-phosphate species. *FEBS Lett* 212: 45–48
- Anderson LE and Pacold I (1972) Chloroplast and cytoplasmic enzymes. IV. Pea leaf fructose 1,6-diphosphate aldolase. *Plant Physiol* 49: 393–397
- Anderson LE, Goldhaber-Gordon IM, Li D, Tang X-Y, Xiang M and Prakash N (1995) Enzyme-enzyme interaction in the chloroplast: glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase and aldolase. *Planta* 196: 245–255
- Anderson LE, Huppe HC, Li AD and Stevens FJ (1996) Identification of a potential redox-sensitive interdomain disulfide in the sedoheptulose bisphosphatase of *Chlamydomonas reinhardtii*. *Plant J* 10: 553–560
- Andersson I (1996) Large structures at high resolution: the 1.6 Å crystal structure of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase complexed with 2-carboxyarabinitol bisphosphate. *J Mol Biol* 259: 160–174
- Andrews TJ, Hudson GS, Mate CJ, Von Caemmerer S, Evans JR and Arvidsson YBC (1995) Rubisco: The consequences of altering its expression and activation in transgenic plants. *J Expt Bot* 46: 1293–1300
- Antia NJ (1967) Comparative studies on aldolase activity in marine planktonic algae and their evolutionary significance. *J Phycol* 3: 81–84
- Ashton AR (1998a) A simple procedure for purifying the major chloroplast fructose-1,6-bisphosphatase from spinach (*Spinacia oleracea*) and characterization of its stimulation by sub-femtomolar mercuric ions. *Arch Biochem Biophys* 357: 207–224
- Ashton AR (1998b) Sedoheptulose-1,7-bisphosphate phosphatase activity of chloroplast fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphate in stromal extracts from chloroplasts of spinach (*Spinacia oleracea*). *Austr J Plant Physiol* 25: 531–537
- Assali N-E, Martin, W and Loiseaux-de Goér S (1991) Evolution of the Rubisco operon from prokaryotes to algae: Structure and analysis of the *rbcS* gene of the brown alga *Pylaiella littoralis*. *Plant Mol Biol* 17: 853–863
- Avilan L, Gontero B, Lebreton S and Ricard J (1997) Memory imprinting effects in multienzyme complexes. I. Isolation, dissociation and reassociation of a phosphoribulokinase-glyceraldehyde-3-phosphate dehydrogenase complex from *Chlamydomonas reinhardtii* chloroplasts. *Eur J Biochem* 246: 74–84
- Baalmann E, Backhausen JE, Kitzmann C and Scheibe R (1994) Regulation of NADP-dependent glyceraldehyde 3-phosphate dehydrogenase activity in spinach chloroplasts. *Bot Acta* 107: 313–320
- Baalmann E, Backhausen JE, Rak C, Vetter S and Scheibe R (1995) Reductive modification and nonreductive activation of purified spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. *Arch Biochem Biophys* 324: 201–208
- Baalmann E, Scheibe R, Cerff R and Martin W (1996) Functional studies of chloroplast glyceraldehyde-3-phosphate dehydrogenase subunits A and B expressed in *Escherichia coli*: Formation of highly active **A₄** and **B₄** homotetramers and evidence that aggregation of the **B₄** complex is mediated by the B subunit carboxyterminus. *Plant Mol Biol* 32: 505–514

- Babadzhanova MA and Bakaeva NP (1987) Quaternary structure and some properties of ribophosphate isomerase from the leaves of *Arabidopsis thaliana* of Enkheim race and its mutants triplex and 58/15. *Biokhimiya* 52: 146–153
- Backhausen JE, Vetter S, Baalmann E, Kitzmann C and Scheibe R (1998) **NAD⁺-dependent** malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase isoenzymes play an important role in dark metabolism of various plastid types. *Planta* 205: 359–366
- Bailey RW and Staehelin LA (1968) The chemical composition of isolated cell walls of *Cyanidium caldarium*. *J Gen Microbiol* 54: 269–276
- Banks FM, Driscoll SP, Parry MAJ, Lawlor DW, Knight JS, Gray JC and Paul MJ (1999) Decrease in phosphoribulokinase activity by antisense RNA in transgenic tobacco. Relationship between photosynthesis, growth, and allocation at different nitrogen levels. *Plant Physiol* 119: 1125–1136
- Beh M, Strauss G, Huber R, Stetter KO and Fuchs G (1993) Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and in the archaeabacterium *Thermoproteus neutrophilus*. *Arch Microbiol* 160: 306–311
- Berger C and Feierabend J (1967) Plastidenentwicklung und Bildung von Photosynthese-Enzymen in etiolierten Roggengemülingen. *Physiol Vég* 5: 109–122
- Bernacchia G, Schwall G, Lottspeich F, Salamini F and Bartels D (1995) The transketolase gene family of the resurrection plant *Craterostigma plantagineum*: differential expression during the rehydration phase. *EMBO J* 14: 610–618
- Bernstein BE, Michels PA and Hol WG (1997) Synergistic effects of substrate-induced conformational changes in phosphoglycerate kinase activation. *Nature* 385: 275–278
- Bertsch U, Schlicher TB, Schröder I and Soll J (1993) Sequence of mature phosphoglycerate kinase from spinach chloroplasts. *Plant Physiol* 103: 1449–1450
- Besford RT (1990) The greenhouse effect: Acclimation of tomato plants growing in high CO₂, relative changes in Calvin cycle enzymes. *J Plant Physiol* 136: 458–463
- Beuf L, Bedu S, Durand MC and Joset F (1994) A protein involved in co-ordinated regulation of inorganic carbon and glucose metabolism in the facultative photoautotrophic cyanobacterium *Synechocystis* PCC6803. *Plant Mol Biol* 25: 855–864
- Biesecker G, Harris JI, Thierry JC, Walker JE and Wonacott AJ (1977) Sequence and structure of D-glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. *Nature* 266: 328–333
- Bilger W, Fisahn J, Brummet W, Kossmann J and Willmitzer L (1995) Violaxanthin cycle pigment contents in potato and tobacco plants with genetically reduced photosynthetic capacity. *Plant Physiol* 108: 1479–1486
- Blom N and Sygusch J (1997) Product binding and role of the C-terminal region in class I D-fructose 1,6-bisphosphate aldolase. *Nat Struct Biol* 4: 36–39
- Boldt R, Börner T and Schnarrenberger C (1992) Repression of the plastidic isoenzymes of aldolase, 3-phosphoglycerate kinase, and triosephosphate isomerase in the barley mutant ‘albostrians.’ *Plant Physiol* 99: 895–900
- Boldt R, Pelzer-Reith B, Börner T and Schnarrenberger C (1994) Aldolases in barley (*Hordeum vulgare* L.): Properties and repression of the plastid enzyme in the plastome mutant ‘albostrians.’ *J Plant Physiol* 144: 282–286
- Boldt R, Koshuchova S, Gross W, Börner T and Schnarrenberger C (1997) Decrease in glycolate pathway enzyme activities in plastids and peroxisomes of the *albostrians* mutant of barley (*Hordeum vulgare* L.). *Plant Sci Lett* 124: 33–40
- Bommer D, Schäferjohann J and Bowien B (1996) Identification of *cbbBc* as an additional distal gene of the chromosomal *cbb* CO₂ fixation operon from *Ralstonia eutropha*. *Arch Microbiol* 166: 245–251
- Börner T, Schumann B and Hagemann R (1976) Biochemical studies on plastid ribosome-deficient mutant of *Hordeum vulgare*. In: Bucher T, Neupert W, Sebald W and Wern S (eds) *Genetics and Biogenesis of Chloroplasts and Mitochondria*, pp 42–48. Elsevier/North-Holland Biomedical Press, Amsterdam
- Bovarnick JG, Schiff JA, Freedman Z and Egan JM (1974) Events surrounding the early development of Euglena chloroplasts: Cellular origins of chloroplast enzymes in *Euglena*. *J Gen Microbiol* 83: 63–71
- Bowien B, Bednarski R, Kusian B, Windhövel U, Freter A, Schäferjohann J and Yoo J-G (1993) Genetic regulation of CO₂ assimilation in chemoautotrophs. In: Murrell JC, Kelley DP (eds) *Microbial Growth on Cl Compounds*, pp 481–491. Intercept Scientific, Andover
- Bradbeer JW (1969) The activities of the photosynthetic carbon cycle enzymes of greening bean leaves. *New Phytol* 68: 233–245
- Bradbeer JW (1971) Plastid development in primary leaves of *Phaseolus vulgaris*. The effects of short blue, red, far-red and white light treatments on dark-grown plants. *J Exp Bot* 22: 382–390
- Bradbeer JW and Börner T (1978) Activities of glyceraldehyde-phosphate dehydrogenase (NADP⁺) and phosphoribulokinase in two barley mutants deficient in chloroplast ribosomes. In: Akoyunoglou G and Argyroundi-Akoyunoglou JH (eds) *Chloroplast Development*, pp 727–732. Elsevier/North-Holland Biomedical Press, Amsterdam
- Bradbeer JW, Gyldenholm AO, Wallis ME and Whatley FR (1969) Studies on the biochemistry of chloroplast development. *Progr Photosynth Res* 1: 272–279
- Bradbeer JW, Atkinson YE, Börner T and Hagemann R (1979) Cytoplasmic synthesis of plastid polypeptides may be controlled by plastid synthesized RNA. *Nature* 279: 816–817
- Bränden C-I and Eklund H (1980) Structure and mechanism of liver alcohol dehydrogenase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. In: Jeffrey J (ed) *Dehydrogenases Requiring Nicotinamide Coenzymes*, pp 40–84. Birkhäuser Verlag, Basel
- Brandes HK, Hartman FC, Lu T-YS and Larimer FW (1996a) Efficient expression of the gene for spinach phosphoribulokinase in *Pichia pastoris* and utilization of the recombinant enzyme to explore the role of regulatory cysteinyl residue by site-directed mutagenesis. *J Biol Chem* 271: 6490–6496
- Brandes HK, Larimer FW and Hartman FC (1996b) The molecular pathway for the regulation of phosphoribulokinase by thioredoxin f. *J Biol Chem* 271: 3333–3335
- Brandt P and Wilhelm C (1990) The light-harvesting system of *Euglena gracilis* during the cell cycle. *Planta* 180: 293–296
- Breazeale VD, Buchanan BB and Wolosiuk RA (1978) Chloroplast sedoheptulose 1,7-bisphosphatase: Evidence for regulation by the ferredoxin/thioredoxin system. *Z Naturforsch* 33c: 521–528

- Bringloe DH, Rao SK, Dyer TA, Raines CA and Bradbeer JW (1996) Differential gene expression of chloroplast and cytosolic phosphoglycerate kinase in tobacco. *Plant Mol Biol* 30: 637–640
- Brinkmann H and Martin W (1996) Higher plant chloroplast and cytosolic 3-phosphoglyceratekinases: A case of endosymbiotic gene replacement. *Plant Mol Biol* 30: 65–75
- Brooks K and Criddle RS (1966) Enzymes of the carbon cycle of photosynthesis I. Isolation and properties of spinach chloroplast aldolase. *Arch Biochem Biophys* 117: 650–659
- Brüggemann W, Klaucke S and Maas-Kantel K (1994) Long-term chilling of young tomato plants under low light. V. Kinetic and molecular properties of two key enzymes of the Calvin cycle in *Lycopersicon esculentum* Mill, and *L. peruvianum* Mill. *Planta* 194: 160–168
- Buchanan BB (1980) Role of light in the regulation of chloroplast enzymes. *Ann Rev Plant Physiol* 31: 341–374
- Buchanan BB (1991) Regulation of CO₂ assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. *Arch Biochem Biophys* 288: 1–9
- Büker M, Schünemann D and Borchert S (1998) Enzymic properties and capacities of developing tomato (*Lycopersicon esculentum* L.) fruit plastids. *J Exp Bot* 49: 681–691
- Bukowiecki AC and Anderson LE (1974) Multiple forms of aldolase and triosephosphate isomerase in diverse plant species. *Plant Sci Lett* 3: 381–386
- Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD (35 other coauthors) and Venter JC (1996) Complete genome sequence of the methanogenic Archeon, *Methanococcus jannaschii*. *Science* 273: 1058–1073
- Cadet F and Meunier J-C (1988a) Spinach chloroplast (*Spinacia oleracea*) sedoheptulose-1,7-bisphosphatase. *Biochem J* 253: 243–248
- Cadet F and Meunier J-C (1988b) pH and kinetic studies of chloroplast sedoheptulose-1,7-bisphosphatase from spinach (*Spinacia oleracea*). *Biochem J* 253: 249–254
- Cadet F, Meunier JC and Ferte N (1987) Isolation and purification of chloroplastic spinach (*Spinacia oleracea*) sedoheptulose-1,7-bisphosphatase. *Biochem J* 241: 71–74
- Calvin M (1956) The photosynthetic carbon cycle. *J Chem Soc* 1956:1895–1915
- Camara B, Hugueny P, Bouvier F, Kuntz M and Moneger R (1995) Biochemistry and molecular biology of chromoplast development. *Int Rev Cytol* 163: 175–247
- Cerff R (1973) Glyceraldehyde 3-phosphate dehydrogenases and glyoxylate reductase. I. Their regulation under continuous red and far red light in the cotyledons of *Sinapis alba* L. *Plant Physiol* 51: 76–81
- Cerff R (1978a) Glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Sinapis alba*: steady state kinetics. *Phytochemistry* 17: 2061–2067
- Cerff R (1978b) Glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Sinapis alba* L. NAD(P)-induced conformation changes of the enzyme. *Eur J Biochem* 82: 45–53
- Cerff R (1982) Separation and purification of NAD- and NADP-linked glyceraldehyde-3-phosphate dehydrogenases from higher plants. In: M Edelmann, RB Hallick and N-H Chua (eds) *Methods in Chloroplast Molecular Biology*, pp 683–694. Elsevier Biomedical Press, Amsterdam
- Cerff R and Chambers S (1979) Subunit structure of higher plant glyceraldehyde-3-phosphate dehydrogenases (EC 1.2.1.12 and 1.2.1.13). *J Biol Chem* 254: 6094–6098
- Cerff R and Kloppstech K (1982) Structural diversity and differential light control of mRNAs coding for angiosperm glyceraldehyde-3-phosphate dehydrogenases. *Proc Natl Acad Sci USA* 79: 7624–7628
- Cerff R and Quail PH (1974) Glyceraldehyde 3-phosphate dehydrogenases and glyoxylate reductase. II. Far red light-dependent development of glyceraldehyde 3-phosphate dehydrogenase isoenzyme activities in *Sinapis alba* cotyledons. *Plant Physiol* 54: 100–104
- Chan RL, Keller M, Canaday J, Weil J-H and Imbault P (1990) Eight small subunits of *Euglena* ribulose-1,5-bisphosphate carboxygenase/oxygenase are translated from a large mRNA as a polyprotein. *EMBO J* 9: 333–338
- Chao S, Raines CA, Longstaff M, Sharp PJ, Gale MD and Dyer TA (1989) Chromosomal location and copy number in wheat and some of its close relatives of genes for enzymes involved in photosynthesis. *Mol Gen Genet* 218: 423–430
- Charles SA and Halliwell B (1980) Properties of freshly purified and thiol-treated spinach chloroplast fructose bisphosphatase. *Biochem J* 185: 689–693
- Chen YR, Hartman FC, Lu TY, Larimer FW (1998) D-Ribulose-5-phosphate 3-epimerase: cloning and heterologous expression of the spinach gene, and purification and characterization of the recombinant enzyme. *Plant Physiol* 118: 199–207
- Chen Z, Silva H and Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262: 1883–1886
- Chory J, Chatterjee M, Cook RK, Elich T, Fankhauser C, Li J, Nagpal P, Neff M, Pepper A, Poole D, Reed J and Vitart V (1996) From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proc Natl Acad Sci USA* 93: 12066–12071
- Clasper S, Easterby JS and Powls R (1991) Properties of two high-molecular-mass forms of glyceraldehyde-3-phosphate dehydrogenase from spinach leaf, one of which also possesses latent phosphoribulokinase activity. *Eur J Biochem* 202: 1239–1246
- Clasper S, Chelvarajan RE, Easterby JS and Powls R (1994) Isolation of multiple dimeric forms of phosphoribulokinase from an alga and a higher plant. *Biochim Biophys Acta* 1209: 101–106
- Clermont S, Corbier C, Mely Y, Gerard D, Wonacott A and Branlant G (1993) Determinants of coenzyme specificity in glyceraldehyde-3-phosphate dehydrogenase: Role of the acidic residue in the fingerprint region of the nucleotide binding fold. *Biochemistry* 32: 10178–10184
- Conley TR and Shih M-C (1995) Effects of light and chloroplast functional state on expression of nuclear genes encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase in hypocotyl (hy) mutants and wild-type *Arabidopsis thaliana*. *Plant Physiol* 108: 1013–1022
- Conley TR, Park S-C, Kwon H-B, Peng H-P and Shih M-C (1994) Characterization of *cis*-acting elements in light regulation of the nuclear gene encoding the A subunit of chloroplast isozyme of glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. *Mol Cell Biol* 14: 2525–2533
- Cooper SJ, Leonard GA, McSweeney SM, Thompson AW, Naismith JH, Qamar S, Plater A, Berry A and Hunter WN (1996) The crystal structure of a class II fructose-

- 1,6-bisphosphate aldolase shows a novel binuclear metal-binding active site embedded in a familiar fold. Structure 4: 1303–1315
- Criqui MC, Durr A, Marbach J, Fleck J and Jamet E (1992) How are photosynthetic genes repressed in freshly-isolated mesophyll protoplasts of *Nicotiana sylvestris*? Plant Physiol Biochem 30: 597–601
- Delwiche CF and Palmer JD (1996) Rampant horizontal transfer and duplication of Rubisco genes in eubacteria and plastids. Mol Biol Evol 13: 873–882
- Dennis ES, Gerlach WL, Walker JC, Lavin M and Peacock WJ (1988) Anaerobically regulated aldolase gene of maize. J Mol Biol 202: 759–767
- Derocher EJ, Quigley F, Mache R and Bohnert HJ (1993) The six genes of the Rubisco small subunit multigene family from *Mesembryanthemum crystallinum*, a facultative CAM plant. Mol Genet 239: 450–462
- Dewdney J, Conley TR, Shih M-C and Goodman HM (1993) Effects of blue and red light on the expression of the nuclear genes encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase of *Arabidopsis thaliana*. Plant Physiol 103: 1115–1121
- Dockerty A and Merret MJ (1979) Isolation and enzymatic characterization of *Euglena* proplasts. Plant Physiol 63: 468–473
- Donath M, Mendel R, Cerff R and Martin W (1995) Intron-dependent transient expression of the maize *GapA* 1 gene. Plant Mol Biol 28: 667–676
- Douglas SE and Dunford DG (1989) The small subunit of ribulose-1,5-bisphosphate carboxylase is plastid-encoded in the chlorophyll *c*-containing alga *Cryptomonas phi*. Plant Mol Biol 13: 13–20
- Dunford RP, Catley MA, Raines CA, Lloyd JC and Dyer TA (1998) Purification of active chloroplast sedoheptulose-1,7-bisphosphatase expressed in *Escherichia coli*. Protein Expr Purif 14: 139–145
- Eckardt NA, Snyder GW, Portis AR Jr and Ogren WL (1997) Growth and photosynthesis under high and low irradiance of *Arabidopsis thaliana* antisense mutants with reduced ribulose-1,5-bisphosphatecarboxylase/oxygenase activase content. Plant Physiol 113: 575–586
- Eklund H, Gleason FK and Holmgren A (1991) Structural and functional relations among thioredoxins of different species. Proteins: Struct Funct Gen 11: 13–28
- Ernes MJ and Fowler MW (1978) Location of transketolase and transaldolase in apical cells of pea roots. Biochem Soc Transactions 6: 203–205
- Eraso JM and Kaplan S (1994) *prrA*, a putative response regulator involved in oxygen regulation of photosynthesis gene expression in *Rhodobacter sphaeroides*. J Bacteriol 176: 32–43
- Escoubas JM, Lomas M, LaRoche J and Falkowski PG (1995) Light intensity regulation of *cab* gene transcription is signaled by the redox state of the plastoquinone pool. Proc Natl Acad Sci USA 92: 10237–10241
- Evans JR, Von Caemmerer S, Setchell BA and Hudson GS (1994) The relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. Austr J Plant Physiol 21: 475–495
- Evans MCW, Buchanan BB and Arnon DI (1966) A new ferredoxin-dependent carbon reduction cycle in a photo-
- synthetic bacterium. Proc Natl Acad Sci USA 55: 928–934
- Fabry S and Hensel R (1988) Primary structure of glyceraldehyde-3-phosphate dehydrogenase deduced from the nucleotide sequence of the thermophilic archaebacterium *Methanothermus fervidus*. Gene 64: 189–197
- Fagan T, Hastings JW and Morse D (1998) The phylogeny of glyceraldehyde-3-phosphate dehydrogenase indicates lateral gene transfer from cryptomonads to dinoflagellates. J Mol Evol 47: 633–639
- Fahey RC, Hunt JS and Windham GC (1977) On the cysteine and cystine content of proteins. Differences between intracellular and extracellular proteins. J Mol Evol 10: 155–160
- Falcone DL and Tabita FR (1991) Expression of endogenous and foreign ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) genes in a RuBisCO deletion mutant of *Rhodobacter sphaeroides*. J Bacteriol 173: 2099–2108
- Falkenstein E, von Schaewen A and Scheibe R (1994) Full-length cDNA sequences for both ferredoxin-thioredoxin reductase subunits from spinach (*Spinacia oleracea* L.). Biochim Biophys Acta 1185: 252–254
- Faske M, Holtgrefe S, Ocheretina O, Meister M, Backhausen JE and Scheibe R (1995) Redox equilibria between the regulatory thiols of light/dark-modulated chloroplast enzymes and dithiothreitol: Fine-tuning by metabolites. Biochim Biophys Acta 1247: 135–142
- Feierabend J (1966) Enzymbildung in Roggenkeimlingen während der Umstellung von heterotrophem auf autotrophes Wachstum. Planta 71: 326–355
- Feierabend J (1979) Role of cytoplasmic protein synthesis and its coordination with the plastidic protein synthesis in the biogenesis of chloroplasts. Ber Deutsch Bot Ges 92: 553–574
- Feierabend J (1986) Investigation of the site of synthesis of chloroplastic enzymes of nitrogen metabolism by the use of heat-treated 70S ribosome-deficient rye leaves. Physiol Plant 67: 145–150
- Feierabend J and Gringel G (1983) Plantransketolase: subcellular distribution, search for multiple forms, site of synthesis. Z Phanzenphysiol 110: 247–258
- Feierabend J and Pirson A (1966) Die Wirkung des Lichts und die Bildung von Photosyntheseenzymen in Roggenkeimlingen. Z Pflanzenphysiol 55: 235–245
- Feierabend J and Schrader-Reichhardt U (1976) Biochemical differentiation of plastids and other organelles in rye leaves with a high-temperature-induced deficiency of plastid ribosomes. Planta 129: 133–145
- Ferri G, Stoppini MC, Meloni ML, Zapponi MC and Iadarola M (1990) Chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP): Amino acid sequence of the subunits from isoenzyme I and structural relationship with isoenzyme II. Biochim Biophys Acta 1041: 36–42
- Fichtner K, Quick WP, Schulze ED, Mooney HA, Rodermel SR, Bogorad L and Stitt M (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ *rbcS*. Planta 190: 1–9
- Fickenscher K and Scheibe R (1988) Limited proteolysis of inactive tetrameric chloroplast NADP-malate dehydrogenase produces active dimers. Arch Biochim Biophys 260: 711–779
- Fisahn J, Kossmann J, Matzke G, Fuss H, Bilger W and Willmitzer L (1995) Chlorophyll fluorescence quenching and violaxanthin deepoxidation of FBPase antisense plants at low light intensities and low temperatures. Physiol Plant 95: 1–10

- Fischer KH and Latzko E (1979) Chloroplastribulose-5-phosphate kinase: light-mediated activation and detection of both soluble and membrane-associated activity. *Biochem Biophys Res Commun* 89: 300–306
- Flechner A, Dreßen U, Westhoff P, Henze K, Schnarrenberger C and Martin W (1996) Molecular characterization of transketolase (EC 2.2.1.1) active in the Calvin cycle of spinach chloroplasts. *Plant Mol Biol* 32: 475–484
- Flechner A, Gross W, Martin W and Schnarrenberger C (1999) Chloroplast class I and class II aldolases are bifunctional for fructose-1,6-bisphosphate and sedoheptulose-1,7-bisphosphate cleavage in the Calvin cycle. *FEBS Lett* 447: 200–202
- Fluhr R, Moses P, Morelli G, Coruzzi G and Chua N-H (1986) Expression dynamics of the pea *rbcS* multigene family and organ distribution of the transcripts. *EMBO J* 5: 2063–2072
- Fothergill-Gilmore LA and Michels PAM (1993) Evolution of glycolysis. *Progr Biophys Mol Biol* 59: 105–238
- Fourcroy P (1986) Phytochrome control of gene expression in radish (*Raphanus sativus*) seedlings: III. Evidence for a rapid control of the ribulose-1,5-bisphosphate carboxylase small subunit gene expression by red light. *Plant Sci* 44: 183–190
- Frederick JF (1968) The polyglucoside and enzymes of *Cyanidium caldarium*. *Phytochemistry* 7, 1573–1576
- Fridlyand LE, Backhausen JE, Holtgrefe S, Kitzmann C and Scheibe R (1997) Quantitative evaluation of the role of 3-phosphoglycerate reduction in chloroplasts. *Plant Cell Physiol* 38: 1177–1186
- Fritz CC, Wolter FP, Schenkemeyer V, Herget T and Schreier PH (1993) The gene family encoding the ribulose-(1,5)-bisphosphate carboxylase/oxygenase (Rubisco) small subunit of potato. *Gene* 137: 271–274
- Frommer WB and Sonnewald U (1995) Molecular analysis of carbon partitioning in solanaceous species. *J Exp Bot* 46: 587–730
- Fuchs G and Stupperich E (1986) Carbon assimilation pathways in archaeabacteria. *System Appl Microbiol* 7: 364–369
- Furbank RT and Taylor WC (1995) Regulation of photosynthesis in C3 and C4 plants. *Plant Cell* 7: 797–805
- Furbank RT, Chitty JA, Von Caemmerer S and Jenkins CLD (1996) Antisense RNA inhibition of *RbcS* gene expression reduces rubisco level and photosynthesis in the C4 plant *Flaveria bidentis*. *Plant Physiol* 111: 725–734
- Gardemann A, Stitt M and Heldt HW (1983) Control of CO₂ fixation. Regulation of spinach ribulose-5-phosphate kinase by stromal metabolite levels. *Biochim Biophys Acta* 722: 51–60
- Gatenby AA and Viitanen PV (1994) Structural and functional aspects of chaperonin-mediated protein folding. *Ann Rev Plant Physiol Plant Mol Biol* 45: 469–491
- Geiger DR and Servaites JC (1994) Diurnal regulation of photosynthetic carbon metabolism in C3 plants. *Annu Rev Plant Physiol Plant Mol Biol* 45: 235–256
- Gerbling KP, Steup M and Latzko E (1986) Fructose 1,6-bisphosphatase form B from *Synechococcus leopoliensis* hydrolyzes both fructose and sedoheptulose bisphosphate. *Plant Physiol* 80: 716–720
- Gibbs SH (1978) The Chloroplast of *Euglena* may have evolved from symbiotic green algae. *Can J Bot* 56: 2883–2889
- Gibson JL and Tabita FR (1996) The molecular regulation of the reductive pentose phosphate pathway in proteobacteria and cyanobacteria. *Arch Microbiol* 166: 141–150
- Gibson JL and Tabita FR (1997) Analysis of the *cbbXYZ* operon in *Rhodobacter sphaeroides*. *J Bacteriol* 179: 663–669
- Gilbert HF (1984) Redox control of enzyme activities by thiol/disulfide exchange. *Methods Enzymol* 107: 330–351
- Gilmartin PM, Memelink J, Hiratsuka K, Kay SA and Chua NH (1992) Characterization of a gene encoding a DNA binding protein with specificity for a light-responsive element. *Plant Cell* 4: 839–849
- Gilmartin PM, Sarokin L, Memelink J and Chua N-H (1990) Molecular light switches for plant genes. *Plant Cell* 2: 369–378
- Goloubinoff P, Christeller JT, Gatenby AA and Lorimer GH (1989) Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature* 342: 884–889
- Gontero B, Cárdenas ML and Ricard J (1988) A functional five-enzyme complex of chloroplasts involved in the Calvin cycle. *Eur J Biochem* 173: 437–443
- Gontero B, Mulliert G, Rault M, Guidici-Orticoni MT and Ricard J (1993) Structural and functional properties of a multi-enzyme complex from spinach chloroplasts: 2. Modulation of the kinetic properties of enzymes in the aggregated state. *Eur J Biochem* 217: 1075–1082
- Gontero B, Guidici-Orticoni MT and Ricard J (1994) The modulation of enzyme reaction rates within multi-enzyme complexes. 2. Information transfer within a Chloroplast multi-enzyme complex containing ribulose bisphosphate carboxylase-oxygenase. *Eur J Biochem* 226: 999–1006
- Graham D, Grieve AM and Smillie R (1968) Phytochrome as the primary photoregulator of the synthesis of Calvin cycle enzymes in etiolated pea seedlings. *Nature* 218: 89–90
- Gray JC, Paul MJ, Barnes SA, Knight JS, Loynes A, Habash D, Parry MAJ and Lawlor DW (1995) Manipulation of phosphoribulokinase and phosphate translocator activities in transgenic tobacco plants. *J Exp Bot* 46: 1309–1315
- Grisson FE and Kahn JS (1974) Glyceraldehyde-3-phosphate dehydrogenases from *Euglena gracilis*. Purification and physical and chemical characterization. *Arch Biochem Biophys* 171: 444–458
- Gross W and Schnarrenberger C (1995) Heterotrophic growth of two strains of the acidothermophilic red alga *Galdieria sulphuraria*. *Plant Cell Physiol* 36: 633–638
- Gross W, Bayer MG, Schnarrenberger C, Gebhart UB, Maier TL and Schenck HEA (1994) Two distinct aldolases of class II type in the cyanoplasts and in the cytosol of the alga *Cyanophora paradoxa*. *Plant Physiol* 105: 1393–1398
- Gross W, Lenze D, Nowitzki U, Weiske J, Schnarrenberger C (1999) Characterization, cloning, and evolutionary history of the Chloroplast and cytosolic class I aldolases of the red alga *Galdieria sulphuraria*. *Gene* 230: 7–14
- Grotjohann N (1997) Activation of Chloroplast fructose-1,6-bisphosphatase of *Pisum sativum* by mole mass change. *Bot Acta* 110: 323–327
- Gunasekera D and Berkowitz GA (1993) Use of transgenic plants with ribulose-1,5-bisphosphate carboxylase/oxygenase antisense DNA to evaluate the rate limitation of photosynthesis under water stress. *Plant Physiol* 103: 629–635
- Gutteridge S and Gatenby AA (1995) Rubisco synthesis, assembly, mechanism and regulation. *Plant Cell* 7: 809–819
- Haake V, Zrenner R, Sonnewald U and Stitt M (1998) A moderate decrease of plastid aldolase activity inhibits photosynthesis,

- alters the levels of sugars and starch, and inhibits growth of potato plants. *Plant J* 14: 147–157
- Hagemann R and Scholz F (1962) Ein Fall geninduzierter Mutationen des Plasmotypus bei Gerste. *Der Züchter* 32: 50–58
- Hahn D, Bennoun P and Kück U (1996) Altered expression of nuclear genes encoding chloroplast polypeptides in non-photosynthetic mutants of *Chlamydomonas reinhardtii*: Evidence for post-transcription regulation. *Mol Gen Genet* 252: 362–370
- Hahn D, Kaltenbach C and Kück U (1998) The Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase is encoded by a light-regulated gene in *Chlamydomonas reinhardtii*. *Plant Mol Biol* 36: 929–934
- Harbinson J and Foyer CH (1991) Relationships between the efficiencies of Photosystem I and II and stromal redox state in CO_2 -free air. *Plant Physiol* 97: 41–49
- Harbinson J, Genty B and Foyer CH (1990) Relationship between photosynthetic electron transport and stromal enzyme activity in pea leaves. *Plant Physiol* 94: 545–553
- Hariharan Tara, Johnson PJ and Cattolico RA (1998) Purification and characterization of phosphoribulokinase from the marine chromophytic alga *Heterosigma carterae*. *Plant Physiol* 117: 321–329
- Harris GC and Koniger M (1997) The ‘high’ concentrations of enzymes within the chloroplast. *Photosynth Res* 54: 5–23
- Harrison DHT, Runquist JA, Holub A and Mizorko HM (1998) The crystal structure of phosphoribulokinase from Rhodobacter sphaeroides reveals a fold similar to that of adenylate kinase. *Biochemistry* 37: 5074–5085
- Harrison EP, Willingham NM, Lloyd JC and Raines CA (1998) Reduced sedoheptulose-1,7-bisphosphatase levels in transgenic tobacco lead to decreased photosynthetic capacity and altered carbohydrate accumulation. *Planta* 204: 27–36
- He Z, Von Caemmerer S, Hudson GS, Price GD, Badger MR and Andrews TJ (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase activase deficiency delays senescence of ribulose-1,5-bisphosphate carboxylase/oxygenase but progressively impairs its catalysis during tobacco leaf development. *Plant Physiol* 115: 1569–1580
- Heber U and Walker D (1992) Concerning a dual function of coupled cyclic electron transport in leaves. *Plant Physiol* 100: 1621–1626
- Heber U, Pon NG and Heber M (1963) Localization of carboxydismutase and triosephosphate dehydrogenases in chloroplasts. *Plant Physiol* 38: 355–360
- Heber U, Hallier UW and Hudson MA (1967) Untersuchungen zur intrazellulären Verteilung von Enzymen und Substraten in der Blattzelle. II. Lokalisation von Enzymen des reduktiven und dem oxidativem Pentosephosphat-Zyklus in den Chloroplasten und Permeabilität der Chloroplasten-Membran gegenüber Metaboliten. *Z Naturforsch* 22b: 1200–1215
- Hensel R, Laumann S, Lang J, Heumann H and Lottspeich F (1987) Characterisation of two D-glyceraldehyde-3-phosphate dehydrogenases from the thermophilic archaebacterium *Thermoproteus tenax*. *Eur J Biochem* 170: 325–333
- Henze K (1997) Molekulare Studien zu den Enzymen des Calvin-Zyklus aus *Spinacia oleracea*. Ph.D. thesis. Technische Universität Braunschweig.
- Henze K, Schnarrenberger C, Kellermann J and Martin W (1994) Chloroplast and cytosolic triosephosphate isomerases from spinach: Purification, microsequencing and cDNA cloning of the chloroplast enzyme. *Plant Mol Biol* 26: 1961–1973
- Henze K, Badr A, Wettern M, Cerff R and Martin W (1995) A nuclear gene of eubacterial origin in *Euglena gracilis* reflects cryptic endosymbioses during protist evolution. *Proc Natl Acad Sci USA* 92: 9122–9126
- Henze K, Morrison HG, Sogin ML, Müller M (1998) Sequence and phylogenetic position of a class II aldolase gene in the amitochondriate protist, *Giardia lamblia*. *Gene* 222: 163–168
- Hermoso R, Castillo M, Chueca A, Lazaro JJ, Sahrawy M and Lopez-Gorgé J (1996) Binding site on pea chloroplast fructose-1,6-bisphosphatase involved in the interaction with thioredoxin. *Plant Mol Biol* (1996) 30: 455–465
- Hess WR, Schendel R, Börner T and Rudiger (1991) Reduction in mRNA level for two nuclear encoded light regulated genes in the barley mutant ‘albostrians’ is not correlated with phytochrome content and activity. *J Plant Physiol* 138: 292–298
- Hess WR, Linke B and Börner T (1997) Impact of plastid differentiation on transcription of nuclear and mitochondrial genes. In: Schenk HEA, Herrmann RG, KW Jeon, Müller NE, Schwemmler W (eds) *Eukaryotism and Symbiosis*, pp 233–242. Springer, Heidelberg
- Hess WR, Müller A, Nagy F and Börner T (1994) Ribosome-deficient plastids affect transcription of light-induced nuclear genes: Genetic evidence for a plastid-derived signal. *Mol Gen Genet* 242: 305–312
- Himmo SD, Thomson M and Gubler CJ (1988) Isolation of transketolase from human erythrocytes. *Prep Biochem* 18: 261–276
- Hirsch D (1994) Operons in eukaryotes follow the spliced leader. *Nature* 372: 222–223
- Hoefgen R, Streber WR and Pohlenz HD (1995) Antisense gene expression as a tool for evaluating molecular herbicide targets. *Pesticide Science* 43: 175–177
- Holzfrefe S, Backhausen JE, Kitzmann C, and Scheibe R (1997) Regulation of steady-state photosynthesis in isolated intact chloroplasts under constant light: Responses of carbon fluxes, metabolite pools and enzyme-activation states to changes of electron pressure. *Plant Cell Physiol* 38: 1207–1216
- Houlné G and Schantz R (1988) Characterization of cDNA sequences for LHCl apoprotein in *Euglena gracilis*: The mRNA encodes a large precursor containing several consecutive polypeptides. *Mol Gen Genet* 213: 479–486
- Houlné G and Schantz R (1993) Expression of polyproteins in *Euglena*. *Crit Rev Plant Sci* 12: 1–17
- Hove-Jensen B and Maigaard M (1993) *Escherichia coli rpiA* gene encoding ribose phosphate isomerase A. *J Bacteriol* 175: 5628–5635
- Hovenkamp-Obbema R and Stegwee D (1974) Effect of chloramphenicol on the development of proplastids in *Euglena gracilis*. I. The synthesis of ribulosediphosphate carboxylase, NADP-linked glyceraldehyde-3-phosphate dehydrogenase and aminolaevulinate dehydratase. *Z Pflanzenphysiol* 73: 430–438
- Hudson GS, Evans JR, Von Caemmerer S, Arvidsson YBC and Andrews TJ (1992) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. *Plant Physiol* 98: 294–302
- Huffaker RC, Obendorf RL, Keller CJ and Kleinkopf GE (1966)

- Effects of light intensity on photosynthetic carboxylative phase enzymes and chlorophyll synthesis in greening leaves of *Hordeum vulgare* L. *Plant Physiol.* 41: 913–918
- Hughes J, Lamparter T, Mittmann F, Hartmann E, Gartner W, Wilde A and Börner T (1997) A prokaryotic phytochrome. *Nature* 386: 663–663
- Ireland HMM and Bradbeer JW (1971) Plastid development in primary leaves of *Phaseolus vulgaris*. The effects of D-threo- and L-threo-chloramphenicol on the light-induced formation of enzymes of the photosynthetic carbon pathway. *Planta* 96: 254–261
- Ishii M, Miake T, Satoh T, Sugiyama H, Kodama T and Igarashi Y (1996) Autotrophic carbon dioxide fixation in *Acidianus brierleyi*. *Arch Microbiol.* 166: 368–371
- Jacquot J-P, Lopez-Jaramillo J, Chueca A, Cherfils J, Lemaire S, Chedozeau B, Miginiac-Maslow M, Decottignies P, Wolosiuk RA and Lopez-Gorgé J (1995) High-level expression of recombinant pea chloroplast fructose-1,6-bisphosphatase and mutagenesis of its regulatory site. *Eur J Biochem* 229: 675–681
- Jacquot J-P, Lopez-Jaramillo J, Miginiac-Maslow M, Lemaire S, Cherfils J, Chueca A and Lopez-Gorgé J (1997a) Cysteine-153 is required for redox regulation of pea chloroplast fructose-1,6-bisphosphatase. *FEBS Lett* 401: 143–147
- Jacquot J-P, Lancelin JM and Meyer Y (1997b) Thioredoxins: Structure and function in plant cells. *New Phytol* 136: 543–570
- Jang J-C and Sheen J (1997) Sugar sensing in higher plants. *Trends Plant Sci* 2: 208–214
- Jebarathirajah JA and Coleman JR (1998) Association of carbonic anhydrase with a Calvin cycle enzyme complex in *Nicotiana tabacum*. *Planta* 204: 177–182
- Jiang CZ and Rodermel SR (1995) Regulation of photosynthesis during leaf development in RbcS antisense DNA mutants of tobacco. *Plant Physiol.* 107: 215–224
- Jiang CZ, Quick WP, Alred R, Kliebenstein D and Rodermel SR (1994) Antisense RNA inhibition of Rubisco activase expression. *Plant J.* 5: 787–798
- Jones PG, Lloyd JC and Raines CA (1996) Glucose feeding of intact wheat plants represses the expression of a number of Calvin cycle genes. *Plant Cell Environ* 19: 231–236
- Joshi HM and Tabita FR (1996) A global two component signal transduction system that integrates the control of photosynthesis, carbon dioxide assimilation, and nitrogen fixation. *Proc Natl Acad Sci USA* 93: 14515–14520
- Kagaya K, Nakamura H, Hidaka S, Ejiri S-I and Tsutsumi K (1995) The promoter from the rice nuclear gene encoding chloroplast aldolase confers mesophyll-specific and light-regulated expression in transgenic tobacco. *Mol Gen Genet* 248: 668–674
- Kaldenhoff R, Henningsen U and Richter G (1994) Gene activation in suspension-cultured cells of *Arabidopsis thaliana* during blue-light-dependent plantlet regeneration. *Planta* 195: 182–187
- Kamber L and Feller U (1998) Influence of the activation status and of ATP on phosphoribulokinase degradation. *J Exp Bot* 49: 139–144
- Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpou S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M and Tabata S (1996a) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* 3: 109–136
- Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpou S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M and Tabata S (1996b) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). *DNA Res* 3: 185–209
- Karlan AW and Russell GK (1976) Aldolase levels in wild type and mutant *Euglena gracilis*. *J Protozool* 23: 176–179
- Karmali A, Drake AF and Spencer N (1983) Purification, properties and assay of D-ribulose-5-phosphate 3-epimerase from human erythrocytes. *Biochem J* 211: 617–623
- Karpinski S, Escobar C, Karpinska B, Creissen G and Mullineaux PM (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. *Plant Cell* 9: 627–640
- Keeling PJ and Doolittle WF (1997) Eukaryotic triosephosphate isomerase originated in the mitochondrion. *Proc Natl Acad Sci USA* 94: 1270–1275
- Keller CJ and Huffaker RC (1967) Evidence for in vivo light-induced synthesis of ribulose-1,5-diP carboxylase and phosphoribulokinase in greening barley leaves. *Plant Physiol* 42: 1277–1283
- Kelley PM and Freeling M (1984) Anaerobic expression of maize fructose-1,6-bisphosphate aldolase. *J Biol Chem* 259: 14180–14183
- Kelley PM and Tolan PR (1986) The complete amino acid sequence for the anaerobically induced aldolase derived from cDNA clones. *Plant Physiol* 82: 1076–1080
- Kennedy RA, Pumpho ME and Fox TC (1992) Anaerobic metabolism in plants. *Plant Physiol* 100: 1–6
- Kern D, Kern G, Neef H, Tittmann K, Killenberg-Jabs M, Wikner C, Schneider G and Hubner G (1997) How thiamine diphosphate is activated in enzymes. *Science* 275: 67–70
- Kersanach R, Brinkmann H, Liaud M-F, Zhang D-X, Martin W and Cerff R (1994) Five identical intron positions in ancient duplicated genes of eubacterial origin. *Nature* 367: 387–389
- Kingston-Smith AH, Harbinson J, Williams J and Foyer CH (1997) Effect of chilling on carbon assimilation, enzyme activation, and photosynthetic electron transport in the absence of photoinhibition in maize leaves. *Plant Physiol* 114: 1039–1046
- Kishore R, Muchhal U and Schwartzbach S (1993) The presequence of *Euglena* LHCII, a cytoplasmically synthesized chloroplast protein, contains a functional endoplasmatic reticulum-targeting domain. *Proc Natl Acad Sci USA* 90: 11845–11849
- Kitaoka S, Nakano Y, Miyatake K and Yokota A (1989) Enzymes and their functional location. In: Buetow DE (ed) *The Biology of Euglena*, Vol 6, Subcellular Biochemistry and Molecular Biology, pp 2–135. Acad Press, San Diego
- Kitayama M, Togasaki RK (1995) Purification and cDNA isolation of chloroplastic phosphoglycerate kinase from *Chlamydomonas reinhardtii*. *Plant Physiol* 107: 393–400
- Kloppstech K (1997) Light regulation of photosynthetic genes.

- Physiol Plant 100: 739–747
- Knaff DB and Hirasawa M (1991) Ferredoxin-dependent chloroplast enzymes. *Biochim Biophys Acta* 1056: 93–125
- Koch KE (1996) Carbohydrate-modulated gene expression in plants. *Ann Rev Plant Physiol Mol Biol* 47: 509–540
- Köhler U, Mendel RR, Cerff R and Hehl R (1996a) A promoter for strong and ubiquitous anaerobic gene expression in tobacco. *Plant J* 10: 175–183
- Köhler U, Donath M, Mendel RR, Cerff R and Hehl R (1996b) Intron-specific stimulation of anaerobic gene expression and splicing efficiency in maize cells. *Mol Gen Genet* 251: 252–258
- Kohlhoff M, Dahm A and Hensel R (1996) Tetrameric triosephosphate isomerase from hyperthermophilic Archaea. *FEBS Lett* 383: 245–250
- Koksharova O, Schubert M, Shestakov S and Cerff R (1998) Genetic and biochemical evidence for distinct key functions of two highly divergent GAPDH genes incatabolic and anabolic carbon flow of the cyanobacterium *Synechocystis* sp. PCC6803. *Plant Mol Biol* 36: 183–194
- Köpke-Seconde E, Molnar I and Schnarrenberger C (1990) Isolation and characterization of the cytosolic and chloroplastic 3-phosphoglycerate kinase from spinach leaves. *Plant Physiol* 93: 40–47
- Kossmann J, Mueller-Roeber B, Dyer T A, Raines C A, Sonnewald U and Willmitzer L (1992) Cloning and expression analysis of the plastidic fructose-1,6-bisphosphatase coding sequence from potato: Circumstantial evidence for the import of hexoses into chloroplasts. *Planta* 188: 7–12
- Kossmann J, Sonnewald U and Willmitzer L (1994) Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth. *Plant J* 6: 637–650
- Kozaki A and Takeba G (1996) Photorespiration protects C₃ plants from photooxidation. *Nature* 384: 557–560
- Kramer DM, Wise RR, Frederick JR, Alm DM, Hesketh JD, Ort DR and Crofts AR (1990) Regulation of coupling factor in field-grown sunflower: A redox model relating coupling factor activity to the activities of other thioredoxin-dependent chloroplast enzymes. *Photosynth Res* 16: 213–222
- Krämer S, Stitt M and Heldt HW (1988) Mitochondrial oxidative phosphorylation participating in photosynthetic metabolism of a leaf cell. *FEBS Lett* 226: 352–356
- Krapp A, Quick WP and Stitt M (1991) Ribulose-1,5-bisphosphate carboxylase-oxygenase, other Calvin cycle enzymes, and chlorophyll decrease when glucose is supplied to mature spinach leaves via the transpiration stream. *Planta* 186: 58–69
- Krapp A, Hoffmann B, Schaefer C and Stitt M (1993) Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: A mechanism for the sink regulation of photosynthesis. *Plant J* 3: 817–828
- Krapp A, Chaves MM, David MM, Rodrigues ML, Pereira JS and Stitt M (1994) Decreased ribulose-1,5-bisphosphate carboxylase/oxygenase in transgenic tobacco transformed with ‘antisense’ rbcS: VIII. Impact on photosynthesis and growth in tobacco growing under extreme high irradiance and high temperature. *Plant Cell Environ* 17: 945–953
- Krishnan G and Altekar W (1991) An unusual class I (Schiff base) fructose-1,6-bisphosphate aldolase from the halophilic archaeabacterium *Haloarcula vallismortis*. *Eur J Biochem* 195: 343–350
- Krüger I and Schnarrenberger C (1983) Purification, subunit structure and immunochemical properties of fructose-bisphosphate aldolases from spinach and corn leaves. *Eur J Biochem* 136: 101–106
- Krüger I and Schnarrenberger C (1985) Development of cytosol and chloroplast aldolases during germination of spinach seeds. *Planta* 164: 109–114
- Kurzok H-G and Feierabend J (1984) Comparison of a cytosolic and a chloroplast triosephosphate isomerase isoenzyme from rye leaves. I. Purification and catalytic properties. *Biochim Biophys Acta* 788: 214–221
- Kurzok H-G and Feierabend J (1986) Comparison of the development and site of synthesis of a cytosolic and a chloroplast isoenzyme of triosephosphate isomerase in rye leaves. *J Plant Physiol* 126: 207–212
- Kusian B and Bowien B (1997) Organization and regulation of *ebb* CO₂ assimilation genes in autotrophic bacteria. *FEMS Microbiol Rev* 21: 135–155
- Kusian B, Yoo JG, Bednarski R and Bowien B (1992) The Calvin cycle enzyme pentose-5-phosphate 3-epimerase is encoded within the *cfx* operons of the chemoautotroph *Alcaligenes eutrophus*. *J Bacteriol* 174: 7337–7344
- Kwon H-B, Park S-C, Peng H-P, Goodman HM, Dewdney J and Shih M-C (1994) Identification of a light responsive region of the nuclear gene encoding the B subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. *Plant Physiol* 105: 357–367
- Lam E and Chua N-H (1990) GT-1 binding site confers light responsive expression in transgenic tobacco. *Science* 248: 471–474
- Lambert DH, Bryant DA, Stirewalt VL, Dubbs JM, Stevens SE Jr and Porter RD (1985) Gene map for the *Cyanophora paradoxa* cyanelle genome. *J Bacteriol* 164: 659–664
- Latzko E and Gibbs M (1968) Distribution and activity of enzymes of the reductive pentose phosphate cycle in spinach leaves and in chloroplasts isolated by different methods. *Z Pflanzenphysiol* 59: 184–194
- Latzko E and Gibbs M (1969) Enzyme activities of the carbon reduction cycle in some photosynthetic organisms. *Plant Physiol* 44: 295–300
- Latzko E and Kelly GJ (1979) Enzymes of the reductive pentose phosphate cycle. *Encyclopedia Plant Physiol (New Series)* 6: 239–250
- Lauerer M, Saftic D, Quick WP, Labate C, Fichtner K, Schulze E-D, Rodermel SR, Bogorad L and Stitt M (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ rbcS. *Planta* 190: 332–345
- Lebherz HG, Leadbetter MM and Bradshaw RA (1984) Isolation and characterization of the cytosol and chloroplast form of spinach leaf fructose diphosphate aldolase. *J Biol Chem* 259: 10111–10117
- Lebreton S, Gontero B, Avilan L and Ricard J (1997) Memory imprinting effects in multienzyme complexes. II. Kinetics of the bienzyme complex from *Chlamydomonas reinhardtii* and hysteretic activation of chloroplast oxidized phosphoribulokinase. *Eur J Biochem* 246: 85–91
- Leegood RC (1990) Enzymes of the Calvin cycle. *Methods Plant Biochem* 3: 15–37
- Li L-A and Tabita FR (1994) Transcription control of ribulose bisphosphate carboxylase/oxygenase activase and adjacent

- genes *mAnabaena* species. J Bacteriol 176: 6697–6706
- Li L-A and Tabita FR (1997) Maximum activity of recombinant ribulose 1,5-bisphosphate carboxylase/oxygenase of *Anabaena* sp. strain CA requires the product of the *rbcX* gene. J Bacteriol 179: 3793–3796
- Liaud MF, Brandt U, Scherzinger M and Cerff R (1997) Evolutionary origin of cryptomonad microalgae: Two novel chloroplast/cytosol-specific GAPDH genes as potential markers of ancestral endosymbiont and host cell components. J Mol Evol 44 :S28–37
- Lloyd JC, Horsnell PR, Dyer TA and Raines CA (1991a) Structure and sequence of a wheat phosphoribulokinase gene. Plant Mol Biol 17: 167–168
- Lloyd JC, Raines CA, John UP and Dyer TA (1991b) The chloroplast FBPase gene of wheat: Structure and expression of the promoter in photosynthetic and meristematic cells of transgenic tobacco plants. Mol Gen Genet 225: 209–216
- Loferer H and Hennecke H (1994) Protein disulfide oxidoreductases in bacteria. Trends Biochem Sci 19: 169–171
- Longstaff M, Raines CA, McMorrow EM, Bradbeer JW and Dyer TA (1989) Wheat phosphoglycerate kinase: Evidence for recombination between the genes for the chloroplastic and cytosolic enzymes. Nucl Acid Res 17: 6569–6580
- Lopez-Jaramillo J, Chueca A, Jacquot J-P, Hermoso R, Lazaro JJ, Sahrawy M and Lopez-Gorgé J (1997) High-yield expression of pea thioredoxin m and assessment of its efficiency in chloroplast fructose-1,6-bisphosphatase activation. Plant Physiol 114: 1169–1175
- Lorimer GH and Miziorko H (1980) Carbamate formation of the *ε*-amino group of a lysyl residue as the basis for the activation of ribulose-1,5-bisphosphate carboxylase by CO_2 and Mg^{2+} . Biochemistry 19: 5321–5328
- Macdonald FD and Buchanan BB (1990) The reductive pentose phosphate pathway and its regulation. In: Dennis DT, Turpin DH (eds) Plant Physiology, Biochemistry and Molecular Biology, pp 249–262. Longman, Singapore
- Macioszek J and Anderson LE (1987) Changing kinetic properties of the two-enzyme phosphoglycerate kinase/NADP-linked glyceraldehyde-3-phosphate dehydrogenase couple from pea chloroplasts during photosynthetic induction. Biochim Biophys Acta 892: 185–190
- Macioszek J, Anderson JB and Anderson LE (1987) Isolation of chloroplastic phosphoglycerate kinase/glyceraldehyde-3-phosphate couple. Plant Physiol 94: 291–296
- Maier U-G (1992) The four genomes of the cryptomonad alga *Pyrenomonas salina*. BioSystems 28: 69–74
- Malhorta OP, Kumar A and Tikoo K (1987) Isolation and quaternary structure of a complex of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase. Ind J Biochem Biophys 24: 16–20
- Malik A (1997) Genexpression der Class I- und Class II-Aldolase in *Euglena gracilis*. Thesis, Freie Universität Berlin
- Marcus A (1960) Photocontrol of formation of red kidney bean leaf triphosphopyridine nucleotide linked triosephosphate dehydrogenase. Plant Physiol 35: 126–128
- Marcus F and Harrisch PB (1990) Amino acid sequence of spinach chloroplast fructose-1,6-bisphosphatase. Arch Biochem Biophys 279: 151–157
- Marcus F, Moberly L and Latshaw SP (1988) Comparative amino acid sequence of fructose-1,6-bisphosphatases: Identification of a region unique to the light-regulated chloroplast enzyme. Proc Natl Acad Sci USA 85: 5379–5383
- Markos A, Miretsky A and Müller M (1993) A glyceraldehyde-3-phosphate dehydrogenase with eubacterial features in the amitochondriate eukaryote *Trichomonas vaginalis*. J Mol Evol 37: 631–643
- Marques IA, Ford DM, Muschink G and Anderson LE (1987) Photosynthetic carbon metabolism in isolated pea chloroplasts: metabolite levels and enzyme activities. Arch Biochem Biophys 252: 458–466
- Marsh JJ and Lebherz HG (1992) Fructose-bisphosphate aldolases: an evolutionary history. Trends Biochem Sci 17: 110–113
- Martin W and Herrmann RG (1998) Gene transfer from organelles to the nucleus: How much, what happens and why? Plant Physiol 118: 9–17
- Martin W and Müller M (1998) The hydrogen hypothesis for the first eukaryote. Nature 392: 37–41
- Martin W and Schnarrenberger C (1997) The evolution of the Calvin cycle from prokaryotic to eukaryotic chromosomes: A case study of functional redundancy in ancient pathways through endosymbiosis. Curr Genet 32: 1–18
- Martin W, Brinkmann H, Savona C and Cerff R (1993) Evidence for a chimaeric nature of nuclear genomes: Eubacterial origin of eukaryotic glyceraldehyde-3-phosphate dehydrogenase genes. Proc Natl Acad Sci USA 90: 8692–8696
- Martin W, Mustafa A-Z, Henze K and Schnarrenberger C (1996a) Higher plant chloroplast and cytosolic fructose-1,6-bisphosphatase isoenzymes: Origins via duplication rather than prokaryote-eukaryote divergence. Plant Mol Biol 32: 485–491
- Martin W, Henze K, Kellermann J, Flechner A and Schnarrenberger C (1996b) Microsequencing and cDNA cloning of the Calvin cycle/OPPP enzyme ribose-5-phosphate isomerase (EC 5.3.1.6) from spinach chloroplasts. Plant Mol Biol 30: 795–805
- Martin W, Stoebe B, Goremykin V, Hansmann S, Hasegawa M, and Kowallik KV (1998) Gene transfer to the nucleus and the evolution of chloroplasts. Nature 393: 162–165
- Masle J, Hudson GS and Badger MR (1993) Effects of ambient CO_2 concentration on growth and nitrogen use in tobacco (*Nicotiana tabacum*) plants transformed with an antisense gene to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol 103: 1075–1088
- Mate CJ, Hudson GS, Von Caemmerer S, Evans JR and Andrews TJ (1993) Reduction of ribulose bisphosphate carboxylase activase levels in tobacco (*Nicotiana tabacum*) by antisense RNA reduces ribulose bisphosphate carboxylase carbamylation and impairs photosynthesis. Plant Physiol 102: 1119–1128
- McFadden G, Gilson PR, Hoffmann CJB, Adecock GJ and Maier U-G (1994) Evidence that an amoeba acquired a chloroplast by retaining part of an engulfed eukaryotic alga. Proc Natl Acad Sci USA 91: 3690–3694
- McFadden GI, Gilson PR, Douglas SE, Cavalier-Smith T, Hofmann CJ and Maier UG (1997) Bonsai genomics: Sequencing the smallest eukaryotic genomes. Trends Genet 13: 46–49
- McMorrow EM and Bradbeer JW (1990) Separation, purification, and comparative properties of chloroplast and cytoplasmic phosphoglycerate kinase from barley leaves. Plant Physiol 93: 374–383
- McNeil PH and Walker DA (1981) The effect of magnesium and other ions on the distribution of ribulose 1,5-bisphosphate

- carboxylase in chloroplast extracts. *Arch Biochem Biophys* 208:184–188
- Meijer WG, Van Den Bergh ERE and Smith LM (1996) Induction of the *gap-pfk* operon encoding glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase of *Xanthobacter flavus* requires the LysR-type transcriptional activator CbbR. *J Bacteriol* 178: 881–887
- Melkonian M (1996) Systematics and evolution of the algae: Endocytobiosis and the evolution of the major algal lineages. *Prog Bot* 57: 281–311
- Meyer-Gauen G, Schnarrenberger C, Cerff R and Martin W (1994) Molecular characterization of a novel, nuclear-encoded, NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase in plastids of the gymnosperm *Pinus sylvestris* L. *Plant Mol Biol* 26: 1155–1166
- Meyer-Gauen G, Herbrandt H, Pahnke J, Tholl D, Cerff R and Martin W (1998) Gene structure, expression in *Escherichia coli* and biochemical properties of the **NAD⁺-dependent** glyceraldehyde-3-phosphate dehydrogenase from *Pinus sylvestris* chloroplasts. *Gene* 209: 167–174
- Michalowski CB, Derocher EJ, Bohnert HJ and Salvucci ME (1992) Phosphoribulokinase from ice plant: Transcription, transcripts and protein expression during environmental stress. *Photosyn Res* 31: 127–138
- Michels PAM, Marchand M, Kohl L, Allert S, Vellieux FMD, Wierenga R and Opperdoes F (1991) The cytosolic and glycosomal isozymes of glyceraldehyde-3-phosphate dehydrogenase in *Trypanosoma brucei* have a distant evolutionary relationship. *Eur J Biochem* 198: 421–428
- Michels S, Rogalska E and Branlant G (1996) Phosphate binding sites in phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. *Eur J Biochem* 235: 641–647
- Miles AJ, Potts SC, Willingham NM, Raines CA and Lloyd JC (1993) A light- and developmentally-regulated DNA-binding interaction is common to the upstream sequences of the wheat Calvin cycle bisphosphatase genes. *Plant Mol Biol* 22: 507–516
- Mills JD and Mitchell P (1982) Modulation of coupling factor ATPase activity in intact chloroplasts. Reversal of thiol modulation in the dark. *Biochim Biophys Acta* 679: 75–83
- Mo Y, Harris BG and Gracy RW (1973) Triosephosphate isomerase and aldolases from light and dark-grown *Englea gracilis*. *Arch Biochem Biophys* 157: 580–587
- Mojzsis SJ, Arrhenius G, McKeegan KD, Harrison TM, Nutman AP and Friend CR (1996) Evidence for life on Earth before 3,800 million years ago. *Nature* 384: 55–59
- Moorhead BG and Plaxton WC (1990) Purification and characterization of cytosolic aldolase from carrot storage root. *Biochem J* 269: 133–139
- Mora-Garcia S, Rodriguez-Suarez R and Wolosiuk RA (1998) Role of electrostatic interactions on the affinity of thioredoxin for target proteins. Recognition of chloroplast fructose-1, 6-bisphosphatase by mutant *Escherichia coli* thioredoxins. *J Biol Chem* 273: 16273–16280
- Morse D, Salois P, Markovic P and Hastings JW (1995) A nuclear-encoded form II RuBisCO in dinoflagellates. *Science* 268:1622–1624
- Mosley CS, Suzuki JY and Bauer CE (1994) Identification and molecular genetic characterization of a sensor kinase responsible for coordinately regulating light harvesting and reaction center gene expression in response to anaerobiosis. *J Bacteriol* 176: 7566–7573
- Müller B (1972) A labile CO₂-fixing enzyme complex in spinach chloroplasts. *Z Naturforsch* 27b: 925–932
- Müller B, Ziegler I and Ziegler H (1969) Lichtinduzierte reversible Aktivitätssteigerung der Glyceraldehyd-3-phosphat-Dehydrogenase in Chloroplasten. *Eur J Biochem* 9: 101–106
- Muschak M, Hoffmann-Benning S, Fuss H, Kossmann J, Willmitzer L, and Fisahn J (1997) Gas exchange and ultrastructural analysis of transgenic potato plants expressing mRNA antisense construct targeted to the cp-fructose-1,6-bisphosphate phosphatase. *Photosynthetica* 33: 455–465
- Nagashima H and Fukuda I (1983) Floridosides in unicellular hot spring algae. *Phytochemistry* 22, 1949–1951
- Nakamura H, Satoh W, Hidaka S, Kagaya Y, Ejiri S and Tsutsumi K. (1996) Genomic structure of the rice aldolase isozyme C-1 gene and its regulation through a **Ca²⁺-mediated** protein kinase-phosphatase pathway. *Plant Mol Biol* 30: 381–385
- Nakamura H, Tokairin Y, Tamayama S, Kon S, Hidaka S, Ejiri S and Tsutsumi K (1997) Cell type- and positionally specific regulation of the aldolase P gene expression in rice seedlings. *Biosci Biotechnol Biochem* 61: 256–262
- Neuhaus HE, Batz O, Thorn E and Scheibe R (1993) Purification of highly intact plastids from various heterotrophic plant tissues: Analysis of enzymic equipment and precursor dependency for starch biosynthesis. *Biochem J* 296: 395–401
- Nicholson S, Esterby JS and Powls R (1987) Properties of a multimeric protein complex from chloroplasts possessing potential activities of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. *Eur J Biochem* 162: 423–431
- Nikkola M, Lindqvist Y and Schneider G (1994) Refined structure of transketolase from *Saccharomyces cerevisiae* at 2.0 Å resolution. *J Mol Biol* 238: 387–404
- Nilsson U, Meshalkina L, Lindqvist Y and Schneider G (1997) Examination of substrate binding in thiamin diphosphate-dependent transketolase by protein crystallography and site-directed mutagenesis. *J Biol Chem* 272: 1864–1869
- Nishimura M and Beevers H (1981) Isoenzymes of sugar phosphate metabolism in endosperm of germinating castor beans. *Plant Physiol* 67: 1255–1258
- Nishizawa AN and Buchanan BB (1981) Enzyme regulation in C4 photosynthesis: Purification and properties of the thioredoxin-linked fructose bisphosphatase and sedoheptulose bisphosphatase from corn leaves. *J Biol Chem* 256: 6119–6126
- Nowitzki U, Westhoff P, Henze K, Schnarrenberger C and Martin W (1995) Cloning of the amphibolic Calvin cycle/OPPP enzyme D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) from spinach chloroplasts: Functional and evolutionary aspects. *Plant Mol Biol* 29: 1279–1291
- Ocheretina O and Scheibe R (1994) Cysteines of chloroplast NADP-malate dehydrogenase form mixed disulfides. *FEBS Lett* 355: 254–258
- Ocheretina O, Harnecker J, Rother T, Schmid R and Scheibe R (1993) Effects of N-terminal truncations upon chloroplast NADP-malate dehydrogenase from pea and spinach. *Biochim Biophys Acta* 1163: 10–16
- Oelmüller R and Mohr H (1985) Specific action of blue light on phytochrome-mediated enzyme syntheses in the shoot of milo (*Sorghum vulgare*). *Plant Cell Environ* 8: 27–32

- Ostrem JA, Vernon DM and Bohnert HJ (1990) Increased expression of a gene coding for NAD:glyceraldehyde-3-phosphate dehydrogenase during the transition from C-3 photosynthesis to crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *J Biol Chem* 265: 3497–3502
- Otto S and Feierabend J (1989) Enzymes of starch and sugar phosphate metabolism in achlorophyllous ribosome-deficient plastids from high-temperature-grown rye leaves. *Physiol Plant* 76: 65–73
- Pacold ME and Anderson LE (1975) Chloroplast and cytosolic enzymes. VI. Pea leaf 3-phosphoglycerate kinases. *Plant Physiol* 55: 168–171
- Pacold ME, Anderson LE, Li D and Stevens FJ (1995) Redox sensitivity and light modulation of enzyme activity in the rhodophytes *Gracilaria tikvahiae* and *Chondrus crispus*. *J Phycol* 31:297–301
- Paoli GC, Morgan NS, Tabita FR and Shively JM (1995) Expression of the *cbbLcbbS* and *cbbM* genes and distinct organization of the cbb Calvin cycle structural genes of *Rhodobacter capsulatus*. *Arch Microbiol* 164: 396–405
- Park SC, Kwon HB and Shih MC (1996) Cis-acting elements essential for light regulation of the nuclear gene encoding the A subunit of chloroplast glyceraldehyde 3-phosphate dehydrogenase in *Arabidopsis thaliana*. *Plant Physiol* 112: 1563–1571
- Paul MJ, Knight JS, Habash D, Parry MAJ, Lawlor DW, Barnes SA, Loynes A and Gray JC (1995) Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: Effect on CO_2 assimilation and growth in low irradiance. *Plant J* 7: 535–542
- Pelzer-Reith B, Freund S, Schnarrenberger C, Yatsuki H and Hori K (1995) The plastid aldolase gene from *Chlamydomonas reinhardtii*: Intron/exon organization, evolution, and promoter structure. *Mol Gen Genet* 248: 481–486
- Pelzer-Reith B, Malik A, Wiegand S and Schnarrenberger C (1994a) Post-transcriptional expression of class I and class II aldolase in synchronized *Euglena gracilis*. In: Markert CL, Scandalios JG, Lim HA and Serov OL (eds) Isozymes: Organization and Roles in Evolution, Genetics and Physiology, pp 179–190. World Scientific Publishing, Singapore
- Pelzer-Reith B, Wiegand S and Schnarrenberger C (1994b) Plastid class I and cytosol class II aldolase of *Euglena gracilis*. Purification and characterization. *Plant Physiol* 106: 1137–1144
- Pettersson G and Ryde-Pettersson U (1989) Dependence of the Calvin cycle activity on kinetic parameters for the interaction of non-equilibrium cycle enzymes with their substrates. *Eur J Biochem* 186: 683–687
- Pfannschmidt T, Nilsson A, and Allen JF (1999) Photosynthetic control of chloroplast gene expression. *Nature* 397: 625–628
- Plaumann M, Pelzer-Reith B, Martin W and Schnarrenberger C (1997) Cloning of fructose-1,6-bisphosphate aldolases from *Euglena gracilis*: Multiple recruitment of class I aldolase to chloroplasts and eubacterial origin of eukaryotic class II aldolase genes. *Curr Genet* 31: 430–438
- Plaxton WC (1996) The organization and regulation of plant glycolysis. *Ann Rev Plant Physiol Plant Mol Biol* 47:185–214
- Pohlmeyer K, Paap BK, Soll J and Wedel N (1996) CP12: a small nuclear-encoded chloroplast protein provides novel insights into higher-plant GAPDH evolution. *Plant Mol Biol* 32: 969–978
- Porter MA, Milanez S, Stringer CD and Hartman FC (1986) Purification and characterization of ribulose-5-phosphate kinase from spinach. *Arch Biochem Biophys* 245: 14–23
- Porter MA, Stringer CD and Hartman FC (1988) Characterization of the regulatory thioredoxin site of phosphoribulokinase. *J Biol Chem* 263: 123–129
- Portis AR (1992) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity. *Ann Rev Plant Physiol Plant Mol Biol* 43: 415–437
- Pratt LH (1995) Phytochromes: Differential properties, expression pattern and molecular evolution. *Photochem Photobiol* 61: 10–21
- Price GD, Evans JR, Von Caemmerer S, Yu JW and Badger MR (1995) Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO_2 assimilation via a reduction in ribulose biphosphate regeneration in transgenic tobacco plants. *Planta* 195: 369–378
- Prüß B, Meyer HE and Holldorf AW (1993) Characterization of the glyceraldehyde-3-phosphate dehydrogenase from the extremely halophilic archaeabacterium *Haloarcula vallismortis*. *Arch Microbiol* 160: 5–11
- Pupillo P and Faggiani R (1979) Subunit structure of three glyceraldehyde-3-phosphate dehydrogenases of some flowering plants. *Arch Biochem Biophys* 194: 581–592
- Pupillo P and Giuliani-Piccarri G (1975) The reversible depolymerization of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. Interactions with nucleotides and dithiothreitol. *Eur J Biochem* 51: 475–482
- Purcell M, Mabrouk YM and Bogorad L (1995) Red/far-red and blue light-responsive regions of maize rbcS-m3 are active in bundle sheath and mesophyll cells, respectively. *Proc Natl Acad Sci USA* 92: 11504–11508
- Qian Y and Tabita FR (1996) A global signal transduction system regulates aerobic and anaerobic CO_2 fixation in *Rhodobacter sphaeroides*. *J Bacteriol* 178: 12–18
- Quick WP, Schurr U, Scheibe R, Schulze ED, Rodermel SR, Bogorad L and Stitt M (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ rbcS: I. Impact on photosynthesis in ambient growth conditions. *Planta* 183: 542–554
- Quick WP, Fichtner K, Schulze ED, Wendler R, Leegood RC, Mooney H, Rodermel SR, Bogorad L and Stitt M (1992) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ rbcS: IV. Impact on photosynthesis in conditions of altered nitrogen supply. *Planta* 188: 522–531
- Quigley F, Martin W and Cerff R (1988) Intron conservation across the prokaryote-eukaryote boundary: Structure of the nuclear gene for chloroplast glyceraldehyde-3-phosphate dehydrogenase from maize. *Proc Natl Acad Sci USA* 85: 2672–2676
- Ragsdale SW (1991) Enzymology of the acetyl-CoA pathway of CO_2 fixation. *Crit Rev Biochem Mol Biol* 26: 261–300
- Raines CA, Lloyd JC, Longstaff M, Bradley D and Dyer TA (1988) Chloroplast fructose-1,6-bisphosphatase: the product of a mosaic gene. *Nucleic Acid Res* 16: 7931–7942
- Raines CA, Longstaff M, Lloyd JC and Dyer TA (1989) Complete coding sequence of wheat phosphoribulokinase: developmental and light-dependent expression of the mRNA. *Mol Gen Genet* 220: 43–48

- Raines CA, Lloyd JC, Willingham NM, Potts S and Dyer TA (1992) cDNA and gene sequences of wheat chloroplast sedoheptulose-1,7-bisphosphatase reveal homology with fructose-1,6-bisphosphatases. *Eur J Biochem* 205:1053–1059
- Raines CA, Lloyd JC and Dyer TA (1998) New insights into the structure and function of sedoheptulose-1,7-bisphosphatase, an important but neglected Calvin cycle enzyme. *J Exp Bot* 50: 1–8
- Rajagopalan R and Altekar W (1994) Characterisation and purification of ribulose-bisphosphate carboxylase from heterotrophically grown halophilic archaeabacterium, *Haloferax mediterranei*. *Eur J Biochem* 221: 863–869
- Rathnam CKM (1978) Malate and dihydroxyacetone phosphate-dependent nitrate reduction in spinach leaf protoplasts. *Plant Physiol* 62: 220–223
- Rault M, Guidici-Orticoni M-T, Gontero B and Ricard J (1993) Structural and functional properties of a multi-enzyme complex from spinach chloroplasts. I. Stoichiometry of the polypeptide chains. *Eur J Biochem* 217: 1065–1073
- Rawal N, Kelkar SM and Altekar W (1988) Ribulose 1,5-bisphosphate dependent CO_2 fixation in the halophilic archaeabacterium, *Halobacterium mediterranei*. *Biochem Biophys Res Commun* 156: 451–456
- Reddy PS, Ramanjulu S, Sudhakar C and Veeranjaneyulu K (1998) Differential sensitivity of stomatal and non-stomatal components NaCl or Na_2SO_4 salinity in horsegram, *Macrotyloma uniflorum* (Lam.). *Photosynthetica* 35: 99–105
- Reinbothe S, Reinbothe C and Parthier B (1991a) Glucose repression of chloroplast development in *Euglena gracilis*. *Plant Physiol Biochem* 29: 297–307
- Reinbothe S, Reinbothe C, Krauspe R and Parthier B (1991b) Changing gene expression during dark-induced dedifferentiation in *Euglena gracilis*. *Plant Physiol Biochem* 29: 309–318
- Ricard B, Rivoal J and Pradet A (1989) Rice cytosolic glyceraldehyde 3-phosphate dehydrogenase contains two subunits differentially regulated by anaerobiosis. *Plant Mol Biol* 12: 131–139
- Ricard J, Giudici-Orticoni MT and Gontero B (1994) The modulation of enzyme reaction rates within multi-enzyme complexes: 1. Statistical thermodynamics of information transfer through multi-enzyme complexes. *Eur J Biochem* 226: 993–998
- Roberts DL, Runquist JA, Miziorko HM and Kim JJ (1995) Crystallization and preliminary X-ray crystallographic analysis of phosphoribulokinase from *Rhodobacter sphaeroides*. *Protein Sci* 4: 2442–2443
- Robinson BH and Chun K. (1993) The relationships between transketolase, yeast pyruvate decarboxylase and pyruvate dehydrogenase of the pyruvate dehydrogenase complex. *FEBS Lett* 328: 99–102
- Robinson JM, Smith MG and Gibbs M (1980) Influence of hydrogen peroxide upon carbon dioxide photoassimilation in the spinach chloroplast. *Plant Physiol* 65: 755–759
- Robinson SP and Walker DA (1981) Photosynthetic carbon reduction cycle. In: Hatch MD and Boardman NK (eds) *Biochemistry of Plants*, Vol 8, *Photosynthesis*, pp 193–236. Academic Press, New York
- Rodermel SR, Abbott MS and Bogorad L (1988) Nuclear-organelle interactions: Nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell* 55: 673–682
- Rodermel SR, Haley J, Jiang CZ, Tsai CH and Bogorad L (1996) A mechanism for intergenomic integration: Abundance of ribulose bisphosphate carboxylase small-subunit protein influences the translation of the large-subunit mRNA. *Proc Natl Acad Sci USA* 93: 3881–3885
- Rodriguez-Suarez RJ and Wolosiuk RA (1995) High level expression in *Escherichia coli*, purification and properties of fructose-1,6-bisphosphatase from rapeseed (*Brassica napus*) leaves. *Photosynth Res* 46: 313–322
- Rodriguez-Suarez RJ, Mora-Garcia S and Wolosiuk RA (1997) Characterization of cysteine residues involved in the reductive activation and the structural stability of rapeseed (*Brassica napus*) chloroplast fructose-1,6-bisphosphatase. *Biochem Biophys Res Commun* 232: 388–393
- Rowan R, Whitney SM, Fowler A and Yellowlees D (1996) Rubisco in marine symbiotic dinoflagellates: form 11 enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. *Plant Cell* 8: 539–553
- Rozario C, Morin L, Roger A, Smith M and Müller M (1996) Primary structure and phylogenetic relationships of glyceraldehyde-3-phosphate dehydrogenase genes of free-living and parasitic diplomonad flagellates. *J Euk Microbiol* 43:330–340
- Runquist JA, Narasimhan C, Wolff CE, Koteiche HA and Miziorko HM (1996) *Rhodobacter sphaeroides* phosphoribulokinase: Binary and ternary complexes with nucleotide substrate analogs and effectors. *Biochemistry* 35: 15049–15056
- Russel DA and Sachs MM (1989) Differential expression and sequence analysis of the maize glyceraldehyde-3-phosphate dehydrogenase gene family. *Plant Cell* 1: 793–803
- Rutner AC (1970) Spinach 5-phosphoribose isomerase. Purification and properties of the enzyme. *Biochemistry* 9: 178–184
- Rutter WJ (1964) Evolution of aldolase. *Fed Proc* 23: 1248–1257
- Sachs MM (1994) Gene expression during anoxia. In: AS Basra (ed) *Stress-Induced-Gene Expression in Plants*, pp 87–102. Horwood Acad Publ, Chur
- Sahrawy M, Chueca A, Hermoso R, Lazaro JJ and Lopez-Gorge J (1997) Directed mutagenesis shows that the preceding region of the chloroplast fructose-1,6-bisphosphatase regulatory sequence is the thioredoxin docking site. *J Mol Biol* 269: 623–630
- Sainis JK and Harris GC (1986) The association of ribulose-1,5-bisphosphate carboxylase-oxygenase with phosphoribulokinase and phosphoribulokinase. *Biochem Biophys Res Commun* 139: 947–954
- Sainis JK and Srinivasan VT (1993) Effect of the state of water as studied by pulsed NMR on the function of RUBISCO in a multienzyme complex. *J Plant Physiol* 142: 564–568
- Sainis JK, Merriam K and Harris GC (1989) The association of D-ribulose-1,5-bisphosphate carboxylase/oxygenase with phosphoribulokinase. *Plant Physiol* 89: 368–374
- Sarokin LP and Chua N-H (1992) Binding sites for two novel phosphoproteins, 3AF5 and 3AF3, are required for rbcS-3A expression. *Plant Cell* 4: 473–483
- Sasajima K, Yoneda M (1974) Simple procedures for the preparation of D-ribose-5-phosphate ketol-isomerase, D-ribulose-5-phosphate 3-epimerase and D-sedoheptulose-1,7-bisphosphate : D-glyceraldehyde-3-phosphate glycoaldehyde transferase. *Agr Biol Chem* 7: 1297–303

- Sassenrath-Cole GF, Pearcy RW and Steinmaus S (1994) The role of enzyme activation state in limiting carbon assimilation under variable light conditions. *Photosynth Res* 41: 295–302
- Scagliarini S, Trost P, Pupillo P and Valenti V (1993) Light activation and molecular mass changes of NAD(P)-glyceraldehyde 3-phosphate dehydrogenase of spinach and maize leaves. *Planta* 190: 313–319
- Scagliarini S, Trost P and Pupillo P (1998) The non-regulatory isoform of NAD(P)-glyceraldehyde-3-phosphate dehydrogenase from spinach chloroplasts. *J Exp Bot* 49: 1307–1315
- Schaefer DG and Zryd JP (1997) Efficient gene targeting in the moss *Physcomitrella patens*. *Plant J* 11: 1195–1206
- Schäfer S, Barkowski C and Fuchs G (1986) Carbon assimilation by the autotrophic thermophilic archaebacterium *Thermoproteus neutrophilus*. *Arch Microbiol* 146: 301–308
- Scheibe R (1990) Light/dark modulation: Regulation of chloroplast metabolism in a new light. *Bot Acta* 103: 327–334
- Scheibe R (1991) Redox-modulation of chloroplast enzymes. A common principle for individual control. *Plant Physiol* 96: 1–3
- Scheibe R (1995) Light-modulation of stromal enzymes. In: MA Madore and WJ Lucas (eds) *Carbon Partitioning and Sucrose-Sink Interactions in Plants*, pp 13–22. American Society of Plant Physiologists, Rockville
- Scheibe R and Beck E (1994) The malate valve: Flux control at the enzymic level. In: Schulze ED (ed) *Flux Control in Biological Systems*, pp 3–11. Academic Press, San Diego
- Scheibe R, Kampfenkel K, Wessels R and Tripier D (1991) Primary structure and analysis of the location of the regulatory disulfide bond of pea chloroplast NADP-malate dehydrogenase. *Biochim Biophys Acta* 1076: 1–8
- Scheibe R, Baumann E, Backhausen JE, Rak C and Vetter S (1996) C-terminal truncation of spinach chloroplast NADP-dependent glyceraldehyde 3-phosphate dehydrogenase prevents inactivation and reaggregation. *Biochim Biophys Acta* 1296: 228–234
- Schidlowski M (1988) A 3,800 million year isotopic record of life from carbon sedimentary rocks. *Nature* 333: 313–318
- Schmidt G and Lyman H (1974) Photocontrol of chloroplast enzyme synthesis in mutant and wild-type *Euglena gracilis*. *Proc 3rd Intern Congr Photosynthesis* 3: 1755–1764
- Schmidt M, Svendsen I and Feierabend J (1995) Analysis of the primary structure of the chloroplast isozyme of triosephosphate isomerase from rye leaves by protein and cDNA sequencing indicates a eukaryotic origin of its gene. *Biochim Biophys Acta* 1261: 257–264
- Schmidt S, Drumm-Herrel H, Oelmueller R and Mohr H (1987) Time course of competence in phytochrome-controlled appearance of nuclear-encoded plastidic proteins and messenger RNA species. *Planta* 170: 400–407
- Schnarrenberger C (1987) Regulation and structure of isozymes of sugar phosphate metabolism in plants. In: Rattazzi MC, Scandalios JG and Whitt GS (eds) *Isozymes: Current Topics in Biological and Medical Research*, Vol. 16, Agriculture, Physiology, and Medicine, pp 223–240. Alan R Liss, New York
- Schnarrenberger C and Krüger I (1986) Distinction of cytosol and chloroplast fructose-bisphosphate aldolases from pea, wheat and corn leaves. *Plant Physiol* 80: 301–304
- Schnarrenberger C and Martin W (1997) The Calvin cycle: A historical perspective. *Photosynthetica* 33: 331–345
- Schnarrenberger C, Oeser A and Tolbert NE (1972) Isolation of plastids from sunflower cotyledons during germination. *Plant Physiol* 50: 55–59
- Schnarrenberger C, Tetour M and Herbert M (1975) Development and intracellular distribution of the oxidative pentose phosphate cycle in radish cotyledons. *Plant Physiol* 56: 836–840
- Schnarrenberger C, Jacobshagen S, Müller B and Krüger I (1990) Evolution of isozymes of sugar phosphate metabolism in green algae. In: Ogita S, Scandalios JS (eds) *Isozymes: Structure, Function, and Use in Biology and Medicine*, pp 743–746. Wiley-Liss, NY
- Schnarrenberger C, Pelzer-Reith B, Yatsuki H, Freund S, Jacobshagen S and Hori K (1994) Expression and sequence of the only detectable aldolase in *Chlamydomonas reinhardtii*. *Arch Biochem Biophys* 313: 173–178
- Schnarrenberger C, Flechner A and Martin W (1995) Enzymatic evidence for a complete oxidative pentose phosphate pathway in chloroplasts and an incomplete pathway in the cytosol of spinach leaves. *Plant Physiol* 108: 609–614
- Schönheit P and Schäfer T (1995) Metabolism of hyperthermophiles. *World J Microbiol Biotechnol* 11: 26–57
- Schopfer P (1977) Phytochrome control of enzymes. *Annu Rev Plant Physiol* 28: 223–253
- Sganga MW and Bauer CE (1992) Regulatory factors controlling photosynthetic reaction center and light-harvesting gene expression in *Rhodobacter capsulatus*. *Cell* 68: 945–954
- Sheen J (1990) Metabolic repression of transcription in higher plants. *Plant Cell* 2: 1027–1038
- Sheen J (1994) Feedback control of gene expression. *Photosynth Res* 39: 427–438
- Shen B, Jensen RG and Bohnert HJ (1997) Mannitol protects against oxidation by hydroxyl radicals. *Plant Physiol* 115: 527–532
- Shibata N, Inoue T, Fukuhara K, Nagara Y, Kitagawa R, Harada S, Kasai N, Uemura K, Kato K, Yokota A and Kai Y (1996) Orderly disposition of heterogeneous small subunits in D-ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach. *J Biol Chem* 271: 26449–26452
- Shih M-C and Goodman HM (1988) Differential light regulated expression of the nuclear genes encoding chloroplast and cytosolic glyceraldehyde-3-phosphate dehydrogenase on *Nicotiana tabacum*. *EMBO J* 7: 893–898
- Shih M-C, Heinrich P and Goodman HM (1992) Cloning and chromosomal mapping of nuclear genes encoding chloroplast and cytosolic glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. *Gene* 119: 317–319
- Shively JM, van Keulen G, Meijer WG (1998) Something from almost nothing: carbon dioxide fixation in chemoautotrophs. *Annu Rev Microbiol* 52: 191–230
- Sicher RC, Kremer DF and Rodermel SR (1994) Photosynthetic acclimation to elevated CO₂ occurs in transformed tobacco with decreased ribulose-1,5-bisphosphate carboxylase/oxygenase content. *Plant Physiol* 104: 409–415
- Skrubrud CL, Gordon IM, Dorwin S, Yuan X-H, Johansson G and Anderson LE (1991) Purification and characterization of pea chloroplastic phosphoribosomerase. *Plant Physiol* 97: 730–735
- Smillie R (1963) Formation and function of soluble proteins in chloroplasts. *Can J Bot* 41: 123–137
- Smillie RM (1968) The Biochemistry of *Euglena*. In: DE Buetow (ed) *The Biology of Euglena*, Vol. II, Biochemistry, pp 1–54. Academic Press, New York

- Sonnewald U, Lerchl J, Zrenner R and Frommer W (1994) Manipulation of sink-source relations in transgenic plants. *Plant Cell Environ* 17: 649–658
- Sørensen KI and Hove-Jensen B (1996) Ribose catabolism in *Escherichia coli*: Characterization of the *rpiB* gene encoding ribose phosphate isomerase B and the *rpiR* gene, which is involved in regulation of *rpiB* expression. *J Bacteriol* 178: 1003–1011
- Steiger HM and Beck E (1981) Formation of hydrogen peroxide and oxygen dependence of photosynthetic CO₂ assimilation by intact chloroplasts. *Plant Cell Physiol* 22: 561–576
- Steiger HM, Beck E and Beck R (1977) Oxygen concentration in isolated chloroplasts during photosynthesis. *Plant Physiol* 60: 903–906
- Stitt M (1990a) Fructose-2,6-bisphosphate as a regulatory molecule in plants. *Ann Rev Plant Physiol Plant Mol Biol* 41: 153–85
- Stitt M (1990b) The flux of carbon between the chloroplast and the cytosol. In: Dennis DT, Turpin DH (eds) *Plant Physiology, Biochemistry and Molecular Biology*, 309–326. Longman, Singapore
- Stitt M (1999) The first will be last and the last will be first: Non-regulated enzymes call the tune? In: Bryant JA, Burrell MM, Kruger NJ (eds) *Plant Carbohydrate Biochemistry*, pp 1–16. BIOS Scientific Publishers, Oxford
- Stitt M and Schulze D (1994) Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant Cell Environ* 17: 465–487
- Stitt M and Sonnewald U (1995) Regulation of metabolism in transgenic plants. *Ann Rev Plant Physiol Plant Mol Biol* 46: 341–368
- Stitt M, von Schaewen A and Willmitzer L (1990) Sink regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell wall involves a decrease in the Calvin-cycle enzymes and an increase in glycolytic enzymes. *Planta* 183: 40–50
- Stoebe B, Martin W and Kowallik KV (1998) Distribution and nomenclature of protein-coding genes in 12 sequenced chloroplast genomes. *Plant Mol Biol Rept* 16: 243–255
- Stoner MT and Shively JM (1993) Cloning and expression of the D-ribulose-1,5-bisphosphate carboxylase/oxygenase form II gene from *Thiobacillus intermedius* in *Escherichia coli*. *FEMS Microbiol Lett* 107: 287–292
- Strauss G and Fuchs G (1993) Enzymes of a novel CO₂ fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle. *Eur J Biochem* 215: 633–643
- Sulli C and Schwartzbach SD (1996) A soluble protein is imported into *Euglena* chloroplasts as a membrane-bound precursor. *Plant Cell* 8: 34–53
- Surek B, Heilbronn A, Austen A and Latzko E (1985) Purification and characterization of phosphoribulokinase from wheat (*Triticum aestivum* cultivar Arkas) leaves. *Planta* 165: 507–512
- Süss K-H, Arkona C, Manteuffel R and Adler K (1993) Calvin cycle multienzyme complexes are bound to chloroplast thylakoid membranes of higher plants in situ. *Proc Acad Sci USA* 90: 5514–5518
- Süss K-H, Prokhorenko I and Adler K (1995) In situ association of Calvin cycle enzymes, ribulose-1,5-bisphosphate carboxylase/oxygenase activase, ferredoxin-NADP⁺ reductase, and nitrite reductase with thylakoid and pyrenoid membranes of *Chlamydomonas reinhardtii* chloroplasts as revealed by immunoelectron microscopy. *Plant Physiol* 107: 1387–1397
- Tabita FR (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiol Rev* 52: 155–189
- Tabita FR (1994) The biochemistry and molecular regulation of carbon dioxide metabolism in cyanobacteria. In: Bryant DA (ed) *The Molecular Biology of Cyanobacteria*, pp 437–467. Kluwer Academic Publishers, Dordrecht
- Tamoi M, Ishikawa T, Takeda T and Shigeoka S (1996) Molecular characterization and resistance to hydrogen peroxide of two fructose-1,6-bisphosphatases from *Synechococcus* PCC 7942. *Arch Biochem Biophys* 334: 27–36
- Teige M, Kopriva S, Bauwe H and Süss K-H (1995) Chloroplast ribulose-5-phosphate 3-epimerase from potato: Cloning, cDNA sequence, and tissue-specific enzyme accumulation. *FEBS Lett* 377: 349–352
- Teige M, Kopriva S, Bauwe H and Süss K-H (1996) Primary structure of chloroplast transketolase from potato. *Plant Physiol* 112: 1735
- Teige M, Melzer M and Süss K-H (1998) Purification, properties and *in situ* localization of the amphibolic enzymes D-ribulose 5-phosphate 3-epimerase and transketolase from spinach chloroplasts. *Eur J Biochem* 252: 237–244
- Tessier LH, Keller M, Chan RL, Fournier R, Weil J-H and Imbault P (1991) Short leader sequences may be transferred from small RNAs to pre-mature mRNAs by *trans-splicing* in *Euglena*. *EMBO J* 10: 2621–2625
- Tessier LH, Paulus F, Keller M, Vial C and Imbault P (1995) Structure and expression of *Euglena gracilis* nuclear rbcS genes encoding the small subunit of the ribulose 1,5-bisphosphate carboxylase/oxygenase: A novel splicing process for unusual intervening sequences. *J Mol Biol* 245: 22–33
- Theiss-Seuberling B (1974) Gelchromatische Untersuchungen an der NADP- und NAD-abhängigen Glycerinaldehyd-3-phosphat Dehydrogenase in etiolierter und ergrünter *Euglena gracilis*. *Ber Deutsch Bot Ges* 94: 733–743
- Theiss-Seuberling B (1981) Zur Regulation der NADPH-abhängigen Glycerinaldehyd-3-phosphat Dehydrogenase in *Euglena gracilis*. *Ber Deutsch Bot Ges* 94: 733–743
- Theiss-Seuberling B (1984) Purification and immunochemical characterization of NADP-dependent glycerinaldehyde 3-phosphate dehydrogenase of *Euglena gracilis*. *Plant Cell Physiol* 25: 601–609
- Trost P, Scaliarini S, Valenti V and Pupillo P (1993) Activation of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. Effect of glycerate 1,3-bisphosphate. *Planta* 190:330–326
- Tsugita A, Yano K, Gardet-Salvi L and Schürmann P (1991) Characterization of spinach ferredoxin-thioredoxin reductase. *Protein Seq Data Anal* 4: 9–13
- Tsutsumi K, Kagaya Y, Hidaka S, Suzuki J, Tokairin Y, Hirai T, Hu D-L, Ishikawa K and Ejiri S (1994) Structural analysis of the chloroplastic and cytoplasmic aldolase-encoding genes implicated the occurrence of multiple loci in rice. *Gene* 141: 215–220
- Turpin DH and Weger HG (1990) Interactions between photosynthesis, respiration and nitrogen metabolism. In: Dennis DT, Turpin DH (eds) *Plant Physiology, Biochemistry and Molecular Biology*, pp 422–433. Longman, Singapore

- Van de Peer Y, Rensing S, Maier U-G and De Wachter R (1996) Substitution rate calibration of small subunit RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. Proc Natl Acad Sci USA 93: 7744–7748
- Van den Bergh ER, van der Kooij TA, Dijkhuizen L and Meijer WG (1995) Fructosebisphosphatase isoenzymes of the chemoautotroph *Xanthobacter flavus*. J Bacteriol 177: 5860–5864
- van Oosten JJ and Besford RT (1994) Sugar feeding mimics effect of acclimation to high CO₂—rapid down regulation of RuBisCO small subunit transcripts but not of the large subunit transcripts. J Plant Physiol 143: 306–312
- van Oosten JJ and Besford RT (1995) Some relationships between the gas exchange, biochemistry and molecular biology of photosynthesis during leaf development of tomato plants after transfer to different carbon dioxide concentrations. Plant Cell Environ 18: 1253–1266
- van Oosten JJ and Besford RT (1996) Acclimation of photosynthesis to elevated CO₂ through feedback regulation of gene expression: Climate of opinion. Photosynth Res 48: 353–365
- van Oosten JJ, Wilkins D, Besford RT (1994) Regulation of the expression of photosynthesis nuclear genes by CO₂ is mimicked by regulation by carbohydrates: A mechanism for the acclimation of photosynthesis to high CO₂? Plant Cell Environ 17: 913–923
- van Oosten JJ, Gerbaud A, Huijser C, Dijkwel PP, Chua N-H, Smeekens SCM (1997) An *Arabidopsis* mutant showing reduced feedback inhibition of photosynthesis. Plant J 12: 1011–1020
- Velanker SS, Ray SS, Gokhale RS, Balaram-Suma SH, Balaram P and Murthy MR (1997) Triosephosphate isomerase from *Plasmodium falciparum*: The crystal structure provides insights into antimalarial drug design. Structure 5: 751–761
- Vernon DM and Bohnert HJ (1992) Increased expression of a myo-inositol methyl transferase in *Mesembryanthemum crystallinum* is part of a stress response distinct from Crassulacean acid metabolism induction. Plant Physiol 99: 1695–1698
- Vernon DM, Ostrem JA and Bohnert HJ (1993) Stress perception and response in a facultative halophyte: The regulation of salinity-induced genes in *Mesembryanthemum crystallinum*. Plant Cell Environ 16: 437–444
- Villeret V, Huang S, Zhang Y, Xue Y and Lipscomb WN (1995) Crystal structure of spinach chloroplast fructose-1,6-bisphosphatase at 2.8 Å resolution. Biochemistry 34: 4299–4306
- Von Caemmerer S, Evans JR, Hudson GS and Andrews TJ (1994) The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. Planta 195: 88–97
- Wadano A, Kamata Y, Iwaki T, Nishikawa K and Hirashiki T (1995) Purification and characterization of phosphoribulokinase from the cyanobacterium *Synechococcus* PCC7942. Plant Cell Physiol 36: 1381–1385
- Wagner J, Lerner RA and Barbas III CF (1995) Efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. Science 270: 1797–1800
- Wara-Aswapati O, Kemble RJ and Bradbeer JW (1980) Activation of glyceraldehyde-phosphate dehydrogenase (NADP) and phosphoribulokinase in *Phaseolus vulgaris* leaf extracts involves the dissociation of oligomers. Plant Physiol 66: 34–
- Watson GM and Tabita FR (1996) Regulation, unique gene organization, and unusual primary structure of carbon fixation genes from a marine phycoerythrin-containing cyanobacterium. Plant Mol Biol 32: 1103–1115
- Watson GM, Yu JP and Tabita FR (1999) Unusual ribulose 1,5-bisphosphate Carboxylase/Oxygenase of anoxic archaea. J Bacteriol 181: 1569–1575
- Weber JP and Berhard SA (1982) Transfer of 1,3-diphosphoglycerate between glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase via an enzyme-substrate-enzyme complex. Biochemistry 21: 4189–4194
- Wedel N and Soil J (1998) Evolutionary conserved light regulation of Calvin cycle activity by NADPH-mediated reversible phosphoribulokinase/CP12/glyceraldehyde-3-phosphate dehydrogenase complex dissociation. Proc Natl Acad Sci USA 95: 9699–9704
- Wedel N, Soll J and Paap BK (1997) CP12 provides a new mode of light regulation of Calvin cycle activity in higher plants. Proc Natl Acad Sci USA 94: 10479–10484
- Weeden NF (1981) Genetic and biochemical implications of the endosymbiotic origin of the chloroplast. J Mol Evol 17: 133–139
- Wiemer EAC, Hannaert V, van den IJssel PRLA, Van Roy J, Opperdoes F and Michels PAM (1995) Molecular analysis of glyceraldehyde-3-phosphate dehydrogenase in *Trypanoplasma borelli*. Evolutionary scenario of subcellular compartmentation in kinetoplastida. J Mol Evol 40: 443–454
- Wilkins D, Van Oosten JJ and Besford RT (1994) Effects of elevated CO₂ on growth and chloroplast proteins in *Prunus avium*. Tree Physiol 14: 769–779
- Willingham NM, Lloyd JC and Raines CA (1994) Molecular cloning of the *Arabidopsis thaliana* sedoheptulose-1,7-bisphosphatase gene and expression studies in wheat and *Arabidopsis thaliana*. Plant Mol Biol 26: 1191–1200
- Windhövel U and Bowien B (1991) Identification of *cfxR*, an activator gene of autotrophic carbon dioxide fixation in *Alcaligenes eutrophus*. Mol Microbiol 5: 2695–2706
- Wingsle G and Karpinski S (1996) Differential redox regulation by glutathione of glutathione reductase and CuZn-superoxide dismutase gene expression in *Pinus sylvestris* L. needles. Planta 198: 151–157
- Winter U and Feierabend J (1990) Multiple coordinate controls contribute to a balanced expression of ribulose-1,5-bisphosphate carboxylase/oxygenase subunits in rye leaves. Eur J Biochem 187: 445–453
- Witke C and Götz F (1993) Cloning, sequencing, and characterization of the gene encoding class I fructose-1,6-bisphosphate aldolase of *Staphylococcus carnosus*. J Bacteriol 175: 7495–7499
- Wolosiuk RA and Buchanan BB (1978) Activation of chloroplast NADP-linked glyceraldehyde-3-phosphate dehydrogenase by concerted hysteresis. Arch Biochem Biophys 246: 1–8
- Wolosiuk RA, Ballicora MA and Hagelin K (1993) The reductive pentose phosphate cycle for photosynthetic CO₂ assimilation: Enzyme modulation. FASEB J 7: 622–637
- Wolter F, Fritz C, Willmitzer L, Schell J and Schreier P (1988) *rbcS* genes in *Solanum tuberosum*: Conservation of transit peptide and exon shuffling during evolution. Proc Natl Acad Sci USA 85: 846–850
- Woodrow IE and Berry JA (1988) Enzymatic regulation of

- photosynthetic CO₂ fixation in C3 plants. *Annu Rev Plant Physiol Plant Mol Biol* 39: 533–594
- Xu HH and Tabita FR (1996) Ribulose-1,5-bisphosphate carboxylase/oxygenase gene expression and diversity of Lake Erie planktonic microorganisms. *Appl Environ Microbiol* 62: 1913–1921
- Yang Y, Kwon H-B, Peng H-P and Shih M-C (1993) Stress responses and metabolic regulation of glyceraldehyde-3-phosphate dehydrogenase genes in *Arabidopsis thaliana*. *Plant Physiol* 101:209–216
- Yeh KC, Wu SH, Murphy JT and Lagarias JC (1997) A cyanobacterial phytochrome two-component light sensory system. *Science* 277: 1505–1508
- Yonuschot GR, Ortwerth BJ and Koeppe OJ (1970) Purification and properties of a nicotinamide adenine dinucleotide phosphate-containing glyceraldehyde 3-phosphate dehydrogenase from spinach leaves. *J Biol Chem* 245: 4193–4198
- Yoo JG and Bowien B (1995) Analysis of the *cbbF* genes from *Alcaligenes eutrophus* that encode fructose-1,6-/sedoheptulose-1,7-bisphosphatase. *Curr Microbiol* 31: 55–61
- Yu JP, Ladapo J and Whitman WB (1994) Pathway of glycogen metabolism in *Methanococcus maripaludis*. *J Bacteriol* 176: 325–332
- Zapponi MC, Iadorola P, Stoppini M and Ferri G (1993) Limited proteolysis of chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Spinacia oleracea*. *Biol Chem Hoppe-Seyler* 374: 395–402
- Zhao G, Pease AJ, Bharani N and Winkler ME (1995) Biochemical characterization of gapB-encoded erythrose 4-phosphate dehydrogenase of *Escherichia coli* K-12 and its possible role in pyridoxal 5'-phosphate biosynthesis. *J Bacteriol* 177:2804–2812
- Ziegler DM (1985) Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu Rev Biochem* 54: 305–329
- Ziegler H and Ziegler I (1965) The influence of light on the **NADP⁺-dependent** glyceraldehyde-3-phosphate dehydrogenase. *Planta* 65: 369–380
- Zimmermann G, Kelly GJ and Latzko E (1976) Efficient purification and molecular properties of spinach chloroplast fructose 1,6-bisphosphatase. *Eur J Biochem* 70: 361–367
- Zrenner R, Krause KP, Apel P and Sonnewald U (1996) Reduction of the cytosolic fructose-1,6-bisphosphatase in transgenic potato plants limits photosynthetic sucrose biosynthesis with no impact on plant growth and tuber yield. *Plant J* 9: 671–681
- Zwickl P, Fabry S, Bogedian C, Haas A and Hensel R (1990) Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaeabacterium *Pyrococcus woesei*: Characterisation of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*. *J Bacteriol* 172: 4329–4338

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Chapter 3

Rubisco: Assembly and Mechanism

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Summary

Ribulose-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39) is unique to photosynthetic metabolism. Two intensively studied aspects of Rubisco physiology are covered in this chapter, its post-translational assembly and its mechanism of action. Bacterial Rubisco can be assembled in vitro and in bacterial hosts but, as yet, assembly in vitro of higher-plant Rubiscos has not been reported. This focuses attention on the assembly pathway for higher plant Rubisco, which has been known for some time to be related to the presence of molecular chaperones in chloroplasts. Analysis of mutants, transformation of plants and bacteria with chloroplast chaperones, and the development of in vitro translation and assembly systems based on chloroplast extracts, have been directed at resolving this problem. It appears from these data that certain bacterial chaperones do not interfere with the assembly of higher plant Rubisco. As in cyanobacterial systems, the absence of S subunits leads to the accumulation of L₈-like particles whose subunits can later be recruited to form Rubisco. Subtle differences between the way S subunits assemble with higher-plant and cyanobacterial L₈-like particles suggest that this process may be concerted with assembly of L₈ in the case of the higher-plant enzyme. The catalytic mechanism of Rubisco depends on two co-factors; a divalent metal ion, usually Mg²⁺, and a CO₂ molecule that carbamylates a specific lysyl residue, K201, in the active site. This carbamate plays a crucial role in initiating catalysis by abstracting the C3 proton of ribulose bisphosphate and it may also act as a general-base catalyst for succeeding steps. So far, Rubisco's use of a carbamate as a base appears to be unique among enzymes. The catalytic sequences of both the carboxylation reaction, and the oxygenation reaction that competes with it, proceed through multiple steps, each of a complexity rivaling that of the complete reaction of many other enzymes. The structure of the active site must change subtly between steps. Selectivity between CO₂ and O₂, of paramount importance to photosynthetic efficiency, is determined by the relative reactivity of the enediol(ate) form of the substrate for the two gases.

Abbreviations: 2' (or 4')-carboxyarabinitol-P₂ – 2' (or 4')-carboxyarabinitol-1,5-bisphosphate; carboxytetritol-P₂ – 2'-carboxytetritol-1,4-bisphosphate; Cpn60 – chaperonin 60; pentodiulose-P₂ – D-glycero-2,3-pentodiulose-1,5-bisphosphate; P-glycerate – 3-phospho-D-glycerate; P-glycolate – 2-phosphoglycolate; ribulose-P₂ – D-ribulose-1,5-bisphosphate; Rubisco-ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39); xylulose-P₂ – D-xylulose-1,5-bisphosphate

I. Introduction

Many enzymes of photosynthetic metabolism are analogous to enzymes in other pathways and may have been recruited from them (Chapter 2, Martin et al.) but a few are known exclusively for their role in photosynthesis. Unquestionably, the most important

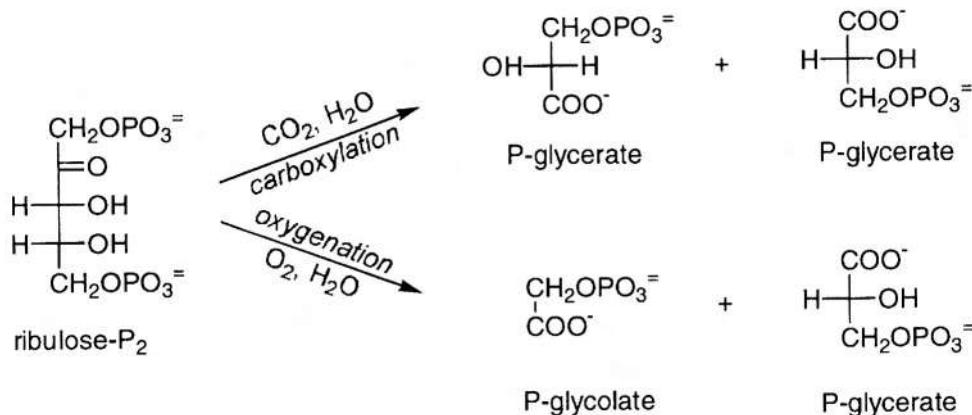


Fig. 1. The carboxylation and oxygenation of ribulose-P₂ catalyzed by Rubisco.

of these is the central CO₂-fixing enzyme ribulose-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39). Rubisco is believed to be the most abundant protein on earth (Ellis, 1979). Its catalytic effectiveness is quite feeble both in terms of its substrate-saturated k_{cat} (2–12 s⁻¹) and its $k_{cat}/K_m(CO_2)$ ratio ($5 - 40 \times 10^4 M^{-1} s^{-1}$) and this necessitates that photosynthetic organisms invest enormous amounts of protein in it (often approximately 50% of soluble leaf protein) to support acceptable rates of photosynthesis.

Rubisco catalyses both the carboxylation and the oxygenation of ribulose-P₂ within the same active site (Fig. 1). The carboxylation reaction, which produces two molecules of P-glycerate, is the CO₂-acquiring reaction upon which the Calvin cycle (Chapter 2, Martin et al.) is based. The oxygenation reaction, which produces a molecule each of P-glycerate and P-glycolate, gives rise to photorespiratory metabolism (Chapter 5, Douce and Heldt). P-glycolate production results from Rubisco's difficulty in discriminating between CO₂ and O₂. The attendant photorespiration wastes carbon and light energy during photosynthesis.

Wildman and Bonner (1947) first discovered Rubisco as a major soluble protein detected by electrophoresis of extracts of plant leaves. Having no information about its function, they called it Fraction I protein. Early ¹⁴CO₂-labeling studies showing that P-glycerate was the first observed labeled product of photosynthesis pointed to the existence of an enzyme that catalyzed the carboxylation of ribulose-P₂ to produce two molecules of P-glycerate (Fig. 1) (Calvin and Massini, 1952; Quayle et al., 1954; Weissbach et al., 1954). Attribution of this enzymatic activity to

Fraction I protein followed a decade later (Trown, 1965). Several more years were required before the second major activity of this protein, the oxygenation of ribulose-P₂ to produce a molecule each of P-glycolate and P-glycerate (Fig. 1) was discovered (Bowes et al., 1971; Andrews et al., 1973; Lorimer et al., 1973). Observation that the plant Rubisco was composed of two dissimilar kinds of subunits (Rutner and Lane, 1967) was followed by discoveries that the two subunits were encoded and synthesized in different cellular compartments (Blair and Ellis, 1973; Gooding et al., 1973; Smith et al., 1974; Roy et al., 1976). This led to the discovery of the mechanism for import of cytoplasmically synthesized proteins into chloroplasts (Chua and Schmidt, 1978). The plastid gene for the large, catalytic subunit was cloned and sequenced first from maize (McIntosh et al., 1980) and eventually from large numbers of other plants, algae and bacteria (Delwiche and Palmer, 1996; Watson and Tabita, 1997).

The physiology of Rubisco has been studied more than that of any other plant enzyme. In addition to the many properties described in other chapters in this volume, two important aspects of Rubisco physiology are covered separately in this chapter. Post-translational assembly of Rubisco has been a major focus of studies of chaperonin function, leading to insights into the folding of many large proteins. The mechanism of Rubisco catalysis has been studied by mutational analysis, X-ray crystallography, other biochemical techniques, and by analysis of the surprising number of side reactions that Rubisco catalyses. This chapter, then, covers the post-translational assembly and mechanism of action of Rubisco, current up to November 1998. The regulation

of Rubisco's synthesis and activity are not covered here. These topics have been reviewed elsewhere in recent years (Hartman and Harpel, 1994; Andrews et al., 1995; Furbank and Taylor, 1995; Gutteridge and Gatenby, 1995; Portis, 1995; Salvucci and Ogren, 1996).

II. Rubisco's Discordant Molecular Phylogeny

There are two major structural forms of Rubisco. Form I Rubisco, found in higher plants, algae, cyanobacteria, and many autotrophic bacteria, consists of eight ca. 53 kDa 'large' or 'L' subunits and eight ca. 14 kDa 'small' or 'S' subunits. Form II Rubisco, found in some dinoflagellates and certain autotrophic bacteria, including the obligate anaerobe, *Rhodospirillum rubrum*, lacks S subunits and contains just L subunits. The crystal structures of several Form I enzymes and the Form II enzyme from *R. rubrum* have been determined, enabling detailed mechanistic studies (see Section IV). In green algae and higher plants, Rubisco occurs in the chloroplast and the L subunit is encoded in the chloroplast genome. A family of nuclear genes encodes the S subunit, which is synthesized as a precursor polypeptide on cytosolic ribosomes and imported into the chloroplast in an ATP-dependent reaction that has been studied intensively (for a review, see Hartman and Harpel, 1994). In non-green algae, both L and S subunits are encoded in the chloroplast genome, confirming the hypothesis of differential migration of genes from the organelle genome to the nucleus that is a corollary of the endosymbiont theory of evolution (for a review, see Shivji et al., 1992). Rubisco L subunit genes of a large number of organisms have been sequenced but the phylogenetic tree resulting from analysis of 48 of these is not congruent with trees resulting from the analysis of rRNA and a variety of other proteins.

Two major classes of Form I Rubisco L subunit genes are distinguished: 'green-like' and 'red-like,' reflecting their predominant associations with plants, cyanobacteria and green algae on the one hand, and red algae on the other. However, both red-like and green-like Rubiscos are found among α , β and γ proteobacteria. In both red-like and green-like classes, two further sub-classes are apparent which again sometimes transgress rRNA-based phylogenies. Either there have been selective losses of some Rubisco genes in different branches of the phylogeny

from an original ancestor that had all types of Rubisco genes or there have been multiple lateral transfers of Rubisco genes across taxonomic lines (Delwiche and Palmer, 1996).

III. The Folding and Assembly of Rubisco

A. Chaperones

Molecular chaperones are proteins that assist in the folding or assembly of other proteins but do not participate in their final structure (Netzer and Hartl, 1998). Examples are Hsp70 in *E. coli* or in the endoplasmic reticulum of eukaryotic cells (so-called BiP) and the GroEL and GroES proteins of *E. coli*. GroEL and GroES support the refolding in vitro of a large number of proteins from diverse sources, most notably the dimeric Rubisco from *Rhodospirillum rubrum* (for a review, see Gatenby, 1992). Proteins related in sequence to the *E. coli* GroEL and GroES proteins are called chaperoning (abbreviated Cpn60 and Cpn10, or in the case of the chloroplast, Cpn60 and Cpn21). The numerals in these abbreviations correspond approximately to the monomer molecular weight of the polypeptides. The native state of Cpn60 is a tetradecamer consisting of two layers, each containing a ring of seven subunits. The native state of Cpn10 is one ring of seven subunits. One Cpn10 ring binds to each Cpn60 ring (Hartl et al., 1994; Xu et al., 1997). Like the *E. coli* GroEL protein, the chloroplast Cpn60 is able to facilitate the refolding of dimeric *Rhodospirillum rubrum* Rubisco in vitro (Viitanen et al., 1995). The chloroplast Cpn21 homolog of Cpn10 has a similar structure, but the subunits are larger (Baneyx et al., 1995). Together with chloroplast Cpn60, it supports the refolding of mitochondrial malate dehydrogenase in vitro (Viitanen et al., 1995). It is clear from this that the chloroplast chaperonins indeed function as molecular chaperones. In vivo, it has been estimated from quantitative considerations that the chloroplast chaperonins are principally concerned with the synthesis and folding of the large subunit of chloroplast Rubisco (Lorimer, 1996). Kessler and Blobel (1996) reported that Cpn60 associates with IAP100, a component of the chloroplast protein import machinery, and that it can associate with imported mature Rubisco S subunits. This confirms an earlier report that Cpn60 can associate with imported chloroplast proteins (Lubben et al., 1989).

Apparently these turn over rapidly, because they do not represent an appreciable amount of radiolabeled material associated with Cpn60 in extracts derived from chloroplasts of pulse-labeled plants (Roy et al., 1982). The chloroplast Cpn 60 differs from that of *E. coli* in that it is encoded by two related genes rather than one (Hemmingsen et al., 1988). Also Na^+ can replace K^+ during refolding (Viitanen et al., 1995) and the tetradecameric form of the chloroplast protein is much less stable in the presence of ATP (Hubbs et al., 1988). Thus, although there has been great progress in the analysis of prokaryotic chaperonin, it is likely that important aspects of the plant system can only be revealed by direct studies of the plant protein (for a recent review, see Boston et al., 1996).

We understand the chaperonin reaction mechanism in broad outline. Unfolded or partially folded polypeptides have a strong tendency to aggregate homotypically. They contain hydrophobic sequences that are exposed to the solvent. These proteins can bind to hydrophobic chaperonin surfaces that face toward the center of the heptameric rings of Cpn60 (Braig et al., 1994; Xu et al., 1997). After this, the details of the mechanism are controversial. One school of thought holds that the bound protein remains in the cavity formed by Cpn60 and Cpn 10, and undergoes a series of conformational shifts there (Ellis and Hartl, 1996; Martin and Hartl, 1997). Agents simulating the crowding effects of the high protein concentrations found in vivo appear to suppress the dissociation of folding proteins without inhibiting chaperone assisted folding (Martin and Harl, 1997). Also, several proteins appear to fold completely while bound to mutant chaperonins that cannot form a double ring structure and thus do not dissociate in the normal manner (Rye et al., 1997). Another view holds that in the presence of K^+ , MgATP and Cpn 10 cooperatively induce release of the bound polypeptide, which then can either aggregate, fold properly, or re-bind (Horwich et al., 1995; Burston et al., 1996; Török et al., 1996). If it aggregates, it may not fold; if it folds, it does not re-bind. If it re-binds, it has an indefinite number of opportunities to dissociate and rebind until it is either aggregated or folded. The net accumulation of folded polypeptide is often enhanced over that which occurs in the absence of chaperonins, in large part because aggregation is suppressed (Todd et al., 1994). However, there is sometimes a rate enhancement of folding, suggesting that the folding process itself is being catalyzed. One way to explain this is to suppose

that protein folding occurs through a matrix of states of differing degrees of stability (Sfatos et al., 1996). Proteins arrested in an unfavorable configuration would be rescued if they could escape that configuration. The chaperonins may provide this opportunity by fostering actively the occurrence of conformational shifts (Todd et al., 1996). The idea is that, due to the energetics of nucleotide binding and hydrolysis, the chaperonin's interior walls change shape, inducing conformational changes in the bound polypeptide in situ. In this model, a very critical part of the folding process (escape from an unfavorable configuration) is envisioned to take place while the protein is bound to the chaperonin complex, even if the protein is released and rebound before complete folding takes place.

B. Chaperonin-Mediated Folding and Assembly of Chloroplast Proteins

The *E. coli* chaperonins support the folding of the dimeric Rubisco of *Rhodospirillum rubrum*, either in vitro or in vivo (for reviews, see Roy, 1989, 1992; Ellis and van der Vies, 1991; Gatenby, 1992). The *E. coli* chaperonins also support the assembly of the hexadecameric cyanobacterial Rubisco in vivo (Ellis and van der Vies, 1991) but so far not in vitro. There is very strong evidence that the chloroplast Cpn60 normally participates in the assembly of hexadecameric higher plant Rubisco (discussed below). Chloroplast Cpn60 and Cpn21, and chloroplast Hsp70, have been found together to facilitate in vitro the reconstitution of the chloroplast coupling factor CF1, which has five different types of subunits (Chen and Jagendorf, 1994). They also support the folding of bacterial Rubisco and malate dehydrogenase (Viitanen et al., 1995). In the latter study, Cpn21 was found not to be essential for refolding of the proteins, and Na^+ could replace K^+ .

C. The Mechanism of Rubisco Assembly

Rhodospirillum rubrum Rubisco assembles as the result of a dimerization reaction that occurs very rapidly once the monomer is properly folded (for a review, see Hartman and Harpel, 1994). Hexadecameric Rubiscos from cyanobacteria dissociate into L₈ cores and free S subunits upon acid treatment. After neutralization, they can reassociate to form active enzyme (Andrews and Ballment, 1983). When cyanobacterial L subunits are cloned in *E. coli*, they

form either insoluble material or soluble L_8 core particles. In the latter case, S subunits can be added to reconstitute active enzyme. Alternately, the S subunits can be expressed in the same cell, resulting in the formation of active enzyme. Thus it is thought that after folding, the monomeric L subunits form dimers. These then aggregate to form the L_8 core, followed by binding of tetramers of S subunits to the top and bottom of the core. However, higher plant Rubisco subunits expressed in *E. coli* do not form active enzyme (for reviews, see Ellis and van der Vies, 1991; Gatenby, 1992; Roy, 1992). It is surprising that even when large subunits from higher plants bind to GroEL chaperonin, they fail to assemble properly. The reason for this is not understood.

In extracts of isolated pea chloroplasts, newly synthesized L subunits are found associated with chloroplast Cpn60. This association could in principle be a result of post-lysis binding of L subunits by Cpn60 (Lorimer, 1996). However, when chloroplasts pulse-labeled with ^{35}S -methionine are incubated in the light before lysis, there is a time-dependent decrease in the extent of binding of radioactive L subunits to Cpn60 in the extracts, which is not accompanied by any degradation of radioactive L subunits (Roy et al., 1988). Therefore, the binding must occur in the chloroplast before lysis. After synthesis and binding to Cpn60, Rubisco L subunits assemble into holoenzyme (Barracough and Ellis, 1980). This assembly is itself dependent on continued post-translational illumination of the chloroplasts and can be detected within about 20 min after the onset of protein synthesis in the presence of radioactive amino acids (Roy et al., 1982). This result would not be expected if folding and assembly both were spontaneous processes. Alternately, if the chloroplasts are broken and the membranes removed, one can monitor the assembly reaction *in vitro* in the presence of MgATP, K^+ , and soluble S subunits (Milos and Roy, 1984; Roy and Gilson, 1996). The L subunits detach from Cpn60 and form binary complexes with each other or with Cpn60 monomers that dissociate from the Cpn60 tetradecamer. (The release of Cpn60 monomers does not seem to occur readily with *E. coli* Cpn60). The released L subunits can assemble into Rubisco *in vitro*. The assembly reaction itself is stimulated by ATP and can be inhibited specifically by antibody directed against chloroplast Cpn60 (Cannon, Wang, and Roy, 1986).

It is also possible to carry out translation of endogenous *rbcL* mRNA in chloroplast extracts,

followed by Rubisco assembly *in vitro*. This reaction is completely dependent on added S subunits and is therefore more likely to be due to de novo assembly, rather than to subunit exchange (Hubbs and Roy, 1992). When protein synthesis was conducted over a range of temperatures in chloroplast extracts, followed by a constant temperature incubation, Rubisco assembly occurred only if translation had taken place at less than 32 °C (Hubbs and Roy, 1993a). Again, this result is not expected if protein folding is a spontaneous process with 100% yield. However, Rubisco synthesis *in vivo* occurs at higher temperature (Weidner and Fehling, 1985). Apparently disrupting the chloroplast dilutes all components or releases or activates proteases or interferes with post-translational processing (Houtz et al., 1992).

Hubbs and Roy (1993b) found that during Rubisco L subunit synthesis at low salt without small subunits, a previously undescribed oligomer accumulates. This oligomer shares electrophoretic and sedimentation properties with L_8 particles that have been detected in cloning experiments involving cyanobacterial Rubisco genes (Andrews, 1988; Goloubinoff et al., 1989a; Lee and Tabita, 1990). However, this oligomer does not bind S subunits at low KCl concentrations. At high KCl concentrations, it dissociates and the L subunits released then appear to assemble into holoenzyme. The results suggest that the Rubisco assembly mechanism in higher plants may be different from that expected based on work with cyanobacterial Rubisco, where it seems quite clear that stable L_8 cores bind S subunits to give rise to active enzyme *in vitro* (see Section IV.M.1). Could it be that L_8 cores are the products of side reactions, which occur to a greater extent in the absence of S subunits, and that the assembly of Rubisco normally proceeds through a variety of intermediates in the presence of S subunits? One likely intermediate in the assembly process is an L_2 dimer, based on the existence of form II Rubisco in prokaryotes, the presence of intimate L-L bonds in crystals of cyanobacterial and higher plant form I Rubisco, and on the presence of 7S (ca 110 kDa) L subunits in pea chloroplast extracts (Roy et al., 1982). The structure of unactivated tobacco Rubisco was described as four $(\text{LS})_2$ dimers substantially stabilized by S subunits (Curmi et al., 1992). One of many possible assembly pathways then would be the formation of $(\text{LS})_2(\text{LS})_2$ structures, followed by aggregation of L_4S_4 units to reach the L_8S_8 structure.

D. Post-translational Modification of L Subunits

A number of post-translational modifications occur in the Rubisco large subunits of several higher plants, including pea (Houtz et al., 1992). These include trimethylation of lysine 14, and truncation and acetylation of the N terminus at proline 3. The enzyme catalyzing the trimethylation reaction has been cloned and sequenced (Klein and Houtz, 1995). Although there are some plants that do not carry out these modifications, it is possible that they play a role in assembly. Detailed molecular studies are needed to see whether this is the case.

E. Possible Involvement of Lipid-Protein Particles in Rubisco Assembly

Smith et al. (1997) reported that L subunits are associated with lipid-protein particles that are produced in light-dependent reactions by isolated thylakoids. No S subunits are associated with these particles, thus showing that these L subunits are not due simply to Rubisco holoenzyme sticking to the particles. Chloroplast chaperonin 60 subunits are also found associated with these lipid-protein particles. The authors suggested that the lipid protein particles might be coordinated with the assembly of Rubisco. However, no kinetic data concerning this hypothesis are available, so it is uncertain whether these L subunits are on the main pathway of assembly, a side pathway, or a degradative pathway.

F. A Model of Rubisco Assembly and the Limits of Our Understanding

A model incorporating most of the foregoing analysis is presented in Fig. 2. The model shows the unfolded L subunit, u-L, after having left the ribosome and any other unknown components with which it may have interacted, binding to the Cpn60-Cpn21 complex. In the presence of ATP, this complex equilibrates with unfolded L subunits and folded L subunits. Early immunological data (Cannon et al., 1986) indicated that it is possible that some of the L subunits associate with monomers of Cpn60. This type of intermediate has been suggested independently by studies of the effect of urea on the chaperonin-mediated refolding of rhodanese (Mendoza et al., 1994), by the observation that truncated monomers of Cpn60 can promote the folding of rhodanese (Makino et al., 1993), and very recently by the demonstration of

Possible Intermediates

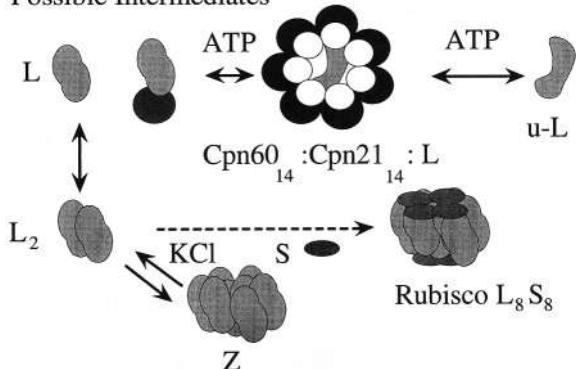


Fig. 2. Putative intermediates in the proposed assembly mechanism of Rubisco in chloroplasts. The unfolded L subunit (u-L) may interact with a variety of factors (e.g. proteolytic cleavage, trimethylation of lysine, or lipid-protein particles) that could lead to posttranslational modification or partial folding (not shown), followed by interaction with the Cpn60-Cpn21 complex. ATP hydrolysis results in release of folded L subunits (L), which probably dimerize to form L₂, and subsequently form L₈S₈ holoenzyme, either via L₈ or concertedly. The S subunits are imported into the chloroplast from a cytosolically synthesized precursor.

chaperone activity by a monomeric unit of GroEL (Zahn et al., 1996). An alternate interpretation of the results of Cannon et al. (1986) is that even in the absence of ATP, unfolded L subunits can bind to and dissociate from Cpn60 in chloroplast extracts. If this is the case, then the L subunit pool seen in chloroplast extracts might consist principally of L subunit dimers and partly folded soluble monomers, also shown in the diagram. The soluble L₈-like oligomer, called Z in the diagram, can accumulate in the absence of S subunits but dissociates in high KCl concentration. The high-salt reactions may not be significant in vivo. However, the binding of S subunits to L subunits leads to the formation of holoenzyme. Although not shown, it is possible that S subunits also interact with the chaperone system, although they are not observed to accumulate under steady state conditions (Roy et al. 1982).

G. The Holy Grail of Rubisco Assembly

Despite the above evidence, so far nobody has demonstrated an absolute requirement for chloroplast Cpn60 in the assembly of higher plant Rubisco. Even when the genes for higher plant Rubisco are co-expressed in *E. coli* with the chloroplast Cpn60 genes, no assembly of Rubisco occurs (Cloney et al.,

1992a,b; 1993). This might have been thought to be due to the formation of mixed oligomers of chloroplast GroEL and *E. coli* Cpn60. However, the expression of *E. coli* Cpn60 in the chloroplast does not prevent assembly of higher plant Rubisco (Wu et al., 1993). An attempt to address this issue with anti-sense RNA in tobacco plants showed that the synthesis of the β subunit of Cpn60 could be inhibited and the plants showed a variety of defects, which nonetheless did not prevent Rubisco synthesis (Zabaleta et al., 1994). Recent work indicates that the β subunit is self-sufficient to form an active chloroplast Cpn60-like protein, but the α subunit is not (cited in Boston et al., 1996). The α subunit is nonetheless important: anti-sense RNA for the α subunit produces plants showing a similar phenotype to those with anti-sense RNA for the β subunit (L. Herrera-Estrella, personal communication). These results suggest that a depleted or abnormal chaperonin system, while deleterious, could still be functional in higher plants, or that an alternative backup chaperone system exists. It seems improbable that higher plant Rubisco folds by itself *in vivo*, simply because it does not do so *in vitro*. Because Rubisco L subunits associate so strongly with Cpn60, it seems unlikely that Cpn60 is not involved in Rubisco assembly, but it may well be the case that other proteins besides Cpn60 and Cpn21 are also able to assist or even substitute for them in folding Rubisco L subunits. Buchberger et al. (1996) found that proteins shuttle between DnaK and GroEL, rather than passing through a definite sequence from one to the next as originally proposed by Langer et al. (1992). They proposed that proteins may interact with a network of chaperones during folding. Freeman et al. (1996) suggested that different chaperones may play somewhat distinct roles in the folding pathway, such as maintenance of a protein in the unfolded state, or facilitation of folding. Checa and Viale (1997) reported that *R. rubrum* Rubisco and the hexadecameric Rubiscos of *Chromatium vinosum* and *Synechococcus PCC7942* required the DnaK protein for productive folding in *E. coli*. These observations, together with earlier work, suggest that a cascade of chaperones may operate in the folding of Rubisco as has been reported for other proteins in the *E. coli* system. Ideas like this might also help reconcile the results of Zabaleta et al. (1994) with the evident involvement of chloroplast Cpn60 in the folding of higher plant Rubisco.

Kanevski and Maliga (1994) reported the transformation of *rbcL*-negative tobacco with a

nuclear-located chimeric gene containing the RbcS transit sequence and tobacco *rbcL* coding sequence. About 3% of normal Rubisco activity was observed in the transformed cells and the seedlings that they raised from these. These experiments show that the cytoplasmic environment permits cytosolic synthesis and transport of the modified *rbcL* polypeptide to the chloroplast. It has been shown as well that a mutant tobacco, Sp25, harbors a mutation in the *rbcL* gene that blocks Rubisco assembly (Shikanai et al., 1996). This mutant may be a suitable host for expression of modified Rubisco genes. Recently, Kanevski et al. (1999) replaced the *rbcL* gene of tobacco chloroplasts with the analogous gene from sunflower and succeeded in forming a hybrid Rubisco with sunflower L subunits and tobacco S subunits in the resulting transgenic plants. Although the enzyme was not active enough to sustain photosynthetic growth, the authors were able to purify and characterize it. This achievement demonstrates the feasibility of chloroplast transformation for cloning and expressing genetically engineered Rubisco L subunits in higher plants.

All attempts at *in vitro* reconstitution of unfolded hexadecameric Rubisco from any source have failed (Lorimer, 1996). Fortunately, it is still possible to study the assembly of hexadecameric Rubisco in chloroplast extracts. Building on this may enable a more refined system to be developed.

IV. The Catalytic Mechanism of Rubisco

A. Reversible Activation

Once the polypeptides are assembled correctly, Rubisco must be reversibly activated before catalysis can occur. Demonstrations that pre-incubation with $\text{CO}_2/\text{HCO}_3^-$ and Mg^{2+} before assay *in vitro* increased activity (Pon et al., 1963; Andrews et al., 1975), that $\text{CO}_2/\text{HCO}_3^-$ became bound to the protein (Akoyunoglou and Calvin, 1963; Mizorko and Mildvan, 1974), and that activity and catalytic properties changed rapidly following extraction from chloroplasts and leaves (Badger and Andrews, 1974; Bahr and Jensen, 1974), led to the discovery that sequential binding of CO_2 and a divalent metal ion (physiologically Mg^{2+}) is a prerequisite for catalysis (Lorimer et al., 1976; Laing and Christeller, 1976). The oxygenase activity also requires this activation (Badger and Lorimer, 1976).

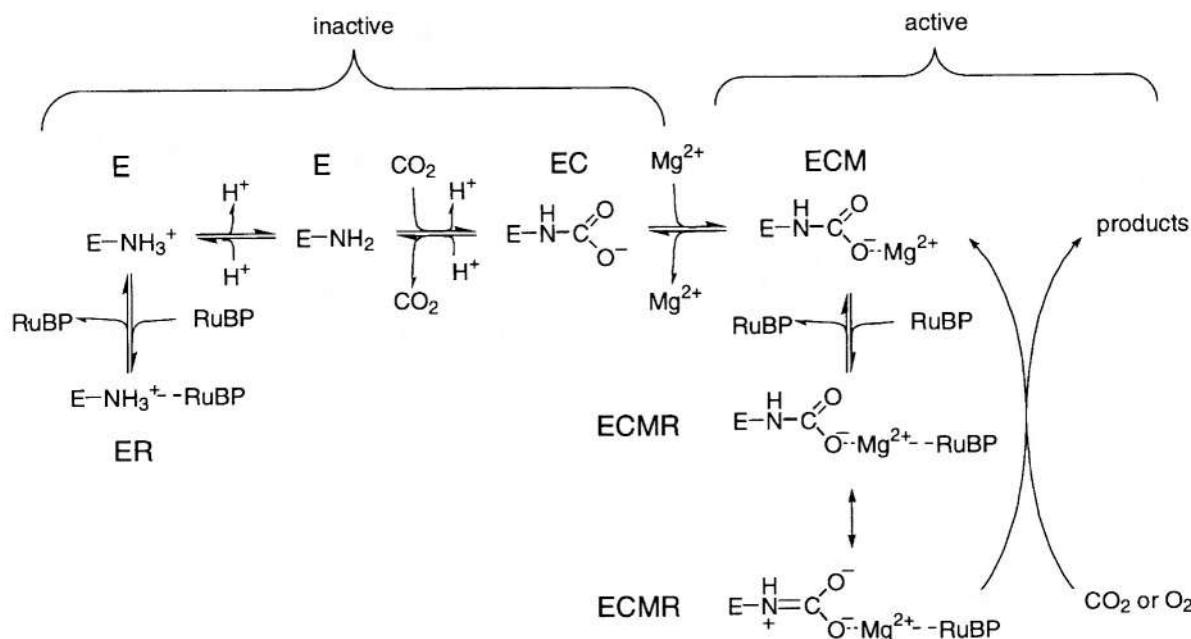


Fig. 3. The activation of Rubisco (E) by carbamylation of K201 (producing EC) followed by binding of Mg^{2+} (producing ECM). While ribulose-P₂ (RuBP) may bind to either the carbamylated form (producing ECMR) or the non-carbamylated form (producing ER), only the carbamylated form is able to carry out catalysis. Access of the carbamate to a resonant form carrying a negative charge on both O atoms may be essential to its catalytic function.

B. An Unusual Cofactor— CO_2

1. Carbamylation Chemistry

The CO_2 molecule involved in the activation process, which binds in a reversible and rate limiting process before subsequent rapid binding of Mg^{2+} (Lorimer et al., 1976), is distinct from the substrate CO_2 molecule fixed during carboxylation (Lorimer, 1979). It becomes covalently attached as a carbamate to the ε amino group of a specific lysyl residue, K201¹, within the active site (Lorimer, 1981). As discussed later (Section IV.F.2), this carbamate is now thought to play an instrumental role in catalysis and therefore qualifies as a cofactor in every sense of the word. Only after the metal-stabilized carbamate is in place

is the active site able to convert ribulose-P₂ to products (Fig. 3).

2. Structure of the Carbamylated Active Site

Crystallographic studies have revealed the structural basis of the synergy between CO_2 and Mg^{2+} binding (Taylor and Andersson, 1996; Taylor and Andersson, 1997) (Fig. 4). The dispositions of the active site residues involved in carbamylation and Mg^{2+} binding are similar in the ER, ECM, ECMR and ECM-analog complexes. Carbamylation converts the side chain of K201 from a positive to a negative charge and the metal coordinates to one of the resulting carbamino oxygen atoms. Therefore, it is not surprising that carbamylation must occur before the metal can bind. Two protons are released during carbamylation (Fig. 3) and this is reflected in the pH response of carbamylation (Lorimer et al., 1976). The slowness of CO_2 addition relative to Mg^{2+} binding may be a result of scarcity of the unprotonated form of K201.

In addition to carbamylated K201, the protein provides two other ligands to the metal. These are carboxyl oxygen atoms of D203 and E204. In the

¹ The numbering of amino acid residues in both Rubisco subunits is based on that of the spinach enzyme. The alignments of Schneider et al. (1990) and Hudson et al. (1990) may be used to convert the residue numbers of the *R. rubrum* and *Synechococcus* PCC6301 enzymes, respectively, to that of spinach. Amino acid residues are represented by their single-letter codes followed by the sequence number. A mutant residue is represented by the sequence number with the natural residue as a prefix and the mutant substitution as a suffix.

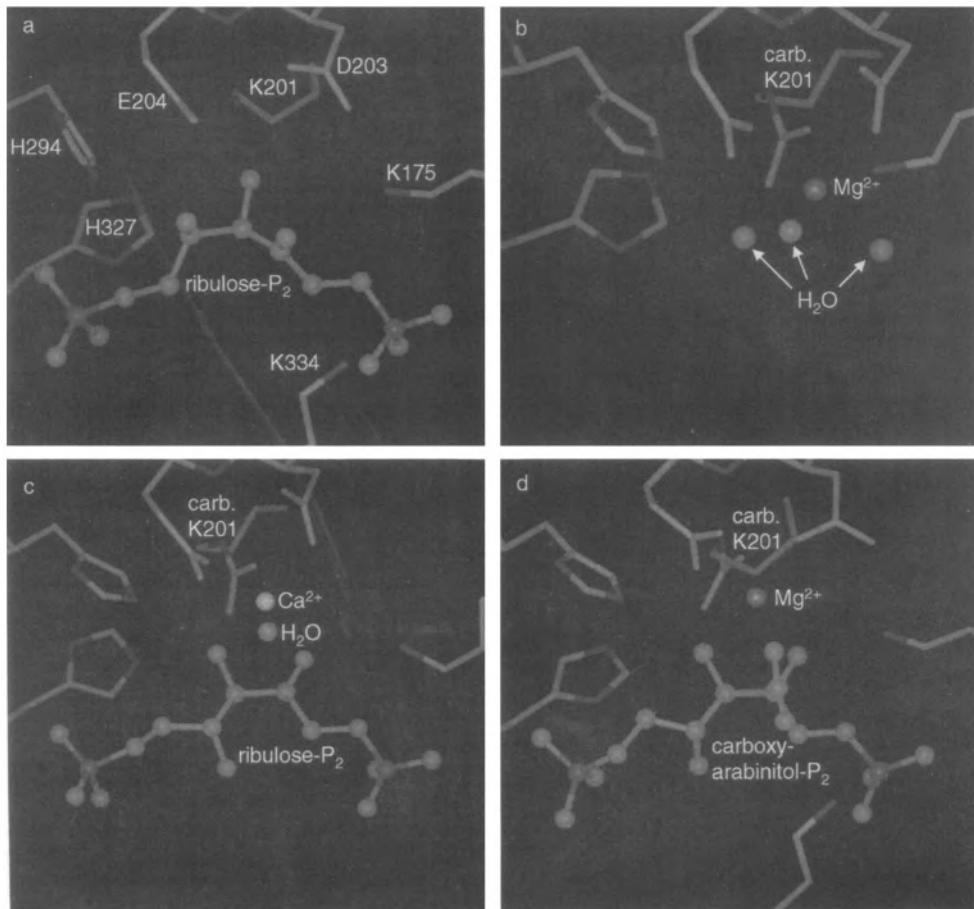


Fig. 4. Views of the active site of spinach Rubisco showing residues involved in catalysis and binding of the metal ion (stick rendering). a, ribulose-P₂ bound to the decarbamylated (inactive), metal-free site (ER) (Taylor and Andersson, 1997); b, the carbamylated, Mg²⁺-activated site without substrate (ECM) (Taylor and Andersson, 1996); c, the carbamylated site with Ca²⁺ and ribulose-P₂ bound (ECMR) (Taylor and Andersson, 1997); d, the carbamylated, Mg²⁺-activated site with 2'-carboxyarabinitol-P₂ bound (ECM-analog) (Andersson, 1996). Heavy atoms of the phosphorylated ligands, metal ions and water molecules are rendered as balls. Also see Color Plate 1.

absence of substrate, water molecules (Fig. 4b) occupy the other three metal-coordination positions. When ribulose-P₂ binds, two of these water molecules are displaced by the O-2 and O-3 atoms of the substrate (Fig. 4c). Ribulose-P₂ can also bind to the uncarbamylated, metal-free active site (Fig. 4a). This blocks the access of the metal to the active site, thus explaining observations that pre-exposure of Rubisco to ribulose-P₂ in the absence of metal seriously retards the carbamylation process (Jordan and Chollet, 1983). Since ribulose-P₂ binds to E three orders of magnitude more tightly than its K_m for catalytic conversion by ECM (Jordan and Chollet, 1983), carbamylation is effectively prevented when ribulose-P₂ is present. In vivo, this impasse is broken by the action of Rubisco

activase, which mediates the release of ribulose-P₂ from its dead-end complex with uncarbamylated Rubisco in an ATP-dependent reaction (for reviews, see Portis, 1992; Andrews et al., 1995; Portis, 1995; Salvucci and Ogren, 1996).

C. The Sequence of Catalytic Events

1. Substrates React in an Ordered Sequence

Once activated, with the lysyl carbamate and the metal in place, the stage is set for catalysis. The reaction is ordered with ribulose-P₂ binding before addition of the gaseous substrates, CO₂ or O₂. This is established by two complementary lines of evidence

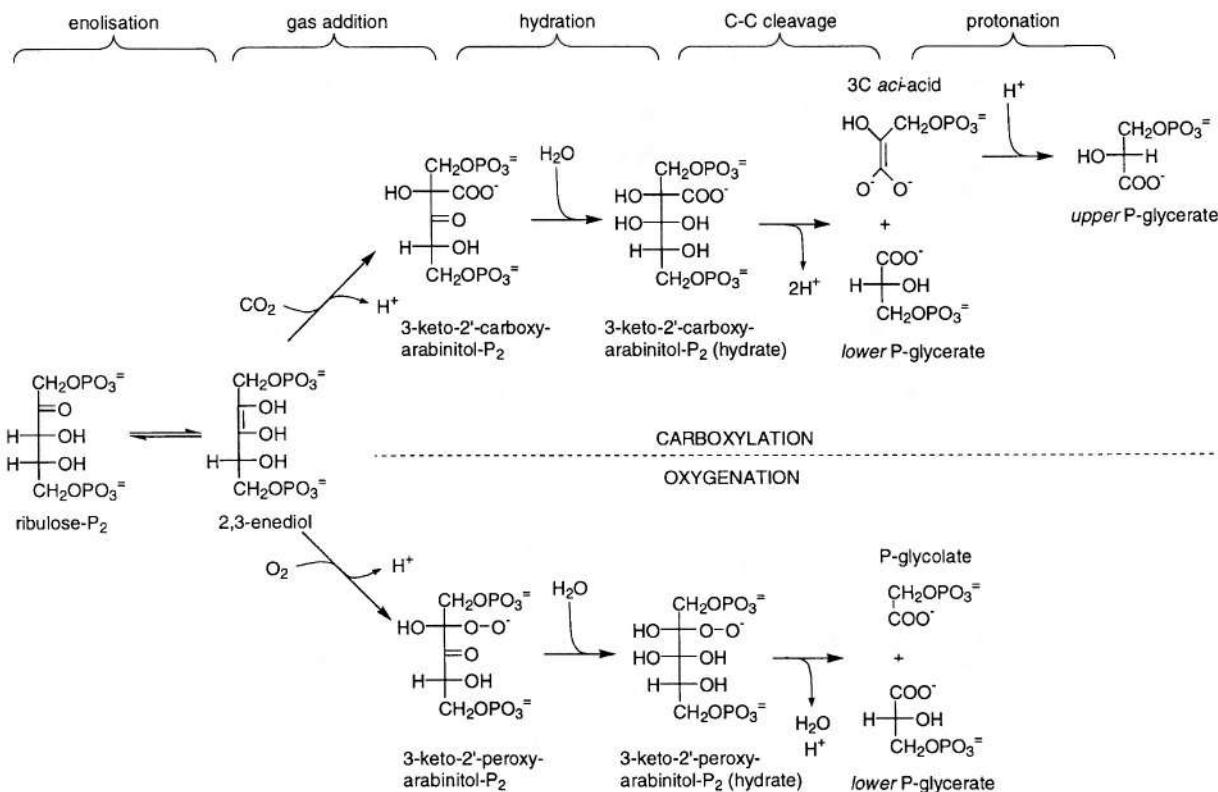


Fig. 5. The sequence of catalytic intermediates involved in carboxylation and oxygenation.

(Pierce et al., 1986). First, NMR studies revealed no enhancement of the relaxation rate of ¹³CO₂ by Mn²⁺ bound at the active site although similar evidence of binding was easy to observe with HCO₃⁻, which binds loosely in the sites normally occupied by the phosphate groups of ribulose-P₂. Even when xylulose-P₂ was present, no CO₂ binding could be detected. Second, pre-exposure of Rubisco to ¹⁴CO₂ followed by simultaneous addition of ribulose-P₂ and an excess of unlabelled CO₂ did not result in the production of labeled P-glycerate. Equilibrium binding experiments also showed no sign of O₂ binding in the absence of ribulose-P₂, even in the presence of xylulose-P₂. Taken together, these observations lead to the conclusion that no binding site for CO₂ or O₂ exists in the absence of ribulose-P₂, as would be expected if the gaseous substrate added first or if the order was random.

2. Sequence of Catalytic Intermediates

The catalytic processes of carboxylation and oxygenation involve a sequence of analogous

intermediates except for a final protonation that is lacking in the case of oxygenation (Fig. 5). The preliminary enolization of ribulose-P₂ is common to both pathways. CO₂ or O₂ then compete for the resulting enediol² producing either a carboxyketone or a peroxyketone, respectively. These ketones are hydrated, either in concert with the addition of the gases or subsequently. The hydrated ketones then cleave heterolytically between C-2 and C-3. In the case of oxygenation, this completes the reaction. Carboxylation involves one further step: a proton must be added to the *Si* face of C-2 of the aci-acid produced from C-1 and C-2 of ribulose-P₂ and the incoming CO₂ molecule in order to produce the second molecule of P-glycerate.

² During the course of the reaction, the enediol intermediate probably exists in three different protonation states depending on whether O₂, O₃ or both are protonated. It is also possible that the enediol is activated for electrophilic attack on C2 by acquiring some carbanionic character at this carbon. Unless otherwise specified, all of these states are referred to genetically as the 'enediol'.

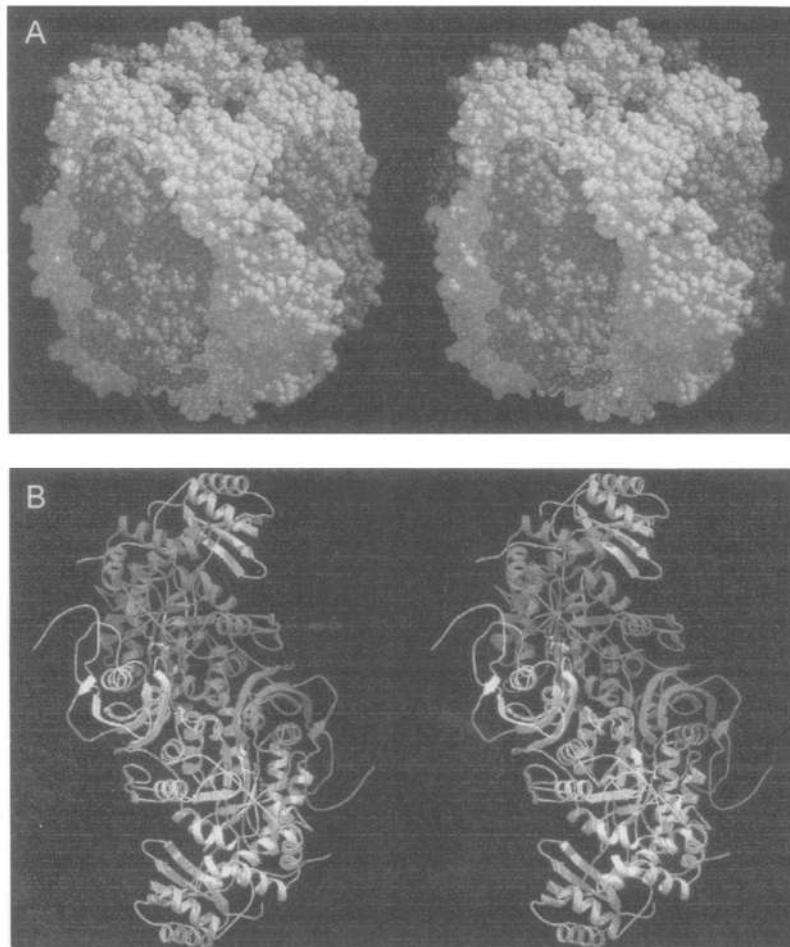


Fig. 6. Stereo views of the structure of spinach Rubisco. Small subunits are colored in yellow in the color plate and large subunits in two shades of grey. One dark grey and one light grey subunit together comprise the basic dimeric unit of large subunits. A, Space-filling rendering of the L₈S₈ hexadecamer showing the small subunits sandwiched between adjacent L₂ dimers and forming S₄ tetramers that cap each pole of the hexadecamer. B, Ribbon rendering of one L₂S₂ unit showing the elements of tertiary structure with bound ligands (Mg²⁺ and 2'-carboxyarabinitol-P₂) rendered in ball and stick and colored by atom in the color plate. Reproduced from Andersson (1996) with permission. Also see Color Plate 2.

D. Structural Transitions Associated with Ribulose-P₂ and Analog Binding

1. Three-Dimensional Structure of Rubisco

Detailed three-dimensional structures have been determined crystallographically for the Form II (L₂) Rubisco of *Rhodospirillum rubrum* and the Form I (L₈S₈) Rubiscos of *Synechococcus* PCC6301 (a cyanobacterium), tobacco and spinach. In each case, structures have been determined with several different ligands occupying the active site. Some structures without ligand, and with or without the activating

carbamate and metal ion, have also been determined. The available structures have been tabulated by (Cleland et al., 1998). Despite the considerable sequence divergence between the large subunits of Form I and Form II Rubiscos (approximately 25% identity clustered in the loop regions that contribute to the active site (Andrews and Lorimer, 1987)), the structures of the two forms are strikingly similar.

The large subunit consists of two main domains (Fig. 6). The N-terminal 150 residues form a five-stranded mixed β sheet with two α helices on one side. The C-terminal domain is a classical eight-stranded α/β barrel. This basic architecture is

maintained in all Rubiscos, both Form I and Form II. Like other α/β barrel enzymes, the active site is located at one end of the barrel, C-terminal with respect to the eight β strands. Most of the residues involved in metal binding and catalysis occur in the loops that connect the β strands to the helices. The others are located in the N-terminal domain of a companion L subunit, making L_2 the minimum functional unit. The *R. rubrum* enzyme consists of this unit alone. Ligands bound at the active site are completely sequestered from solvent by several mobile loops that undergo a disorder-to-order transition as they close over the ligand (Schreuder et al., 1993).

The small subunits, present only in Form I Rubisco, are composed of a four-stranded anti-parallel β sheet flanked on one side by two α helices (Fig. 6). They occur as S_4 clusters, one such cluster capping each pole of the L_8 octamer. They are quite remote from the active sites and this arrangement suggests that the S_4 clusters play a role in stabilizing the octameric core.

2. Binding to Uncarbamylated Site

Ribulose-P₂ and its analogs bind to both uncaramblylated and carbamylated active sites. When the site is not carbamylated, and therefore metal-free, the complex with ribulose-P₂ is unreactive and very stable. This has allowed the structure of this complex with spinach Rubisco to be determined crystallographically (Fig. 4a) (Taylor and Andersson, 1997). Similar structures are also seen with the analogs, xylulose-P₂ and 4'-carboxyarabinitol-P₂, with both *Synechococcus* and spinach Rubiscos (Newman and Gutteridge, 1994; Taylor et al., 1996). These structures are all similar and, except for the absence of the activating carbamate and the metal ion, similar to the structure of the complex of the carbamylated enzyme with 2'-carboxyarabinitol-P₂ (Section IV.D.3). In particular, the active sites in these structures are in a fully ordered, closed conformation with the ligand completely sequestered from solvent.

3. Binding to Carbamylated Site

Binding of 2'-carboxyarabinitol-P₂ to the carbamylated active site also induces closure, as shown by the structures of this complex with the *Synechococcus* (Newman and Gutteridge, 1993), tobacco (Schreuder et al., 1993) and spinach (Knight et al., 1990;

(Andersson, 1996) enzymes. All other structures show the active site in its open form. These include ligand-free structures with (Taylor and Andersson, 1996) or without (Curmi et al., 1992) the carbamate and metal, a carbamylated structure with two molecules of the product, P-glycerate, bound (Taylor and Andersson, 1997) and a complex between carbamylated spinach Rubisco and ribulose-P₂ rendered inactive by substitution of Ca²⁺ for Mg²⁺ (Taylor and Andersson, 1997).

4. Structural Changes Accompanying Closure

Structural differences between the open and closed conformations of the active site provide information about the transitions that must occur during every catalytic cycle (Schreuder et al., 1993; Taylor and Andersson, 1996). The most spectacular is the movement of loop 6 (residues 333–338) of the barrel domain. This region moves to cover the ligand like a hinged lid. The α carbons of the two residues at the apex of this lid move over 10 Å during this closure so that their side chains come into contact with the ligand. Residues 64–68 and the preceding helix of the N-terminal domain of the companion L subunit also move closer to the active site during ligand binding and residues 9–21 become ordered. At the C terminus, two turns of the C-terminal helix unwind so that the C-terminus can extend over loop 6, locking it in the closed position. In concert with these movements, the entire small subunit and the N-terminal domain of one LS pair pivot slightly with respect to the barrel domain.

5. When Does the Active Site Close?

The stage of the catalytic cycle (Fig. 5) at which this closure occurs is not presently known. The structural evidence establishes that it must occur by the time the carboxylated intermediate, 3-keto-2'-carboxy-arabinitol-P₂, has formed and that it reopens for product release. A closed, desolvated active site would also be necessary to protect the enediol intermediate from incorrect protonation (Edmondson et al., 1990d). However, the open state of the activated complex with Ca²⁺ and ribulose-P₂ presents a puzzle (Taylor and Andersson, 1997). Does this mean that active-site closure occurs during the act of enolization? Or is closure hindered by the larger size of Ca²⁺ compared to the functional metals?

E. The Central Role of the Metal

Rubisco's catalytic chemistry is centered on the metal, which pervasively influences all of the steps of the reaction. The 1.6 Å structure of the 2'-carboxy-arabinitol-P₂ complex with activated spinach Rubisco (Andersson, 1996) provides an excellent view of the way the metal is liganded (Fig. 4d). The octahedral coordination arrangement is somewhat distorted with rather long metal-ligand bond distances. Three of the ligands are provided by the protein and three by the analog, all of them O atoms. The four ligands in the equatorial plane (as defined by the shading in Fig. 7) are carboxyl O atoms of D203 and E204 and O2 and O3 of the analog. The ligand above the plane is provided by a carboxyl O atom of the analog; the one below the plane is a carbamino O atom of carbamylated K201. When the substrate analog is missing, water molecules substitute for its three ligands (Fig. 4b). The two water molecules in the equatorial plane are displaced by ribulose-P₂ as it binds (Fig. 4c); the one above the plane is displaced by the incoming CO₂ molecule (and presumably by the incoming O₂ molecule in the case of oxygenation) as the carboxylated (or oxygenated) intermediate is formed.

F. The First Hurdle—Enolization

1. Occurs in the Absence of Gaseous Substrate

The first step of the catalytic sequence is the only one common to both carboxylation and oxygenation. It requires the removal of a proton from C3 of ribulose-P₂ and addition of a proton to O2 (Figs. 5 and 7A,B). Protonation of O2 is essential. If this O atom were left unprotonated, gas addition in the next step would be directed towards C3, not C2. At some stage, deprotonation of O3 is also required to ensure gas addition to C2—see Section IV.F.4.

Enolization can be measured independently of overall catalysis by observing the exchange of the C3 proton with solvent when either the proton or the solvent is suitably labeled (e.g. see (Sue and Knowles, 1982; Gutteridge et al., 1984; Pierce et al., 1986; Lorimer and Hartman, 1988). The exchange rate is highest when CO₂ concentration is low and is suppressed at high CO₂ concentration (Pierce et al., 1986), implying that enolization precedes CO₂ addition and that reprotonation of the enediol and carboxylation of it are competing reactions.

2. Is the Carbamate the Essential Base?

Before the advent of crystallographic structural information about Rubisco's active site, speculation abounded about the identity of the residue that initiated the catalytic sequence by abstracting the C3 proton of ribulose-P₂. Several possibilities were advanced, based on circumstantial evidence. Early crystallographic evidence ruled out some of these notions but did not shed further light on the question because of inadequate resolution and confusion about which O atoms of the substrate were liganded to the metal. The confusion was finally resolved when the structures of the 2'-carboxyarabinitol-P₂ complexes with the carbamylated *Synechococcus* (Newman and Gutteridge, 1993) and spinach (Andersson, 1996) enzymes were determined at high resolution. These established unequivocally that O2 and O3 were the metal-coordinated substrate O atoms and they showed that only one residue was appropriately positioned to act as the proton abstracter. Carbamylated K201 was monodentately coordinated to the metal via one of its carbamate O atoms; the other O atom of the carbamate seemed ideally positioned with respect to C3 of the analog to abstract the C3 proton of the substrate. This was confirmed in the structure of the spinach Rubisco/Ca²⁺/ribulose-P₂ complex where this O atom is 3.1 Å from C3 (Taylor and Andersson, 1997).

Despite this precise structural identification, uncertainty persisted about whether a carbamate O atom so positioned could be basic enough to remove a proton from carbon. Metal coordination of one O atom of a carboxylate, for example, lowers the pK_a of the other O atom so much that it can no longer act as a general base. However, a carbamate has access to a resonant structure where both O atoms are negatively charged and the N atom is positively charged. Proof that such an *aci*-carbamate can exist in proteins was provided by the structures of urease (Jabri et al., 1995) and phosphotriesterase (Benning et al., 1995). In both of these enzymes, a carbamate is monodentately coordinated to each of two divalent metal ions (Ni²⁺ or Zn²⁺), indicating that both carbamate O atoms must bear substantial charge. In the case of Rubisco, a hydrogen bond between the carbamino N atom and the main-chain carbonyl O of residue 202 might assist in stabilizing the *aci*-carbamate (Lundqvist and Schneider, 1991).

Quantum chemical calculations were applied to the enolization step of the Rubisco reaction to assess whether it is likely that the carbamate could be the

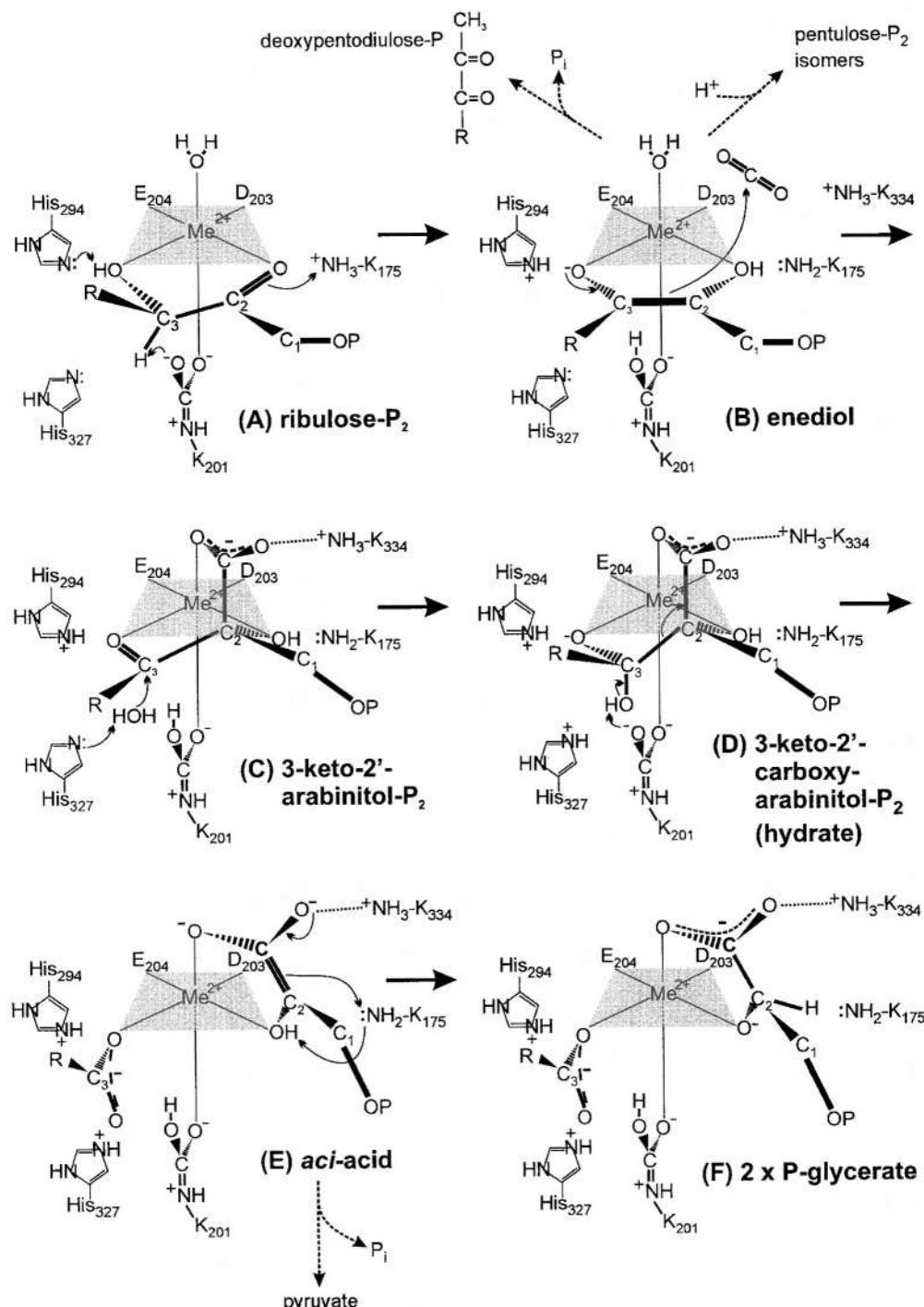


Fig. 7. A proposed catalytic mechanism for the carboxylation of ribulose-P₂ showing the suggested roles of various active-site residues. Compared to Fig. 4, the structures are viewed rotated by approximately 90° (clockwise as viewed from P1) around an axis through the two phosphorus atoms. During enolization (A → B), deprotonation of C3 by the Lys-201 carbamate must occur first but the timing of the subsequent protonation of O₂ and deprotonation of O₃ is not specified. Carboxylation of C2 of the enediol B is shown preceding hydration at C3 but a concerted mechanism without intermediate C is also plausible. Known side reactions of the carboxylation pathway are shown with dashed arrows.

proton abstracter. Using a 29-atom fragment model of the Mg^{2+} -complexed active site in which glycolaldehyde represented C2 and C3 of the substrate, formate ions represented D203 and E204, and methyl carbamate represented carbamylated K201, states along a proposed enolization pathway were calculated (King et al., 1998). Calculations at several levels of theory yielded estimates of the energy of the transition state associated with transfer of the proton from C3 to the carbamate O atom that were low enough to lend credence to the notion that the carbamate could function in this way.

The basicity of the carbamate could be effectively increased in a way not modeled by the quantum chemical calculations. In the spinach Rubisco/ Ca^{2+} /ribulose-P₂ complex, the non-coordinated O atom has access to a hydrogen-bonded network involving H327, Q401, S379, T173, two water molecules and the P1 phosphate group (Taylor and Andersson, 1997). This network could stabilize the protonated carbamate and allow the proton originally derived from C3 to exchange into it, consistent with the observed lack of retention of label derived from this proton during enolization.

3. Role of Lys-175 in Enolization

Another residue is also critical for enolization, K175. Its replacement with other residues caused 99.9% or greater inactivation of enolization and overall catalysis (Hartman et al., 1987; Hartman and Lee, 1989). Furthermore, the impairment appeared to be specific to the enolization partial reaction, the Gly-175 mutant enzyme being capable of binding 2'-carboxyarabinitol-P₂ and cleaving the carboxylated intermediate, 3-keto-2'-carboxyarabinitol-P₂ (Lorimer and Hartman, 1988). K175 also displays the unusually low pK_a of 7.9 in its arylation by trinitrobenzenesulfonate (Hartman et al., 1985), a value close to that of an essential base observed in the pH-dependence of the deuterium isotope effect with [3-²H]ribulose-P₂, (Van Dyk and Schloss, 1986).

Were it not for its totally inappropriate position and orientation (Figs. 4 and 7), all of this evidence might appear to establish K175 as the base that abstracts the C3 proton of ribulose-P₂. Even with such a direct role ruled out, the data nevertheless show that K175 must have a very important role in enolization. What is this role? Participation in a proton relay that transports protons away from the active site has been hypothesized (Cleland et al.,

1998). An alternative role envisages K175 acting as a general acid, not a base (Lundqvist and Schneider, 1991; King et al., 1998). While it certainly must act as a base in the reaction with trinitrobenzenesulfonate, it does not necessarily follow that this must also be true for ribulose-P₂ enolization. If K175 was protonated at the outset of the reaction (and its observed pK_a of 7.9 is consistent with substantial protonation), then it would facilitate enolization first by assisting the metal to polarize the C2 carbonyl and second by protonating the developing negative charge on O₂ (Fig. 7A,B), thus reducing the danger of misdirecting subsequent gas addition to C3. Quantum chemical calculations with the 29-atom representation of the active site, supplemented with either an ammonia molecule or an ammonium ion in the position occupied by the ε amino group of K175, showed that the presence of the ammonium ion (but not the ammonia molecule) stabilized the transition state for C3 proton abstraction by approximately 4 kcal mol⁻¹. This value is quite consistent with the rate enhancement provided by Lys at position 175 compared to other residues. Furthermore, the protonation of O₂ by the ammonium ion strongly stabilized the resultant enediol (King et al., 1998).

4. Deprotonation of O₃

Protonation of O₂ of the enediol is necessary to direct gas addition to C2 but not sufficient. Deprotonation of O₃ is also required. H294 is appropriately positioned to accomplish this task (Figs. 4 and 7) and, in keeping with this assignment, its replacement with Asn or Gln virtually abolished catalysis (Harpel et al., 1998). However, these mutants were also disabled in enolization and in processing 3-keto-2'-carboxyarabinitol-P₂, indicating that this residue influences several different catalytic steps. The near-total dependence of exchange of the C3 proton on H294 is a puzzle not readily explained by any current model of Rubisco's catalytic mechanism. H294 is close to the carbamate and perhaps removal of the imidazole side chain disturbs the hydrogen-bonded network that stabilizes the protonated carbamate and facilitates the exchange of its proton (Section IV.F.2) (Harpel et al., 1998).

Alternatively, if the hydrogen-bonded network can function to traffic the protonated carbamate's proton away from the active site, then the carbamate itself would be available for re-use as a general base. It is also well positioned to deprotonate O₃.

5. Stabilization of the Enediol Intermediate is not Completely Successful

The active site faces a challenging task in stabilizing such a highly reactive enolic nucleophile. It must protect it from attack by electrophiles other than CO_2 and frustrate the intrinsic tendency of enols to eliminate β substituents.

Attack by unwanted nucleophiles—The most notable of these is O_2 which substitutes for CO_2 and causes the oxygenase reaction (Section IV.K). Stereochemically misdirected attack by protons presents another difficulty. Reprotonation of C3 from below the plane of Fig. 7 (i.e. from its *Si* face) reproduces the substrate, ribulose-P₂. Misdirected proton attack from above produces the epimer, xylulose-P₂. For the spinach enzyme, this occurs about once in every 400 turnovers and contributes to progressive inhibition during catalysis (Edmondson et al., 1990a,b,c,d). As expected, this misprotonation reaction is reversible, allowing xylulose-P₂ to serve as a very slowly reacting substrate (Yokota, 1991; Lee et al., 1993; Newman and Gutteridge, 1994). However, xylulose-P₂ eventually forces decarbamylation (Zhu and Jensen, 1991a). In vivo, inhibition by xylulose-P₂ presumably is reversed by Rubisco activase (for reviews, see Portis, 1992, 1995; Andrews et al., 1995; Salvucci and Ogren, 1996). The wild-type *R. rubrum* enzyme, however, does not form appreciable amounts of xylulose-P₂ (Lee et al., 1993). Partitioning of product to xylulose-P₂ is exacerbated with the T65V mutant of *Synechococcus* Rubisco (Morell et al., 1997), several mutants of *R. rubrum* Rubisco (Lee et al., 1993; Larson et al., 1995; Harpel et al., 1998) and with wild-type spinach and *Synechococcus* Rubiscos when CO_2 and O_2 become depleted (Morell et al., 1997).

Misprotonation at C2, rather than C3, is also theoretically possible. Proton attack from the *Si* face (the upper face in Fig. 7) would produce 3-keto-arabinitol-1,5-bisphosphate; from below it would produce 3-keto-ribitol-1,5-bisphosphate. Attack from above, the direction that CO_2 adds from, was speculated to occur (Edmondson et al., 1990d) and detection of arabinitol-1,5-bisphosphate after borohydride reduction of reaction products supported this speculation (Zhu and Jensen, 1991b; Morell et al., 1994, 1997; Zhu et al., 1998). However, under some conditions, arabinitol-1,5-bisphosphate can be produced nearly exclusively by reduction of a

bisphosphorylated, dicarbonyl compound (pentodiulose-P₂) formed as an abortive by-product of the oxygenase reaction by mutants of *R. rubrum* Rubisco (Chen and Hartman, 1995; Harpel et al., 1995) and by non-enzymatic oxidation of ribulose-P₂ (Kane et al., 1998). This compound occurs as a contaminant in ribulose-P₂ preparations and it binds tightly to carbamylated Rubisco. It contributes substantially to the progressive inhibition observed during catalysis and is the dominant cause of this inhibition with poor quality ribulose-P₂ preparations (Kane et al., 1998). In view of this confusion, the actual extent of misprotonation of the enediol at C2 remains uncertain.

β Elimination of the P1 Phosphate—Enols of phosphorylated sugars are very prone to loss of phosphate via β elimination reactions (Richard, 1984). Since these reactions involve elimination, not hydrolysis, desolvation within an active site provides no protection against them. Protection can be provided, however, by maintaining the bond to the β substituent in the same plane as the double bond, thereby minimizing orbital overlap between the substituent bond and the π system of the double bond (Rose, 1981). Although there is no structural information about the way the enediol is bound in the active site, the similarity between the structures of carbamylated spinach Rubisco with ribulose-P₂ and 2'-carboxyarabinitol-P₂ bound suggests that the enediol is bound in a similarly extended configuration. Furthermore, in the carbamylated Rubisco/Ca²⁺/ribulose-P₂ complex, O1, C1, C2 and C3 appear perfectly coplanar (Fig. 4c) (Taylor and Andersson, 1997). If this arrangement persists after formation of the enediol, β elimination would be minimized.

However, this stabilization strategy does not succeed completely. Production of 1-deoxy-D-glycero-2,3-pentodiulose-5-phosphate, the ultimate product of β elimination (Fig. 7B), can be detected with both spinach and *Synechococcus* Rubiscos following depletion of CO_2 and O_2 (Morell et al., 1997). Mutagenic perturbation of the P1-binding site, which would be expected to impair ability to maintain the required planar configuration, drastically increases the proportion of the enediol β -eliminated by both *Synechococcus* and *R. rubrum* Rubiscos (Larimer et al., 1994; Morell et al., 1994). Similar stimulation of the abortive reaction is also caused by replacement, in the *R. rubrum* enzyme, of K334 or deletion of the loop that contains it (Larson et al., 1995). This implies that blockage of carboxylation

of the enediol (Section IV.G.3) results in some of the enediol escaping from the active site to β eliminate in solution.

G. The Main Event—Gas Addition

1. No Michaelis Complex?

The correct tautomeric form of the enediolate (Fig. 7B) encourages CO_2 (or O_2) addition at C2. As discussed in Section IV.C.1, the evidence seems convincing that neither gas binds in the absence of ribulose-P₂. From this, it generally has been inferred that neither CO_2 nor O_2 forms a Michaelis (i.e. non-covalent) complex before reaction. This may well be true. It is difficult to imagine how such featureless molecules could bind non-covalently with substantial affinity. Nevertheless, the possibility of some sort of loose pre-reaction complex of the gases that forms only after ribulose-P₂ has bound (or enolized) and the active site has closed is not yet formally excluded.

2. Is Addition of Gas and Water Concerted or Sequential?

Acid quenching of Rubisco during steady-state carboxylation releases the carboxylated intermediate, 3-keto-2'-carboxyarabinitol-P₂. It has a 1-h half life in neutral solution at 25 °C, decaying by decarboxylation, but can be trapped by borohydride reduction (Schloss and Lorimer, 1982; Lorimer et al., 1986). It exists in solution predominantly as the free carbonyl but on the active site it is fully hydrated (Pierce et al., 1986). When isolated 3-keto-2'-carboxyarabinitol-P₂ (free carbonyl) is supplied to Rubisco, it is processed to P-glycerate but at maximal rate of only one-fiftieth of the maximal rate of carboxylation of ribulose-P₂ (Pierce et al., 1986). This might be interpreted as evidence that the free carbonyl form is not a normal intermediate of the reaction, i.e. that CO_2 and OH^- addition to the enediol are concerted (Cleland, 1990). However, binding of 2'-carboxyarabinitol-P₂ (the analog of the gem-diol form 3-keto-2'-carboxyarabinitol-P₂ lacking one of the gem-diol hydroxyls) is a two-step process and the second, slower step proceeds at a rate comparable to the rate of turnover of 3-keto-2'-carboxyarabinitol-P₂ (Pierce et al., 1980; Pierce et al., 1986). Thus it is likely that processing of 3-keto-2'-carboxyarabinitol-P₂ is limited by a similar slow-binding

step. Even so, this rate is still over 50-fold faster than the uncatalyzed hydration of 3-keto-2'-carboxyarabinitol-P₂ (Lorimer et al., 1986). Therefore we must conclude that Rubisco must bind the free carbonyl form of 3-keto-2'-carboxyarabinitol-P₂ and catalyze its hydration. The reduced rate of these processes compared to catalysis with the natural substrates may be caused by slower closure of the active site with the 2' carboxylate and is not necessarily evidence that the free carbonyl form does not lie on the catalytic pathway.

3. Features in the Active Site that Mediate CO_2 Addition

Regardless of whether CO_2 addition and hydration are concerted or sequential, three active-site features are required to facilitate the combined process.

First, a means of maintaining the negative charge on O3 is necessary to direct CO_2 attack to C2. The interaction of this atom with the metal and with H294 (if it still bears the proton originally derived from O3) will achieve this. If the mechanism is sequential and the charge on O3 transiently disappears (Fig. 7C), the same features will be required to polarize the intermediate carbonyl to assist its hydration.

Second, the nascent carboxylate group at C2 must be stabilized. Structural (Fig. 4d) and mutagenesis and chemical-rescue (Lorimer et al., 1993; Larson et al., 1995) data concur in assigning the ϵ amino group of K334 a central role in this task in concert with the metal. Other side chains in the vicinity, such as Glu-60 (Lee et al., 1993) and L335 (Lee et al., 1993) also assist in maintaining accurate positioning of the K334 amino group.

Third, a general base is required to deprotonate the water molecule that attacks C3. Either H327 or H294 could serve in this way. If H294 is preoccupied accommodating the proton originally derived from O3, then the task might fall to H327. The crippling effects of mutating this side chain in *Synechococcus* Rubisco (Haining and McFadden, 1994) are consistent with such a role. On the other hand, substitution of residue 327 of *R. rubrum* Rubisco with Asn or Gln reduced both catalysis and enolization by only approximately 10-fold (Harrel et al., 1991), arguing against a total dependence on this residue alone. Again, the suspicion arises that the hydrogen-bonded network involving the K201 carbamate, H327,

Q401, S379, T173, two water molecules and the P1 phosphate group (Taylor and Andersson, 1997) collectively functions to absorb protons and transport them from the active site.

4. This Step is Effectively Irreversible

When 3-keto-2'-carboxyarabinitol-P₂ is supplied as substrate to Mg²⁺-activated Rubisco, it is converted nearly exclusively to P-glycerate. Little or no decarboxylation occurs, showing that the reaction is effectively irreversible once CO₂ addition has occurred. If the metal is omitted, only decarboxylation is observed; intermediate partitioning between the forward and reverse pathways occurs when the enzyme is activated with Mn²⁺ or Co²⁺ (Pierce et al., 1986). Reverse partitioning is also seen with some mutants of *R. rubrum* Rubisco, e.g. K201E and H294N (Lorimer et al., 1987; Harpel et al, 1998). Interpretation of the significance of the decarboxylation reaction catalyzed by metal-free enzyme is clouded, however, by structural observations that the analog of the same intermediate, 2'-carboxyarabinitol-P₂, binds to the metal-free active site in reverse orientation, i.e. with the P2 phosphate group in the position normally occupied by P1 and vice versa (Lundqvist and Schneider, 1989; Zhang et al., 1994).

H. Carbon-Carbon Cleavage

1. The Facilitating Base

For cleavage between C2 and C3 to occur so that C3 is converted from a gem diol to a carboxylate, both of the gem-diol O atoms must be deprotonated. According to the mechanism formulated here (Fig. 7D), the metal-coordinated O atom is already deprotonated. Therefore, during C-C cleavage, the non-coordinated O also must lose its proton. This hydroxyl points towards the carbamate, which would be well positioned to acquire the proton (Fig. 7D) provided that it had been able to dispose of previously abstracted protons into its hydrogen-bonded network. For this to occur, the capacity of this network, and the associated histidyl residues 294 and 327, to absorb and/or transport protons must be quite prodigious. All four protons originally associated with C3 and the O atoms attached to it apparently are lost into this network.

2. The Aci-Acid Intermediate and its Stabilization

C-C cleavage produces one molecule of P-glycerate and the *aci*-acid (i.e. enol) form of a second (Fig. 7E). This intermediate has the same propensity to β eliminate the phosphate group that the enediol intermediate has (Section IV.F.5). Presumably the same device stabilizes it; the C1-O bond to the phosphate must be held in the same plane as the carboxyl C-C2 bond (illustrated in Fig. 7E). However, a geometric conflict is now encountered. This is the same C1-O bond that, in the enediol intermediate, must be held co-planar with the C3-C2 bond which is perpendicular to the carboxyl C-C2 bond (compare Fig. 7B and Fig. 7E). To achieve the required planar configuration, either the C1-O bond or the carboxyl C-C2 bond (or both) must rotate on the C2-C1 axis through a total dihedral angle of 90° compared to their dispositions in the hydrated 3-keto-2'-carboxyarabinitol-P₂ intermediate (compare Fig. 7D and Fig. 7E).

This critically important movement to co-planarity must occur within the constraints imposed by the coordination and hydrogen-bonded interactions which tether O2 to the metal, the carboxylate to the metal and to K334, and the phosphate to three Gly residues in loops 7 and 8 and to the T65 hydroxyl of the companion large subunit. These tethering interactions lie at opposite extremities of the *aci*-acid fragment, leaving C1 and C2 free to move. An economical clockwise movement of these two C atoms (as viewed from P1) about a line joining P1 and the metal would bring them into the required plane with O1 and O2 syn to each other. Such a movement involves only modest disturbance of the coordination with the metal of O2 and one carboxyl O and of the interaction of the other carboxyl O with K334. A small movement of O1 and a slight tilt of the plane through the three O atoms of P1 are also required. The structure of carbamylated spinach Rubisco complexed with two P-glycerate molecules reveals the *upper* P-glycerate molecule bound in a manner generally consistent with this interpretation, although in this complex loop 6 has retracted away from the active site and K334 no longer interacts with the carboxyl O atom (Taylor and Andersson, 1997).

As with the enediol intermediate, stabilization of the *aci*-acid is not completely successful. All wild-type Rubiscos studied abort their *aci*-acid inter-

mediate to produce pyruvate once in every 150 turnovers under a wide range of conditions (Fig. 7E) (Andrews and Kane, 1991). Again, this might be the result of inadequate stabilization of the *aci*-acid within the active site or escape of the intermediate into solution. Mutation of any of the residues that tether P1 perturbs this stoichiometry (Morell et al., 1994; Larimer et al., 1994). Replacement of the solvent with $^2\text{H}_2\text{O}$ increases the tendency for the phosphate to be eliminated, presumably by slowing the subsequent protonation reaction (Andrews and Kane, 1991).

I. The Denouement—Stereospecific Protonation

1. Role of K175

To complete the reaction, protonation of C2 of the *aci*-acid intermediate, specifically on its *Si* face, is required. The stereochemical requirement is such that the proton donor cannot be any of the residues that have been involved in deprotonations earlier in the catalytic sequence (Fig. 7). Structural information about the identity of the donor is unambiguous. K175 is ideally placed. The aforementioned movement of C2 following C2-C3 cleavage brings it into optimal proximity to the ϵ amino N of K175 (Taylor and Andersson, 1997). Mutagenesis data are in perfect concordance. While K175 mutants of the *R. rubrum* Rubisco retain ability to process 3-keto-2'-carboxyarabinitol-P₂ (Lorimer and Hartman, 1988), they do not produce the correct product. Pyruvate, instead of the *upper* P-glycerate molecule is a major product (Harrel and Hartman, 1996). Unable to acquire a proton quickly before the active site opens, the unstable *aci*-acid intermediate falls victim to β elimination.

K175 may not be the ultimate source of the proton supplied to C2. It has already donated a proton to O₂ during enolization (Section IV.F.3). However, C2 is now very strongly basic and K175 may simply mediate the transfer of a proton from elsewhere to satisfy this basicity. For example, transfer of the O₂ proton to C2, via K175, would be favorable thermodynamically. Such a close-coupled mechanism might be indifferent to solvent pH, in keeping with the observation that partitioning of product to pyruvate by spinach Rubisco is not influenced by pH between 6.4 and 9.1 (Andrews and Kane, 1991).

J. Product Dissociation

Breakage of the C2-C3 bond probably triggers opening of the active site for release of products. The structure of carbamylated Rubisco with two P-glycerate molecules bound (Taylor and Andersson, 1997) provides an excellent snapshot of this last stage of the catalytic cycle. These crystals were grown in the presence of a very high P-glycerate concentration (100 mM), forcing occupancy despite the poor affinity of the active site for products. The picture shows the active site open with *upper* P-glycerate in the expected position (as depicted in Fig. 7F). The *lower* P-glycerate is bound through its P2 phosphate but the rest of the molecule has swung out towards solvent.

K. The Oxygenase Reaction

The oxygenation reaction is presumed to involve a sequence of intermediates analogous to those of the carboxylase reaction. Presumably, in concert with C2-C3 cleavage, heterolytic cleavage of the peroxy O-O bond yields P-glycolate from C1 and C2 (Fig. 5). In keeping with this mechanism, one of the atoms of the O₂ molecule is incorporated into the carboxylate group of P-glycolate; the other is lost to solvent (Lorimer et al., 1973).

1. Spin Prohibited?

Formally, addition of ground-state, triplet O₂ to a singlet species such as the enediol is a spin-forbidden process. Furthermore, Rubisco lacks common devices for circumventing this barrier, such as redox-active metals or organic cofactors that facilitate radical chemistry. Despite this theoretical impediment, Rubisco manages to catalyze oxygenation of ribulose-P₂ quite successfully and it is not unique in catalyzing O₂ addition to enols or carbanions (Abell and Schloss, 1991; Tse and Schloss, 1993; Hixon et al., 1996). Indeed, such additions are well known in organic chemistry. In Rubisco's case, two mechanisms have been proposed for the O₂-addition step; both are purely conjectural.

2. Radical Mechanism?

A radical mechanism can be envisioned whereby two successive single-electron transfers occur from the C2 carbanion form of the enediol to O₂ (Andrews

and Lorimer, 1987). After the first transfer, a radical pair consisting of a superoxide anion radical and a C2 radical cation form of the enediol would exist in the active site. Interactions of these species with the metal and with the rest of the active site presumably would cage the pair long enough for spin inversion to occur and the peroxyketone intermediate to form.

3. Triplet Enediol?

Alternatively, it has been proposed that the carbanionic enediol has access to a low lying triplet state. Deformation of the enediol in the active site is hypothesized to reduce the energy gap between the singlet ground state and the first excited triplet state sufficiently that the approach of triplet O_2 induces intersystem crossing leading to a reactive supermolecule and eventually to singlet products (Andrés et al., 1992; Tapia and Andrés, 1992; Andrés et al., 1993).

4. The Peroxyketone Intermediate

The carboxyketone intermediate of the carboxylase reaction can be isolated and used as a substrate and its structure in the active site can be inferred from that of its analog, 2'-carboxyarabinitol-P₂. None of these advantages apply to the analogous intermediate in the oxygenase pathway and information about it is therefore largely inferential. Spectroscopic evidence obtained with Cu²⁺-substituted spinach Rubisco favors an interaction of one of the O atoms of this intermediate with the metal (Brändén et al., 1984). If this is correct, then the interaction of the carboxylated intermediate with K334 must not be duplicated by the oxygenated intermediate. A role for this side chain in favoring carboxylation over oxygenation is therefore indicated, in accord with mutagenesis and chemical-rescue data for this residue (Gutteridge et al., 1993; Lorimer et al., 1993).

The most compelling evidence for the involvement of the peroxyketone intermediate comes from study of the E60Q and K334A mutants of the *R. rubrum* enzyme (Chen and Hartman, 1995; Harpel et al., 1995). These produce novel side products that are derived exclusively from the oxygenation pathway. The products are pentodiulose-P₂ and its product of benzylic acid-type rearrangement, carboxytetritol-P₂. Production of the former is accompanied by equimolar amounts of H₂O₂, indicating that it is derived by elimination of H₂O₂ from the peroxyketone

intermediate. In the case of the K334A mutant, rearrangement of pentodiulose-P₂ to carboxytetritol-P₂ within the active site then ensues. This oxidative reaction also occurs non-enzymatically. Ribulose-P₂ is oxidized to pentodiulose-P₂ by molecular O₂ in a reaction catalyzed by transition metals (Kane et al., 1998). Unlike mutant or wild-type *R. rubrum* Rubiscos, spinach Rubisco does not convert pentodiulose-P₂ to carboxytetritol-P₂ or other products. Therefore, the carbamylated enzyme binds it in a very tight, dead-end complex that presumably mimics the complex with the enediol intermediate in the positions of all of the heavy atoms. The complex can, however, be disrupted easily with H₂O₂, yielding the normal oxygenase products, P-glycolate and P-glycerate (Kane et al., 1998). The importance of stabilizing the peroxyketone intermediate, suppressing H₂O₂ elimination from it, and encouraging its cleavage to P-glycolate and P-glycerate is thus underscored, particularly for the higher-plant enzyme. Better to catalyze a wasteful side reaction (P-glycolate production) than to sequester the enzyme in a dead-end complex with the H₂O₂-elimination product.

5. Chemiluminescence with Mn²⁺

Spinach Rubisco activated by Mn²⁺, but not by other metals, emits light while catalyzing oxygenation. This was initially interpreted in terms of the hypothetical radical mechanism of oxygenation (Section IV.K.2) and attributed to the simultaneous decay (the so-called dimol emission) of two molecules of singlet O₂ formed by reversal of the first single-electron transfer (Mogel and McFadden, 1990). However, Lilley et al. (1993) noted that the light emission was proportional to enzyme concentration and the rate of oxygenase catalysis and that it had a rather broad emission spectrum with a maximum at 770 nm. They considered it more likely that the light originated from an outer-orbital transition of the Mn²⁺ ion excited by the presence of an excited species in its ligand field. Candidates for the exciting species might be the hypothetical radical pair of the radical model, the reactive supermolecule of the triplet model, or a species resulting from O-O cleavage of the peroxyketone. Chemiluminescence is also emitted from Mn²⁺-activated *R. rubrum* Rubisco but with an intriguing difference: a pronounced burst of light emission occurs in the first second after ribulose-P₂ is added (Lilley et al., 1993). Perhaps this indicates that *R. rubrum* Rubisco is more limited by a late step

in its catalytic sequence (beyond the one causing light emission) than is the spinach enzyme. While mutation of S379 of *Synechococcus* Rubisco reduced oxygenation and chemiluminescence approximately commensurately (Lee and McFadden, 1992), the ratio between chemiluminescence and oxygenation varied drastically among several L335 mutations (Lee et al., 1993). Apparently, slight changes within the active site can greatly affect the luminescence efficiency.

L. How is Selectivity between CO₂ and O₂ Achieved?

Since the catalytic sequence is ordered with the gases reacting second in a mutually competitive manner, the relative specificity for CO₂ as opposed to O₂ is given by the ratio of the V_{max}/K_m values for the two gases (i.e. $S_{c/o} = (V_c/K_c)/(V_o/K_o)$ where V_c , V_o and K_c , K_o are the V_{max} and K_m values for the CO₂ and O₂, respectively (Laing et al., 1974)). $S_{c/o}$ values for Mg²⁺-activated Rubisco at 25 °C vary over a 20-fold range (Table 1). Form II Rubiscos, which lack small subunits, have the lowest values, clustering around 10⁻¹⁵ except for the curious, nucleus-encoded Form II Rubisco from the dinoflagellate, *Amphidinium carterae*, which has a value approximately twice as high (Whitney and Andrews, 1998). Hexadecameric Form I Rubiscos have values ranging from a low of 35–40 in some bacteria and cyanobacteria up to a high in excess of 200 in some thermophilic rhodophytes. Higher plants cluster between 60 and 100.

As discussed in Section IV.G. 1, it is likely that gas addition occurs without the aid of a Michaelis complex. Furthermore, once formed, the carboxyketone intermediate appears fully committed to product formation. The peroxyketone intermediate is likely to be similarly committed, as it is in analogous organic reactions (Frankvoort, 1978). With the further assumption that both CO₂ and O₂ react with the same form of the enediol, it can be shown that the expression for $S_{c/o}$ simplifies to the ratio of the second-order rate constants for addition of CO₂ and O₂ (Pierce et al., 1986; Lorimer et al., 1993). By this reasoning, the CO₂/O₂ specificity of a particular Rubisco is governed simply by the difference in reactivity of CO₂ and O₂ for the enediol. This is related to the difference in free energy between the transition states involved in the addition of the two gases (i.e. $\Delta G^t_{o-c} = RT \ln S_{c/o}$,

where R is the gas constant, T is the absolute temperature and ΔG^t_{o-c} is the difference in free energy between the two transition states (Chen and Spreitzer, 1991; Lorimer et al., 1993)). It thus becomes apparent that the differences in $S_{c/o}$ between different Rubiscos are the result of very small differences in the relative stabilization of the transition states. The 7-fold difference in $S_{c/o}$ between the higher-plant and *R. rubrum* enzymes, so crucial to the physiology of the organisms, amounts to no more than 1.2 kcal mol⁻¹ difference in relative free energy, approximately that contributed by a single hydrogen bond (Lorimer et al., 1993). An important caveat must be borne in mind when considering this analysis. It could become inappropriate if CO₂ and O₂ react with different species, such as the singlet and triplet states of the enediol discussed in Section IV.K.3. In this case, the interconversion between the two states would also influence the partitioning between carboxylation and oxygenation.

The CO₂/O₂ specificity is 10- to 25-fold greater when Mg²⁺ is the activating metal, compared to Mn²⁺ (Jordan and Ogren, 1983). The reduction caused by Mn²⁺ substitution is much greater than that predicted from the modest decrease in forward partitioning of the carboxyketone intermediate seen with Mn²⁺ (Pierce et al., 1986) and must reflect the role of the metal in stabilizing the transition states leading to carboxylation and oxygenation.

Apart from the obvious role of the K334 side chain in promoting carboxylation (Section IV.K.4), numerous other mutations in *R. rubrum*, *Synechococcus* and *Chlamydomonas reinhardtii* Rubiscos lead to reductions in $S_{c/o}$. (Spreitzer, 1993) and (Wildner et al., 1996) have tabulated many of these. There are also a few examples of mutations leading to slight increases in this parameter, sometimes but not always at the cost of reductions in V_c (Harpel and Hartman, 1992; Parry et al., 1992; Gutteridge et al., 1993; Kostov et al., 1997; Madgwick et al., 1998). Some of these changes can be rationalized in terms of indirect effects that the mutations may have on the crucial positioning of the ε amino N atom of K334. Effects of substitutions in loop 6 are usually so explained. For many others the connection seems quite indirect. Apparently, the slightest perturbation can be transmitted through the complex web of interactions in the active site to alter this crucial selectivity.

Table 1. $S_{c/o}$ values at 25 °C for Rubiscos from various sources

Source	Form ^a	$S_{c/o}$ (Mg^{2+})	$S_{c/o}$ (Mn^{2+})	Reference
C ₃ plants				
<i>Spinacia oleracea</i>	I (green)	80	3.1	Jordan and Ogren (1983)
"	"	82		Kane et al. (1994)
"	"	94		Uemura et al. (1997)
<i>Nicotiana tabacum</i>	I (green)	77	3.2	Jordan and Ogren (1983)
"	"	82		Kane et al. (1994)
<i>Glycine max</i>	I (green)	82	3.9	Jordan and Ogren (1983)
<i>Lolium perenne</i>	I (green)	80		Jordan and Ogren (1981)
<i>Triticum aestivum</i>	I (green)	90		Kane et al. (1994)
<i>Hordeum vulgare</i>	I (green)	87		Kane et al. (1994)
<i>Oryza punctata</i>	I (green)	85		Kane et al. (1994)
C ₄ plants				
<i>Amaranthus hybridus</i>	I (green)	82	3.5	Jordan and Ogren (1983)
<i>Zea mays</i>	I (green)	78	3.3	Jordan and Ogren (1983)
"	"	79		Kane et al. (1994)
<i>Setaria italica</i>	I (green)	58		Jordan and Ogren (1983)
Green algae				
<i>Chlamydomonas reinhardtii</i>	I (green)	61	2.4	Jordan and Ogren (1983)
<i>Scenedesmus obliquus</i>	I (green)	63		Jordan and Ogren (1981)
<i>Coccomyxa</i> sp.	I (green)	83		Palmqvist et al. (1995)
<i>Euglena gracilis</i>	I (green)	54	2.8	Jordan and Ogren (1983)
Non-green eukaryotic algae				
<i>Cylindrotheca</i> N1	I (red)	105		cited in Tabita (1995)
<i>Cylindrotheca fusiformis</i>	I (red)	110		cited in Tabita (1995)
<i>Olisthodiscus luteus</i>	I (red)	100		cited in Tabita (1995)
<i>Porphyridium cruentum</i>	I (red)	130		cited in Tabita (1995)
<i>Porphyridium purpureum</i>	I (red)	144		Uemura et al. (1997)
<i>Cyanidium caldarium</i>	I (red)	225		Uemura et al. (1997)
<i>Galdieria partita</i>	I (red)	238		Uemura et al. (1997)
<i>Amphidinium carterae</i>	II	37 (10°C)		Whitney and Andrews (1998)
Cyanobacteria				
<i>Coccochloris peniocystis</i>	I (green)	47		Jordan and Ogren (1981)
<i>Aphanizomenon flos-aquae</i>	I (green)	48	2.0	Jordan and Ogren (1983)
<i>Synechococcus</i> PCC6301	I (green)	43		Kane et al. (1994)
<i>Anabaena</i> PCC7120	(green)	35		cited in Tabita (1995)
Bacteria				
<i>Chromatium vinosum</i>	I (green)	40		cited in Tabita (1995)
"	"	44		Uemura et al. (1997)
<i>Rhodobacter sphaeroides</i>	I (red)	62		Jordan and Ogren (1981)
<i>Alcaligenes eutrophus</i>	I (red)	75		cited in Tabita (1995)
<i>Bradyrhizobium japonicum</i>	I (red)	75		cited in Tabita (1995)
<i>Xanthobacter flavus</i>	I (red)	40		cited in Tabita (1995)
<i>Rhodobacter sphaeroides</i>	II	9		Jordan and Ogren (1981)
<i>Rhodospirillum rubrum</i>	II	15	1.5	Jordan and Ogren (1983)
"	"	12		Kane et al. (1994)
<i>Thiobacillus denitrificans</i>	II	10		cited in Tabita (1995)

^a Form I Rubiscos are further classified according to whether they fall in the 'green-like' or 'red-like' phylogenies (see Section II).

M. The Role of the Small Subunits

The great majority of Rubiscos in nature are hexadecameric proteins containing small subunits (L_8S_8 , Form I). All eukaryotic Rubiscos, except some from dinoflagellates (Morse et al., 1995; Rowan et al., 1996), fall into this class. However, the existence of bacterial and dinoflagellate Rubiscos that lack

small subunits (Form II) shows that they are dispensable, at least in some circumstances. The primary sequences of the large subunits of Form II Rubiscos are quite divergent from those of Form I enzymes, forming a distinct group deeply divided from the rest of the large-subunit phylogeny (Watson and Tabita, 1997).

1. In Promoting Catalysis

Early studies exploited the ability to reversibly dissociate the small subunits from the L_8 core of some cyanobacterial and bacterial Rubiscos (Andrews and Abel, 1981; Andrews and Bailment, 1983; Incharoensakdi et al., 1985; Jordan and Chollet, 1985). These revealed that all but a trace of activity was lost on removal of small subunits and that partially depleted preparations had activities commensurate with their small-subunit content. Expression of the *rbcL* gene from *Synechococcus* PCC6301 in *E. coli* without the companion *rbcS* gene confirmed that the L_8 core retains approximately 1% of the holoenzyme's activity and that it can be fully activated in vitro if supplied with isolated small subunits (Andrews, 1988; Paul et al., 1991). Heterologous small subunits, even those from spinach Rubisco, partially restore activity (Andrews et al., 1984; Andrews and Lorimer, 1985). The L_8 core is competent in carbamylation and binds divalent metal ions and 2'-carboxyarabinitol-P₂ (Andrews and Bailment, 1984; Jordan and Chollet, 1985; Andrews et al., 1986; Andrews, 1988). Its competence in enolizing ribulose-P₂ and in processing 3-keto-2'-carboxyarabinitol-P₂ matches its limited competence in overall carboxylation, indicating that the impairment is shared between the various steps of the catalytic sequence (Andrews et al., 1986). The influence of the small subunit on the active site's ability to suppress the various side reactions (Fig. 7) is more specific, however. In the absence of small subunits, some 10% of the enediol intermediate eliminates phosphate, rather than being carboxylated, but neither misprotonation of the enediol nor the pyruvate-producing side reaction are exacerbated (Morell et al., 1997).

2. In Determining CO₂/O₂ Specificity

As discussed in Section IV.L, Rubiscos with small subunits discriminate against the oxygenation reaction better than those without. While this might imply a role for the small subunits in determining CO₂/O₂ specificity, initial evidence was to the contrary. The L_8 core of *Synechococcus* Rubisco retains the same S_{co} as its holoenzyme (Gutteridge, 1991), as does its hybrids with spinach (Andrews and Lorimer, 1985) and *Alcaligenes eutrophus* (Lee et al., 1991) small subunits. In contrast, hybridization of *Synechococcus* L₈ with small subunits derived from the diatom *Cylindrotheca* sp. N1, whose Rubisco has

a very high S_{co} (Table 1), results in a hybrid Rubisco with an S_{co} value intermediate between those of the two parent holoenzymes (Read and Tabita, 1992). There is also a report of small-subunit mutations of the *Synechococcus* holoenzyme that alter S_{co} (Kostov et al., 1997). Therefore, the small subunit obviously can have some influence on S_{co} in some instances.

3. In Structure

Despite the integrity of the L_8 core of some cyanobacterial and bacterial Rubiscos in the absence of small subunits, the positions of the small subunits in the structure of the hexadecamer strongly suggest that they have an adhesive role in holding the four dimers of large subunits together. Each small subunit in the S₄ unit which caps each pole of the L_8 core interacts with two of its neighbors and, lying in a crevice between two L_2 units, also interacts with three different large subunits (Fig. 6A) (Knight et al., 1990). However, it is clear that the small subunit must have more than a simple adhesive role. Not only does it induce a 100-fold stimulation of catalysis and alter catalytic parameters such S_{co} , but it also makes the equilibrium of the carbamylation reactions more favorable (Andrews and Bailment, 1984). There is also evidence of synergism between binding of small subunits and binding of 2'-carboxyarabinitol-P₂ (Andrews, 1988). Binding of small subunits must cause structural changes that are communicated to the active site. One possibility, inferred from a comparison of the structures of the spinach and *R. rubrum* enzymes (Schneider et al., 1990), is that contact between a small subunit and helix 8 of the barrel domain of the large subunit induces a change in loop 8 which contributes to the P1 binding site. The stimulation of β elimination of the enediol observed in the absence of small subunits (Morell et al., 1997) accords with this proposal. So too do observations that mutations and truncations of the conserved N-terminal region of the small subunit that contacts loop 8 impair catalysis, loosen inter-subunit binding, and alter S_{co} (Paul et al., 1991; Paul et al., 1993; Kostov et al., 1997). However, the small subunit has several other conserved regions that contact different parts of the large subunit and mutations in these regions also impair catalysis (Voordouw et al., 1987; Lee et al., 1991; Smrcka et al., 1991; Read and Tabita, 1992) and exacerbate xylulose-P₂ production (Flachmann et al., 1997). All such contacts have the potential to transmit structural

information to the active site. Determination of the structure of the L₈ core without small subunits would provide useful further information about structural changes induced by the small subunits.

While structural changes induced in the active site by remote binding of small subunits provide a functional explanation for the role of the small subunit, this does not satisfactorily explain the existence of the small subunits. Form II Rubiscos function adequately without small subunits but have not achieved CO₂/O₂ specificities comparable to those of Form I enzymes. Reasons for the requirement of a separate polypeptide to impart the additional structural information necessary to attain greater specificity remain elusive.

V. Conclusion

The mechanisms of assembly of and catalysis by Rubisco are both characterized by daunting complexity. Despite the wealth of knowledge that exists about both aspects, no eukaryotic Rubisco has yet been assembled successfully in vitro or in any foreign host and the efforts to engineer improvements in catalytic efficiency have had very limited success. While more is now known about Rubisco than any other plant protein, this knowledge provides only incomplete understanding of critical aspects of both assembly and catalysis. Nevertheless, current Rubisco research represents an outstanding synthesis of modern chemical, molecular and structural biology. The objective of understanding Rubisco's assembly and catalysis in sufficient depth to manipulate them presents a tantalizing challenge.

References

- Abell LM and Schloss JV (1991) Oxygenase side reactions of acetolactate synthase and other carbanion-forming enzymes. *Biochemistry* 30: 7883–7887
- Akoyunoglu G and Calvin M (1963) Mechanism of the carboxydismutase reaction. II. Carboxylation of the enzyme. *Biochem Z* 338: 20–30
- Andersson I (1996) Large structures at high resolution: The 1.6 Å crystal structure of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase complexed with 2-carboxyarabinitol bisphosphate. *J Mol Biol* 259: 160–174
- Andrés J, Safont VS and Tapia O (1992) Straining the double bond in 1,2-dihydroxyethylene. A simple theoretical model for the enediol moiety in Rubisco's substrate and analogs. *Chem Phys Lett* 198: 515–520
- Andrés J, Safont VS, Queralt J and Tapia O (1993) A theoretical study of the singlet-triplet energy gap dependence upon rotation and pyramidalization for 1,2-dihydroxyethylene. A simple model to study the enediol moiety in Rubisco's substrate. *J Phys Chem* 97: 7888–7893
- Andrews TJ (1988) Catalysis by cyanobacterial ribulose-bisphosphate carboxylase large subunits in the complete absence of small subunits. *J Biol Chem* 263: 12213–12220
- Andrews TJ and Abel KM (1981) Kinetics and subunit interactions of ribulose bisphosphate carboxylase-oxygenase from the cyanobacterium, *Synechococcus* sp. *J Biol Chem* 256: 8445–8451
- Andrews TJ and Ballment B (1983) The function of the small subunits of ribulose bisphosphate carboxylase-oxygenase. *J Biol Chem* 258: 7514–7518
- Andrews TJ and Ballment B (1984) Active site carbamate formation and reaction-intermediate-analog binding by ribulose bisphosphate carboxylase-oxygenase in the absence of its small subunits. *Proc Natl Acad Sci USA* 81: 3660–3664
- Andrews TJ and Kane HJ (1991) Pyruvate is a by-product of catalysis by ribulosebisphosphate carboxylase/oxygenase. *J Biol Chem* 266: 9447–9452
- Andrews TJ and Lorimer GH (1985) Catalytic properties of a hybrid between cyanobacterial large subunits and higher plant small subunits ofribulosebisphosphate carboxylase-oxygenase. *J Biol Chem* 260: 4632–4636
- Andrews TJ and Lorimer GH (1987) Rubisco: Structure, mechanisms, and prospects for improvement. In: Hatch MD, Boardman NK (eds) *The Biochemistry of Plants: A Comprehensive Treatise*, Vol 10, Photosynthesis, pp 131–218. Academic Press, New York
- Andrews TJ, Lorimer GH and Tolbert NE (1973) Ribulose diphosphate oxygenase. I. Synthesis of phosphoglycolate by fraction-1 protein of leaves. *Biochemistry* 12:11–18
- Andrews TJ, Badger MR and Lorimer GH (1975) Factors affecting interconversion between kinetic forms of ribulose diphosphate carboxylase-oxygenase from spinach. *Arch Biochem Biophys* 171: 93–103
- Andrews TJ, Greenwood DM and Yellowlees D (1984) Catalytically active hybrids formed in vitro between large and small subunits of different procaryotic ribulose bisphosphate carboxylases. *Arch Biochem Biophys* 234: 313–317
- Andrews TJ, Lorimer GH and Pierce J (1986) Three partial reactions of ribulose-bisphosphate carboxylase require both large and small subunits. *J Biol Chem* 261: 12184–12188
- Andrews TJ, Hudson GS, Mate CJ, von Caemmerer S, Evans JR and Arvidsson YBC (1995) Rubisco: The consequences of altering its expression and activation in transgenic plants. *J Exp Bot* 46:1293–1300
- Badger MR and Andrews TJ (1974) Effects of CO₂, O₂ and temperature on a high-affinity form of ribulose diphosphate carboxylase-oxygenase from spinach. *Biochem Biophys Res Commun* 60: 204–210
- Badger MR and Lorimer GH (1976) Activation of ribulose-1,5-bisphosphate oxygenase. The role of Mg²⁺, CO₂ and pH. *Arch Biochem Biophys* 175: 723–729
- Bahr JT and Jensen RG (1974) Ribulose diphosphate carboxylase from freshly ruptured spinach chloroplasts having an in vivo K_m[CO₂]. *Plant Physiol* 53: 39–44
- Baneyx F, Bertsch U, Kalbach CE, Vandervies SM, Soll J and Gatenby AA (1995) Spinach chloroplast Cpn21 co-chaperonin

- possesses two functional domains fused together in a toroidal structure and exhibits nucleotide-dependent binding to plastid chaperonin 60. *J Biol Chem* 270: 10695–10702
- Barracough R and Ellis RJ (1980) Assembly of newly synthesized large subunits into ribulose bisphosphate carboxylase in isolated pea chloroplasts. *Biochim Biophys Acta* 608: 19–31
- Benning MM, Kuo JM, Raushel FM and Holden HM (1995) Three-dimensional structure of the binuclear metal center of phosphotriesterase. *Biochemistry* 34: 7973–7978
- Bertsch U, Soll J, Seetharam R and Viitanen PV (1992) Identification, characterization, and DNA sequence of a functional ‘double’ Cpn 10-like chaperonin from chloroplasts of higher plants. *Proc Natl Acad Sci USA* 89: 8696–8700
- Blair G and Ellis R (1974) Protein synthesis in chloroplasts. I. Light driven synthesis of the large subunit of fraction I protein by isolated pea chloroplasts. *Biochim Biophys Acta* 319: 223–234
- Bloom M, Milos P and Roy H (1983) Light dependent assembly of ribulose 1,5-bisphosphate carboxylase. *Proc Natl Acad Sci USA* 80: 1013–1017
- Bochkareva ES, Lissin NM and Girshovich AS (1988) Transient association of newly synthesized unfolded proteins with the heat-shock groEL protein. *Nature* 336: 254–257
- Boston RS, Viitanen PV and Vierling E (1996) Molecular chaperones and protein folding in plants. *Plant Mol Biol* 32: 191–222
- Bowes G, Ogren WL and Hageman RH. (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. *Biochem Biophys Res Commun.* 45:716–722
- Bradley D, van der Vies S and Gatenby AA (1986) Expression of cyanobacterial and higher-plant ribulose 1,5-bisphosphate carboxylase genes in *Escherichia coli*. *Phil Trans R Soc Lond B* 313:447–458
- Braig K, Simon M, Furuya F, Hainfeld J and Horwich A (1993) A polypeptide bound by the chaperonin GroEL is localized within a central cavity. *Proc Natl Acad Sci USA* 90: 3978–3982
- Brändén R, Nilsson T and Styring S (1984) An intermediate formed by the Cu²⁺-activated ribulose-1,5-bisphosphate carboxylase/oxygenase in the presence of ribulose 1,5-bisphosphate and O₂. *Biochemistry* 23: 4378–382
- Buchberger A, Schroder H, Hesterkamp T, Schonfeld HJ and Bukau B (1996) Substrate shuttling between the DnaK and GroEL systems indicates a chaperone network promoting protein folding. *J Mol Biol* 261: 328–333
- Buchner J (1994) Symmetric complexes of groE chaperonins as part of the functional cycle. *Science* 265: 656–659
- Burston SG, Weissman JS, Farr GW, Fenton WA and Horwich AL (1996) Release of both native and non-native proteins from a *cis*-only GroEL ternary complex. *Nature* 383: 96–99
- Calvin M and Massini P (1952) The path of carbon in photosynthesis. XX. The steady state. *Experientia* 8: 445–457
- Cannon S, Wang P and Roy H (1986) Inhibition of ribulose bisphosphate carboxylase assembly by antibody to a binding protein. *J Cell Biol* 103: 1327–1335
- Checa S and Viale A (1997) The 70-kDa heat-shock protein/DnaK chaperone system is required for the productive folding of ribulose-bisphosphate carboxylase subunits in *Escherichia coli*. *Eur. J. Biochem* 248: 848–855
- Chen GG and Jagendorf AT (1994) Chloroplast molecular chaperone-assisted refolding and reconstitution of an active multisubunit coupling factor CF1 core. *Proc Natl Acad Sci USA* 91: 11497–11501
- Chen Y-R and Hartman FC (1995) A signature of the oxygenase intermediate in catalysis by ribulose-bisphosphate carboxylase/oxygenase as provided by a site-directed mutant. *J Biol Chem* 270: 11741–11744
- Chen Z and Spreitzer RJ (1991) Proteolysis and transition-state-analogue binding of mutant forms of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Chlamydomonas reinhardtii*. *Planta* 183: 597–603
- Chua N-H and Schmidt G (1978) Post-translational import into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc Natl Acad Sci USA* 75:6110–6114
- Cleland WW (1990) Kinetic competence of enzymic intermediates: Fact or fiction. *Biochemistry* 29: 3194–3197
- Cleland WW, Andrews TJ, Gutteridge S, Hartman FC and Lorimer GH (1998) Mechanism of Rubisco—the carbamate as general base. *Chem Rev* 98: 549–561
- Cloney LP, Bekkaoui DR, Wood MG and Hemmingsen SM (1992a) Assessment of plant chaperonin-60 gene function in *Escherichia coli*. *J Biol Chem* 267: 23333–23336
- Cloney LP, Wu HB and Hemmingsen SM (1992b) Expression of plant chaperonin-60 genes in *Escherichia coli*. *J Biol Chem* 267:23327–23332
- Cloney LP, Bekkaoui DR and Hemmingsen S M (1993) Co-expression of plastid chaperonin genes and a synthetic plant rubisco operon in *Escherichia coli*. *Plant Mol Biol* 23:1285–1290
- Curmi PMG, Cascio D, Sweet RM, Eisenberg D and Schreuder H (1992) Crystal structure of the unactivated form of ribulose-1,5-bisphosphate carboxylase/oxygenase from tobacco refined at 2.0 Å resolution. *J Biol Chem* 267: 16980–16989
- Delwiche CF and Palmer JD (1996) Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. *Mol Biol Evol* 13: 873–882
- Edmondson DL, Badger MR and Andrews TJ (1990a) A kinetic characterization of slow inactivation of ribulosebisphosphate carboxylase during catalysis. *Plant Physiol* 93: 1376–1382
- Edmondson DL, Badger MR and Andrews TJ (1990b) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is not due to decarbamylation of the catalytic site. *Plant Physiol* 93: 1383–1389
- Edmondson DL, Badger MR and Andrews TJ (1990c) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is caused by accumulation of a slow, tight-binding inhibitor at the catalytic site. *Plant Physiol* 93: 1390–1397
- Edmondson DL, Kane HJ and Andrews TJ (1990d) Substrate isomerization inhibits ribulosebisphosphate carboxylase-oxygenase during catalysis. *FEBS Lett* 260: 62–66
- Ellis RJ (1979) The most abundant protein in the world. *Trends Biochem Sci* 4: 241–244
- Ellis RJ and Hartl FU (1996) Protein folding in the cell: competing models of chaperonin function. *FASEB J* 10: 20–26
- Ellis RJ and van der Vies SK (1991) Molecular chaperones. *Annu Rev Biochem* 60: 321–347
- Flachmann R, Zhu GH, Jensen RG and Bohnert HJ (1997) Mutations in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase increase the formation of the misfire product xylulose-1,5-bisphosphate. *Plant Physiol* 114: 131–136

- Frankvoort W (1978) The reaction between diacetyl 1 and hydrogen peroxide: Its mechanism and kinetic constants. *Thermochim Acta* 25: 35–49
- Freeman BC and Morimoto RI (1996) The human cytosolic molecular chaperones Hsp90, Hsp70 (Hsc70) and Hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J* 15: 2969–2979
- Frydman J, Nimmagere K, Ohtsuka K and Hartl FU (1994) Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* 370: 111–117
- Furbank RT and Taylor WC (1995) Regulation of photosynthesis in C₃ and C₄ plants: A molecular approach. *Plant Cell* 7: 797–807
- Gatenby AA (1992) Protein folding and chaperonins. *Plant Mol Biol* 19: 677–687
- Gooding L, Roy H and Jagendorf A (1973) Immunological identification of nascent subunits of wheat ribulose bisphosphate carboxylase on ribosomes of both chloroplast and cytoplasmic origin. *Arch Biochem Biophys* 159: 324–335
- Goloubinoff P, Gatenby AA and Lorimer GH (1989a) GroE heat shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in *Escherichia coli*. *Nature* 337: 44–17
- Gutteridge S (1991) The relative catalytic specificities of the large subunit core of *Synechococcus* ribulose bisphosphate carboxylase/oxygenase. *J Biol Chem* 266: 7359–7362
- Gutteridge S and Gatenby AA (1995) Rubisco synthesis, assembly, mechanism, and regulation. *Plant Cell* 7:809–819
- Gutteridge S, Parry MAJ, Schmidt CNG and Feeney J (1984) An investigation of ribulosebisphosphate carboxylase activity by high-resolution ¹H-NMR. *FEBS Lett* 170: 355–359
- Gutteridge S, Rhoades DF and Herrmann C (1993) Site-specific mutations in a loop region of the C-terminal domain of the large subunit of ribulose bisphosphate carboxylase/oxygenase that influence substrate partitioning. *J Biol Chem* 268: 7818–7824
- Haining RL and McFadden BA (1994) Active-site histidines in recombinant cyanobacterial ribulose-1,5-bisphosphate carboxylase/oxygenase examined by site-directed mutagenesis. *Photosynth Res* 41: 349–356
- Harpel MR and Hartman FC (1992) Enhanced CO₂/O₂ specificity of a site-directed mutant of ribulose-bisphosphate carboxylase/oxygenase. *J Biol Chem* 267: 6475–6478
- Harrel MR and Hartman FC (1996) Facilitation of the terminal proton transfer reaction of ribulose 1,5-bisphosphate carboxylase oxygenase by active-site Lys166. *Biochemistry* 35: 13865–13870
- Harrel MR, Larimer FW and Hartman FC (1991) Functional analysis of the putative catalytic bases His-321 and Ser-368 of *Rhodospirillum rubrum* ribulose bisphosphate carboxylase/oxygenase by site-directed mutagenesis. *J Biol Chem* 266: 24734–24740
- Harrel MR, Serpersu EH, Lamerdin JA, Huang ZH, Gage DA and Hartman FC (1995) Oxygenation mechanism of ribulose-bisphosphate carboxylase/oxygenase. Structure and origin of 2-carboxytetritol 1,4-bisphosphate, a novel O₂-dependent side product generated by a site-directed mutant. *Biochemistry* 34: 11296–11306
- Harrel MR, Larimer FW and Hartman FC (1998) Multiple catalytic roles of His 287 of *Rhodospirillum rubrum* ribulose 1,5-bisphosphate carboxylase/oxygenase. *Protein Sci* 7: 730–738
- Hartl FU, Hlodan R and Langer T (1994) Molecular chaperones in protein folding—the art of avoiding sticky situations. *Trends Biochem Sci* 19:20–25
- Hartman FC and Harpel MR (1994) Structure, function, regulation, and assembly of D-ribulose-1,5-bisphosphate carboxylase/oxygenase. *Ann Rev Biochem* 63: 197–234
- Hartman FC and Lee EH (1989) Examination of the function of active site lysine 329 of ribulose-bisphosphate carboxylase/oxygenase as revealed by the proton exchange reaction. *J Biol Chem* 264: 11784–11789
- Hartman FC, Milanez S and Lee EH (1985) Ionization constants of two active-site lysyl ε-amino groups of ribulosebisphosphate carboxylase/oxygenase. *J Biol Chem* 260: 13968–13975
- Hartman FC, Soper TS, Niyogi SK, Mural PJ, Foote RS, Mitra S, Lee EH, Machanof R and Larimer FW (1987) Function of Lys-166 of *Rhodospirillum rubrum* ribulosebisphosphate carboxylase/oxygenase as examined by site-directed mutagenesis. *J Biol Chem* 262: 3496–3501
- Hemmingsen SM and Ellis RJ (1986) Purification and properties of ribulosebisphosphate carboxylase large subunit binding protein. *Plant Physiol* 80: 269–276
- Hixon M, Sinerius G, Schneider A, Walter C, Fessner WD and Schloss JV (1996) Quo vadis photorespiration: A tale of two aldolases. *FEBS Lett* 392: 281–284
- Horwitz AL, Weissman JS and Fenton WA (1995) Kinesis of polypeptide during GroEL-mediated folding. *Cold Spring Harbor Symposia On Quantitative Biology* 60: 435–440
- Houtz, RL Poneleit, L Jones, SB Royer, M and Stults, JT (1992) Posttranslational modifications in the amino-terminal region of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from several plant species. *Plant Physiol* 98:1170–1174
- Hubbs AE (1993) The in vitro synthesis and assembly of Rubisco large subunits: characterization of an unidentified large subunit containing species, and early chaperonin interactions with large subunits. PhD Thesis, Rensselaer Polytechnic Institute, Troy, NY
- Hubbs AE and Roy H (1992) Synthesis and assembly of ribulose bisphosphate carboxylase/oxygenase in chloroplast extracts. *Plant Physiol* 100: 272–281
- Hubbs AE and Roy H (1993a) Assembly of in vitro-synthesized large subunits into ribulose bisphosphate carboxylase/oxygenase is sensitive to Cl[−], requires ATP, and does not proceed when large subunits are synthesized at temperatures ≥32 °C. *Plant Physiol* 101: 523–533
- Hubbs AE and Roy H (1993b) Assembly of in vitro synthesized large subunits into ribulose bisphosphate carboxylase/oxygenase: formation and discharge of an L₈-like species. *J Biol Chem* 268: 13519–13525
- Hudson GS, Mahon JD, Anderson PA, Gibbs MJ, Badger MR, Andrews TJ and Whitfeld PR (1990) Comparisons of *rbcL* genes for the large subunit of ribulose-bisphosphate carboxylase from closely related C₃ and C₄ plant species. *J Biol Chem* 265: 808–814
- Incharoensakdi A, Takabe T and Akazawa T (1985) Structure and functions of chloroplast proteins. 64. Factors affecting the dissociation and reconstitution of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Aphanthece halophytica*. *Arch Biochem Biophys* 237: 445–453

- Jabri E, Carr MB, Hausinger RP and Karplus PA (1995) The crystal structure of urease from *Klebsiella aerogenes*. *Science* 268: 998–1004
- Jordan DB and Chollet R (1983) Inhibition of ribulose bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. *J Biol Chem* 258: 13752–13758
- Jordan DB and Chollet R (1985) Subunit dissociation and reconstitution of ribulose-1,5-bisphosphate carboxylase from *Chromatium vinosum*. *Arch Biochem Biophys* 236: 487–96
- Jordan DB and Ogren WL (1981) Species variation in the specificity of ribulose bisphosphate carboxylase/oxygenase. *Nature* 291: 513–515
- Jordan DB and Ogren WL (1983) Species variation in kinetic properties of ribulose 1,5-bisphosphate carboxylase oxygenase. *Arch Biochem Biophys* 227: 425–433
- Kane HJ, Vilj J, Entsch B, Paul K, Morell MK and Andrews TJ (1994) An improved method for measuring the CO₂/O₂ specificity of ribulosebisphosphate carboxylase-oxygenase. *Aust J Plant Physiol* 21: 449–161
- Kane HJ, Wilkin J-M, Portis AR, Jr. and Andrews TJ (1998) Potent inhibition of ribulose-bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate. *Plant Physiol* 117: 1059–1069
- Kanevski I and Maliga P (1994) Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proc Natl Acad Sci USA* 91: 1969–1973
- Kanevski I, Maliga P, Rhoades DF, Gutteridge S (1999) Plastome engineering of ribulose-1,5-bisphosphate carboxylase/oxygenase in tobacco to form a sunflower large subunit and tobacco small subunit hybrid. *Plant Physiol* 119: 133–141
- Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries in the chloroplast. *Proc Natl Acad Sci USA* 93:7684–7689
- King WA, Gready JE and Andrews TJ (1998) Quantum chemical analysis of the enolization of ribulose-bisphosphate: The first hurdle in the fixation of CO₂ by Rubisco. *Biochemistry* 37: 15414–15422
- Klein RR. and Houtz RL (1995) Cloning and developmental expression of pea ribulose-1,5-bisphosphate carboxylase oxygenase large subunit N-methyltransferase. *Plant Molecular Biology* 27: 249–261
- Knight S, Andersson I and Brändén C-I (1990) Crystallographic analysis of ribulose 1,5-bisphosphate carboxylase from spinach at 2.4 Å resolution. Subunit interactions and active site. *J Mol Biol* 215: 113–160
- Kostov RV, Small CL and McFadden BA (1997) Mutations in a sequence near the N-terminus of the small subunit alter the CO₂/O₂ specificity factor for ribulose bisphosphate carboxylase/oxygenase. *Photosynth Res* 54: 127–134
- Laing WA and Christeller JT (1976) A model for the kinetics of activation and catalysis of ribulose 1,5-bisphosphate carboxylase. *Biochem J* 159: 563–570
- Laing WA, Ogren WL and Hageman RH (1974) Regulation of soybean net photosynthetic CO₂ fixation by the interaction of CO₂, O₂, and ribulose 1,5-bisphosphate carboxylase. *Plant Physiol* 54: 678–685
- Langer T, Lu C, Echols H, Flanagan J, Hayer MK and Hartl FU (1992) Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* 356: 683–689
- Larimer FW, Harpel MR and Hartman FC (1994) β -Elimination of phosphate from reaction intermediates by site-directed mutants of ribulose-bisphosphate carboxylase/oxygenase. *J Biol Chem* 269: 11114–11120
- Larson EM, Larimer FW and Hartman FC (1995) Mechanistic insights provided by deletion of a flexible loop at the active site of ribulose- 1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 34: 4531–4537
- Lee B and Tabita FR (1990) Purification of recombinant ribulose- 1,5- bisphosphate carboxylase/oxygenase large subunits suitable for reconstitution and assembly of L₈S₈ enzyme. *Biochemistry* 29: 9352–9357
- Lee B, Berka RM and Tabita FR (1991a) Mutations in the small subunit of cyanobacterial ribulose- bisphosphate carboxylase/oxygenase that modulate interactions with large subunits. *J Biol Chem* 266: 7417–7422
- Lee B, Read BA and Tabita FR (1991b) Catalytic properties of recombinant octameric, hexadecameric, and heterologous cyanobacterial/bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase. *Arch Biochem Biophys* 291: 263–269
- Lee EH, Harpel MR, Chen Y-R and Hartman FC (1993) Perturbation of reaction-intermediate partitioning by a site-directed mutant of ribulose-bisphosphate carboxylase/oxygenase. *J Biol Chem* 268: 26583–26591
- Lee GJ and McFadden BA (1992) Serine-376 contributes to the binding of substrate by ribulose-bisphosphate carboxylase/oxygenase from *Anacystis nidulans*. *Biochemistry* 31: 2304–2308
- Lee GJ, McDonald KA and McFadden BA (1993) Leucine 332 influences the CO₂/O₂ specificity factor of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Anacystis nidulans*. *Protein Sci* 2: 1147–1154
- Lilley RM, Riesen H and Andrews TJ (1993) The source and characteristics of chemiluminescence associated with the oxygenase reaction catalyzed by Mn²⁺-ribulosebisphosphate carboxylase. *J Biol Chem* 268: 13877–13884
- Lorimer GH (1979) Evidence for the existence of discrete activator and substrate sites for CO₂ on ribulose-1,5-bisphosphate carboxylase. *J Biol Chem* 254: 5599–5601
- Lorimer GH (1981) Ribulosebisphosphate carboxylase—amino-acid sequence of a peptide bearing the activator carbon dioxide. *Biochemistry* 20: 1236–1240
- Lorimer GH (1996) A quantitative assessment of the role of the chaperonin proteins in protein folding in vivo. *FASEBJ* 10: 5–9
- Lorimer G and Hartman FC (1988) Evidence supporting lysine-166 of *Rhodospirillum rubrum* ribulosebisphosphate carboxylase as the essential base which initiates catalysis. *J Biol Chem* 263: 6468–6471
- Lorimer GH, Andrews TJ and Tolbert NE (1973) Ribulose diphosphate oxygenase. II. Further proof of reaction products and mechanism of action. *Biochemistry* 12: 18–23
- Lorimer GH, Badger MR and Andrews TJ (1976) The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism and physiological implications. *Biochemistry* 15: 529–536
- Lorimer GH, Andrews TJ, Pierce J and Schloss JV (1986) 2'-carboxy-3-keto-D-arabinitol-1,5-bisphosphate, the six-carbon intermediate of the ribulose bisphosphate carboxylase reaction. *Phil Trans R Soc Lond B* 313: 397–407

- Lorimer GH, Gutteridge S and Madden M (1987) Partial reactions of ribulose bisphosphate carboxylase: Their utility in the study of mutant enzymes. In: von Wettstein D, Chua N-H (eds) Plant Molecular Biology, pp 21–31. Plenum Press, New York
- Lorimer GH, Chen Y-R and Hartman FC (1993) A role for the ϵ -amino group of lysine-334 of ribulose-1,5-bisphosphate carboxylase in the addition of carbon dioxide to the 2,3-enediol(ate) of ribulose 1,5-bisphosphate. Biochemistry 32: 9018–9024
- Lubben T, Donaldson G, Viitanen P, Gatenby A. (1989) Several proteins imported into chloroplasts form stable complexes with the groEL-related chloroplast molecular chaperone. Plant Cell 1:1223–1230
- Lundqvist T and Schneider G (1989) Crystal structure of the complex of ribulose-1,5-bisphosphate carboxylase and a transition state analogue, 2-carboxy-D- arabinitol 1,5-bisphosphate. J Biol Chem 264: 7078–7083
- Lundqvist T and Schneider G (1991a) Crystal structure of activated ribulose-1,5-bisphosphate carboxylase complexed with its substrate, ribulose-1,5-bisphosphate. J Biol Chem 266: 12604–12611
- Lundqvist T and Schneider G (1991b) Crystal structure of the ternary complex of ribulose-1,5-bisphosphate carboxylase, Mg(II), and activator CO₂ at 2.3-Å resolution. Biochemistry 30: 904–908
- Madgwick PJ, Parmar S and Parry MAJ (1998) Effect of mutations of residue 340 in the large subunit polypeptide of Rubisco from *Anacystis nidulans*. Eur J Biochem 253: 476–479
- Makino Y, Taguchi H and Yoshida M (1993) Truncated GroEL monomer has the ability to promote folding of rhodanese without GroES and ATP. FEBS Lett 336: 363–367
- Martin J and Hartl F-U (1997) The effect of macromolecular crowding on chaperonin-mediated protein folding. Proc. Natl. Acad. Sci. U.S.A. 94: 1107–1112
- McIntosh L, Poulsen C, Bogorad L (1980) Chloroplast gene sequence for the large subunit of ribulosebisphosphate carboxylase of maize. Nature 288:556–560
- Mendoza JA and Horowitz PM (1994) Bound substrate polypeptides can generally stabilize the tetradecameric structure of Cpn60 and induce its reassembly from monomers. J Biol Chem 269: 25963–25965
- Milos P and Roy H (1984) ATP-released large subunits participate in the assembly of ribulose bisphosphate carboxylase. J Cell Biochem 24: 153–162
- Miziorko HM and Mildvan AS (1974) Electron paramagnetic resonance, ¹H, and ¹³C nuclear magnetic resonance studies of the interaction of manganese and bicarbonate with ribulose 1,5-diphosphate carboxylase. J Biol Chem 249: 2743–2750
- Mogel SN and McFadden BA (1990) Chemiluminescence of the Mn²⁺-activated ribulose-1,5-bisphosphate oxygenase reaction: Evidence for singlet oxygen production. Biochemistry 29: 8333–8337
- Morell MK, Paul K, O' Shea NJ, Kane HJ and Andrews TJ (1994) Mutations of an active site threonyl residue promote β elimination and other side reactions of the enediol intermediate of the ribulosebisphosphate carboxylase reaction. J Biol Chem 269: 8091–8098
- Morell MK, Wilkin J-M, Kane HJ and Andrews TJ (1997) Side reactions catalyzed by ribulose-bisphosphate carboxylase in the presence and absence of small subunits. J Biol Chem 272: 5445–5451
- Morse D, Salois P, Markovic P and Hastings JW (1995) A nuclear-encoded form II RuBisCO in dinoflagellates. Science 268: 1622–1624
- Mullet JE, Klein RR and Grossman AR (1986) Optimization of protein synthesis in isolated higher plant chloroplasts. Identification of paused translation intermediates. Eur J Biochem 155: 331–338
- Netzer WJ and Hartl FU (1998) Protein folding in the cytosol - chaperonin-dependent and -independent mechanisms. Trends Biochem Sci 23:68–73
- Newman J and Gutteridge S (1993) The X-ray structure of *Synechococcus* ribulose-bisphosphate carboxylase/oxygenase activated quaternary complex at 2.2-Å resolution. J Biol Chem 268: 25876–25886
- Newman J and Gutteridge S (1994) Structure of an effector-induced inactivated state of ribulose 1,5-bisphosphate carboxylase/oxygenase: The binary complex between enzyme and xylulose 1,5-bisphosphate. Structure 2: 495–502
- Ogren WL (1984) Photorespiration: pathways, regulation, and modification. Annu Rev Plant Physiol 35: 415–442
- Palmqvist K, Süttermeyer D, Baldet P, Andrews TJ and Badger MR (1995) Characterisation of inorganic carbon fluxes, carbonic anhydrase(s) and ribulose-1,5-bisphosphate carboxylase-oxygenase in the green unicellular alga *Coccomyxa*. Comparisons with low-CO₂ cells of *Chlamydomonas reinhardtii*. Planta 197: 352–361
- Parry MAJ, Madgwick P, Parmar S, Cornelius MJ and Keys AJ (1992) Mutations in loop six of the large subunit of ribulose-1,5-bisphosphate carboxylase affect substrate specificity. Planta 187: 109–112
- Paul K, Morell MK and Andrews TJ (1991) Mutations in the small subunit of ribulosebisphosphate carboxylase affect subunit binding and catalysis. Biochemistry 30: 10019–10026
- Paul K, Morell MK and Andrews TJ (1993) Amino-terminal truncations of the ribulose-bisphosphate carboxylase small subunit influence catalysis and subunit interactions. Plant Physiol 102: 1129–1137
- Pierce J, Tolbert NE and Barker R (1980) Interaction of ribulosebisphosphate carboxylase/oxygenase with transition-state analogues. Biochemistry 19: 934–942
- Pierce J, Andrews TJ and Lorimer GH (1986a) Reaction intermediate partitioning by ribulose-bisphosphate carboxylases with differing substrate specificities. J Biol Chem 261: 10248–10256
- Pierce J, Lorimer GH and Reddy GS (1986b) Kinetic mechanism ofribulosebisphosphate carboxylase: Evidence for an ordered, sequential reaction. Biochemistry 25: 1636–1644
- Pon NG, Rabin BR and Calvin M (1963) Mechanism of the carboxydismutase reaction. I. The effect of preliminary incubation of substrates, metal ion and enzyme on activity. Biochem Z 338: 7–19
- Portis AR, Jr. (1992) Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. Annu Rev Plant Physiol Plant Mol Biol 43: 415–437
- Portis AR, Jr (1995) The regulation of Rubisco by Rubisco activase. J Exp Bot 46 (290):1285–1291
- Quayle JR, Fuller RC, Benson AA and Calvin M (1954) Enzymatic carboxylation of ribulose diphosphate. J Amer Chem So. 76:3610–3611
- Read BA and Tabita FR (1992a) A hybrid ribulosebisphosphate carboxylase/oxygenase enzyme exhibiting a substantial increase

- in substrate specificity factor. *Biochemistry* 31: 5553–5560
- Read BA and Tabita FR (1992b) Amino acid substitutions in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase that influence catalytic activity of the holoenzyme. *Biochemistry* 31: 519–525
- Richard JP (1984) Acid-base catalysis of the elimination and isomerization reactions of triose phosphates. *J Amer Chem Soc* 106: 4926–4936
- Rose IA (1981) Chemistry of proton abstraction by glycolytic enzymes (aldolase, isomerases and pyruvate kinase). *Phil Trans R Soc Lond B* 293: 131–143
- Rowan R, Whitney SM, Fowler A and Yellowlees D (1996) Rubisco in marine symbiotic dinoflagellates: Form II enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. *Plant Cell* 8: 539–553
- Roy H (1989) Rubisco assembly: A model system for studying the mechanism of chaperonin action. *Plant Cell* 1: 1035–1042
- Roy H (1992) Chaperonins—what do they really do?. *Plant Physiol (Lif Sci Adv)* 11: 75–78
- Roy H and Gilson M (1996) Rubisco and the chaperonins. In: Passararakli M (ed) *Handbook of Photosynthesis*, pp 295–304. Marcel Dekker, New York
- Roy H, Patterson R and Jagendorf A (1976) Identification of the small subunit of ribulose-1,5-bisphosphate carboxylase as a product of wheat leaf cytoplasmic ribosomes. *Arch Biochem Biophys* 172: 64–73
- Roy H, Adari H and Costa KA (1979) Characterization of free subunits of ribulose-1,5-bisphosphate carboxylase. *Plant Science Letters* 16: 305–318
- Roy H, Bloom M, Milos P and Monroe M (1982) Studies on the assembly of large subunits of ribulose bisphosphate carboxylase in isolated pea chloroplasts. *J Cell Biol* 94: 20–27
- Roy H, Hubbs A and Cannon S (1988) Stability and dissociation of the large subunit RuBisCO binding protein in vitro and in organello. *Plant Physiol* 86: 50–53
- Rutner A and Lane M (1967) Nonidentical subunits of ribulose diphosphate carboxylase. *Biochem Biophys Res Commun* 28: 531–537
- Rye HS, Burston SG, Fenton WA, Beechem JM, Xu ZH, et al. (1997) Distinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL. *Nature* 388: 792–798
- Salvucci ME and Ogren WL (1996) The mechanism of Rubisco activase: Insights from studies of the properties and structure of the enzyme. *Photosynth Res* 47: 1–11
- Schloss JV and Lorimer GH (1982) The stereochemical course of ribulosebisphosphate carboxylase—reductive trapping of the six carbon reaction-intermediate. *J Biol Chem* 257: 4691–4694
- Schmidt M, Rutkat K, Rachel R, Pfeifer G, Jaenicke R, Viitanen P, Lorimer G and Buchner J (1994) Symmetric complexes of groE chaperonins as part of the functional cycle. *Science* 265: 656–659
- Schneider G, Knight S, Andersson I, Brändén C-I, Lindqvist Y and Lundqvist T (1990) Comparison of the crystal structures of L₂ and L₈S₈ Rubisco suggests a functional role for the small subunit. *EMBO J* 9: 2045–2050
- Schreuder HA, Knight S, Curmi PMG, Andersson I, Cascio D, Brändén C-I and Eisenberg D (1993a) Formation of the active site of ribulose-1,5-bisphosphate carboxylase/oxygenase by a disorder-order transition from the unactivated to the activated form. *Proc Natl Acad Sci USA* 90: 9968–9972
- Schreuder HA, Knight S, Curmi PMG, Andersson I, Cascio D, Sweet RM, Brändén C-I and Eisenberg D (1993b) Crystal structure of activated tobacco rubisco complexed with the reaction-intermediate analogue 2-carboxy-arabinitol 1,5-bisphosphate. *Protein Sci* 2: 1136–1146
- Sfatos CD, Gutin AM, Abkevich VI and Shakhnovich EI (1996) Simulations of chaperone-assisted folding. *Biochemistry* 35: 334–339
- Shikanai T, Foyer CH, Dulieu H, Parry MAJ and Yokota A (1996) A point mutation in the gene encoding the Rubisco large subunit interferes with holoenzyme assembly. *Plant Mol Biol* 31: 399–103
- Shivji MS, Li N and Cattolico RA (1992) Structure and organization of rhodophyte and chromophyte plastid genomes: implications for the ancestry of plastids. *Mol Gen Genet* 232: 65–73
- Smith MA, Criddle S, Peterson L and Huffaker RC (1974) Synthesis and assembly of ribulose bisphosphate carboxylase enzyme during greening of barley plants. *Arch Biochem Biophys* 165: 494–504
- Smith MD, Ghosh S, Dumbroff EB and Thompson JE (1997) Characterization of thylakoid-derived lipid-protein particles bearing the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol* 115: 1073–1082
- Spreitzer RJ (1993) Genetic dissection of Rubisco structure and function. *Annu Rev Plant Physiol Plant Mol Biol* 44: 411–434
- Sue JM and Knowles JR (1982) Ribulose-1,5-bisphosphate carboxylase - fate of the tritium label in ³H-3-labeled ribulose 1,5-bisphosphate during the enzyme-catalyzed reaction. *Biochemistry* 21: 5404–5410
- Tabita FR (1995) The biochemistry and metabolic regulation of carbon metabolism and CO₂ fixation in purple bacteria. In: Blankenship RE, Madigan MT and Bauer CE (eds) *Anoxygenic Photosynthetic Bacteria*, pp 885–914. Kluwer Academic Publishers, Dordrecht
- Tapia O and Andrés J (1992) Towards and explanation of the carboxylation/oxygenation bifunctionality in Rubisco. Transition structure for the carboxylation reaction of 2,3,4-pentanetriol. *Mol Eng* 2: 37–41
- Taylor TC and Andersson I (1996) Structural transitions during activation and ligand binding in hexadecameric Rubisco inferred from the crystal structure of the activated unliganded spinach enzyme. *Nature Struct Biol* 3: 95–101
- Taylor TC and Andersson I (1997a) The structure of the complex between rubisco and its natural substrate ribulose 1,5-bisphosphate. *J Mol Biol* 265: 432–144
- Taylor TC and Andersson I (1997b) Structure of a product complex of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 36: 4041–4046
- Taylor TC, Fothergill MD and Andersson I (1996) A common structural basis for the inhibition of ribulose 1,5-bisphosphate carboxylase by 4-carboxyarabinitol 1,5-bisphosphate and xylulose 1,5-bisphosphate. *J Biol Chem* 271: 32894–32899
- Todd MJ, Viitanen P and Lorimer GH (1994) Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding. *Science* 265: 659–666
- Todd MJ, Lorimer GH and Thirumalai D (1996) Chaperonin-facilitated protein folding—Optimization of rate and yield by an iterative annealing mechanism. *Proc Natl Acad Sci USA* 93: 4030–4035
- Török Z, Vigh L and Goloubinoff P (1996) Fluorescence detection

- of symmetric GroEL(14)(GroES(7))(2) heterooligomers involved in protein release during the chaperonin cycle. *J Biol Chem* 271: 16180–16186
- Trown PW (1965) An improved method for the isolation of caboxylidismutase. Probable identity with fraction 1 protein and the protein moiety of protochlorophyll holochrome. *Biochemistry* 4:908–918
- Tse JMT and Schloss JV (1993) The oxygenase reaction of acetolactate synthase. *Biochemistry* 32: 10398–10403
- Uemura K, Anwaruzzaman, Miyachi S and Yokota A (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from thermophilic red algae with a strong specificity for CO₂ fixation. *Biochem Biophys Res Commun* 233: 568–571
- Van Dyk DE and Schloss JV (1986) Deuterium isotope effects in the carboxylase reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 25: 5145–5156
- Viitanen PV, Schmidt M, Buchner J, Suzuki T, Vierling E, Dickson R, Lorimer GH, Gatenby A and Soll J (1995) Functional characterization of the higher plant chloroplast chaperonins. *J Biol Chem* 270: 18158–18164
- Voordouw G, De Vries PA, van den Berg WAM and De Clerck EPJ (1987) Site-directed mutagenesis of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Anacystis nidulans*. *Eur J Biochem* 163: 591–598
- Watson GMF and Tabita FR (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* 146: 13–22
- Webber AN, Nie GY and Long SP (1994) Acclimation of photosynthetic proteins to rising atmospheric CO₂. *Photosynth Res* 39: 413–425
- Weidner M and Fehling E (1985) Heat modification of ribulose-1,5-bisphosphate carboxylase/oxygenase by temperature pretreatment of wheat (*Triticum aestivum* L) seedlings. *Planta* 166: 117–127
- Weissbach A, Smyrniotis PZ and Horecker BL (1954) Pentose phosphate and CO₂ fixation with spinach extracts. *J Amer Chem Soc* 76:3611–3612
- Whitney SM and Andrews TJ (1998) The CO₂/O₂ specificity of single-subunit ribulose-bisphosphate carboxylase from the dinoflagellate, *Amphidinium carterae*. *Aust J Plant Physiol* 25: 131–138
- Wildman S and Bonner J (1947) The proteins of green leaves. I. Isolation, enzymatic properties, and auxin content of spinach cytoplasmic proteins. *Arch Biochem* 14:381–413
- Wildner GF, Schlitter J and Müller M (1996) Rubisco, an old challenge with new perspectives. *Z Naturforsch [C]* 51: 263–276
- Wu HB, Feist GL and Hemmingsen SM (1993) A modified *Escherichia coli* chaperonin (GroEL) polypeptide synthesized in tobacco and targeted to the chloroplasts. *Plant Mol Biol* 22: 1087–1100
- Xu ZH, Horwitz AL and Sigler PB (1997) The crystal structure of the asymmetric GroEL-GroES-(ADP)₇ chaperonin complex. *Nature* 388: 741–750
- Yokota A (1991) Carboxylation and detoxification of xylulose bisphosphate by spinach ribulose bisphosphate carboxylase/oxygenase. *Plant Cell Physiol* 32: 755–762
- Zabaleta E, Oropesa A, Assad N, Mandel A, Salerno G and Herrera-Estrella H (1994) Antisense expression of chaperonin 60 β in transgenic tobacco plants leads to abnormal phenotypes and altered distribution of photoassimilates. *Plant J* 6: 425–432
- Zahn R, Buckle AM, Perrett S, Johnson CM, Corrales FJ, Golbik R and Fersht AR (1996) Chaperone activity and structure of monomeric polypeptide binding domains of GroEL. *Proc Natl Acad Sci USA* 93: 15024–15029
- Zhang KYJ, Cascio D and Eisenberg D (1994) Crystal structure of the unactivated ribulose 1,5-bisphosphate carboxylase/oxygenase complexed with a transition state analog, 2-carboxy-D-arabinitol 1,5-bisphosphate. *Protein Sci* 3: 64–69
- Zhu G and Jensen RG (1991a) Xylulose 1,5-bisphosphate synthesized by ribulose 1,5-bisphosphate carboxylase/oxygenase during catalysis binds to decarbamylated enzyme. *Plant Physiol* 97: 1348–1353
- Zhu G and Jensen RG (1991b) Fallover of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. Decarbamylation of catalytic sites depends on pH. *Plant Physiol* 97: 1354–1358
- Zhu GH, Bohnert HJ, Jensen RG and Wildner GF (1998) Formation of the tight-binding inhibitor, 3-ketoarabinitol-1,5-bisphosphate by ribulose-1,5-bisphosphate carboxylase/oxygenase is O₂-dependent. *Photosynth Res* 55: 67–74

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Chapter 4

Rubisco: Physiology in Vivo

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Summary

A broad overview of the physiology and biochemistry of Rubisco is presented with a comparison of information obtained *in vitro* and *in vivo*. First a brief background to kinetic properties of Rubisco is given and Rubisco's influence on photosynthetic metabolism is reviewed for C₃, C₄, CAM and C₃-C₄ species. The effect of environmental variables such as light and CO₂ are considered for both short and longer term effects on the activity and abundance of Rubisco protein. Over the past few years experiments with transgenic plants with antisense RNA constructs to Rubisco, Rubisco activase and several of the PCR cycle enzymes have added new insights into Rubisco physiology. For example, transgenic tobacco with reduced amount of Rubisco has allowed the identification of environmental conditions where Rubisco exerts maximal control on photosynthesis. These plants have also been used to determine Rubisco kinetic constants *in vivo*. Transgenic plants with reduced amounts of Rubisco activase have been used to elucidate the role of activase *in vivo*.

I. Introduction

In C₃ plants, Rubisco is at the gateway of photosynthesis facilitating the primary carboxylation of atmospheric CO₂. Its role in the photosynthetic carbon reduction (PCR) cycle and its catalytic mechanism have been discussed in previous chapters. The aim of this chapter is to provide a broad overview of the physiology of Rubisco *in vivo* as evident in the CO₂ fixation characteristics of leaves. There are already many excellent reviews discussing this topic such as those by Andrews and Lorimer (1987), Woodrow and Berry (1988), Sharkey (1989), Salvucci (1989) and Portis (1990). Over the past few years experiments with transgenic plants with antisense RNA constructs to Rubisco, Rubisco activase and several of the PCR cycle enzymes have added new insights (Stitt and Schulze, 1994).

In higher plants, the holoenzyme Rubisco is a hexadecamer consisting of eight large and small subunits with eight catalytic sites. To function, Rubisco's catalytic sites must be carbamylated to allow the binding of a catalytically essential Mg²⁺ (for review see Andrews and Lorimer, 1987) and nocturnal inhibitors such as 2' carboxyarabinitiol 1-phosphate (CA1P) need also be removed from catalytic sites (Vu et al., 1984; Seemann et al., 1985). In most instances, Rubisco is fully carbamylated under high light. We will therefore first explore the relationship between CO₂ assimilation rate and fully active Rubisco and then consider the *in vivo* regulation of the activation process. To aid our discussion we use the photosynthetic model of Farquhar et al. (1980) to provide the quantitative link between measurements of CO₂ assimilation rate and Rubisco carboxylation rate.

II. Rubisco and CO₂ Assimilation Rate, a Quantitative Relationship

A. The Model

Here we give a brief description of the key equations of the model by Farquhar et al. (1980). A more detailed discussion of many of the individual parameters follows in later sections.

The CO₂ assimilation rate of leaves with the C₃ photosynthetic pathway is the net result of Rubisco carboxylations and CO₂ evolved during photorespiration and other mitochondrial respiration. The realization that the oxygen sensitivity of CO₂ assimilation could be explained by Rubisco oxygenase activity highlighted the role Rubisco plays in determining the net rate of CO₂ assimilation (Bowes

Abbreviations: Γ – CO₂ compensation point; Γ_* – CO₂ compensation point in the absence of day respiration; A – rate of CO₂ assimilation; C_a , C_c , C_i – partial pressure of CO₂ in the air surrounding the leaf, in the chloroplast and in the intercellular airspace; CABP-2' carboxyarabinitol-1,5-bisphosphate; CA1P – 2' carboxyarabinitol 1-phosphate; gi – internal conductance to CO₂; FBP – fructose-1,6-bisphosphate; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; J – rate of chloroplast electron transport; k_{cat} – catalytic turnover rate; K_c , K_o , K_r – Michaelis Menten constants for CO₂, O₂ and RuBP of Rubisco; K_f – dissociation constant of the inactive Rubisco RuBP complex; PCO – photorespiratory carbon oxidation; PCR – photosynthetic carbon reduction; PEP – phosphoenolpyruvate; PEP case – phosphoenolpyruvate carboxylase; PGA – 3-phosphoglycerate; Pi – inorganic phosphate; R_d – day respiration (mitochondrial respiration other than that associated with photorespiration in the light); RuBP – ribulose-1,5-bisphosphate; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; S_{co} – CO₂/O₂ specificity of Rubisco; V_c , V_o – rate of carboxylation and oxygenation of Rubisco; V_{max} – maximal Rubisco carboxylation rate; V_{oxygen} – maximal Rubisco oxygenation rate

et al., 1971; Laing et al., 1974). We assume that in the photorespiratory carbon oxidation (PCO) cycle, oxygenation of one mole of RuBP leads to the release of 0.5 mole of CO_2 and the net rate of CO_2 assimilation, A , can thus be given by

$$A = V_c - 0.5V_o - R_d, \quad (1)$$

where V_c and V_o are the rates of Rubisco carboxylation and oxygenation, respectively, and R_d denotes mitochondrial respiration in the light other than that associated with the PCO cycle (Farquhar et al., 1980; Farquhar and von Caemmerer, 1982). Since CO_2 and O_2 are competitive alternate substrates of Rubisco (Laing et al., 1974; Chapter 3, Roy and Andrews) it follows that

$$S_{c/o} = \frac{V_c O}{V_o C_c} = \frac{V_{cmax} K_o}{V_{omax} K_c}, \quad (2)$$

where $S_{c/o}$ is the relative specificity of Rubisco for CO_2 as opposed to O_2 . C_c and O are the partial pressures of CO_2 and O_2 at the catalytic site, V_{cmax} and V_{omax} are the maximal rates of carboxylation and oxygenation, and K_c and K_o are the Michaelis-Menten constants for CO_2 and O_2 , respectively.

The CO_2 partial pressure at which the carboxylation rate equals the rate of photorespiratory CO_2 release has been denoted Γ_* (Laisk, 1977). When the partial pressure of CO_2 in the chloroplast, C_c , is at Γ_* , then $A = -R_d$ and $V_c/V_o = 0.5$. Therefore

$$\Gamma_* = \frac{0.5O}{S_{c/o}} \quad (3)$$

and at any C_c , the rate of Rubisco oxygenation can be related to the rate of carboxylation by

$$V_o = \dots \quad (4)$$

and CO_2 assimilation rate can be expressed as a function of V_c , Γ_* , and C_c :

$$A = (1 - \Gamma_*/C_c)V_c - R_d. \quad (5)$$

Farquhar et al. (1980) showed that CO_2 assimilation rate can be given as either a RuBP-saturated rate or RuBP regeneration limited rate. This is because Rubisco is present at very high concentrations in the chloroplast (up to 4 mM) and the K_m for RuBP is

quite low (20 μM , Badger and Collatz, 1977). The RuBP-saturated carboxylation rate of Rubisco is given by

$$V_c = \frac{C_c V_{cmax}}{C_c + K_c(1 + O/K_o)}, \quad (6)$$

which is the general rate equation for an enzyme with two competing substrates (Fersht, 1984). By substituting Eq. (6) into Eq. (5), CO_2 assimilation rate can be given as a function of chloroplastic CO_2 and O_2 partial pressures and Rubisco kinetic parameters

$$A = \frac{(C_c - \Gamma_*)V_{cmax}}{C_c + K_c(1 + O/K_o)} - R_d. \quad (7)$$

To regenerate one mol of RuBP, two mol of NADPH are required in both the PCR and PCO cycle, which in turn requires two mol of electrons per NADPH. The electron transport rate, J , satisfying the rate of NADPH consumption in the PCR and PCO cycle is therefore:

$$J = 4(V_c + V_o) = 4(1 + 2\Gamma_*/C_c)V_c \quad (8)$$

(Farquhar et al., 1980). Thus when the rate of RuBP regeneration is limited by electron transport Farquhar et al. (1980) derived an equation for the electron transport (or RuBP regeneration) limited rate by combining Eqs. (5) and (8).

$$A = \frac{(C_c - \Gamma_*)J}{(4C_c + 8\Gamma_*)} - R_d. \quad (9)$$

Eqs. (7) and (9) are the two equations most important to our analysis. Figure 1 shows the modeled dependence of CO_2 assimilation rate on chloroplastic CO_2 partial pressures. The Rubisco-limited (or RuBP-saturated) rate is linearly dependent on V_{cmax} , the maximal Rubisco activity. When A is limited by the rate of RuBP regeneration, Γ_* determines the partitioning of available energy between carboxylation and oxygenations.

B. Rubisco Specificity and the CO_2 Compensation Point

Rubisco's relative specificity for CO_2 as opposed to O_2 (Eq. (2)) is simply the ratio between the specificity for CO_2 (V_{cmax}/K_c) and the specificity for O_2 (V_{omax}/K_o). $S_{c/o}$ is often used as a performance index of Rubisco

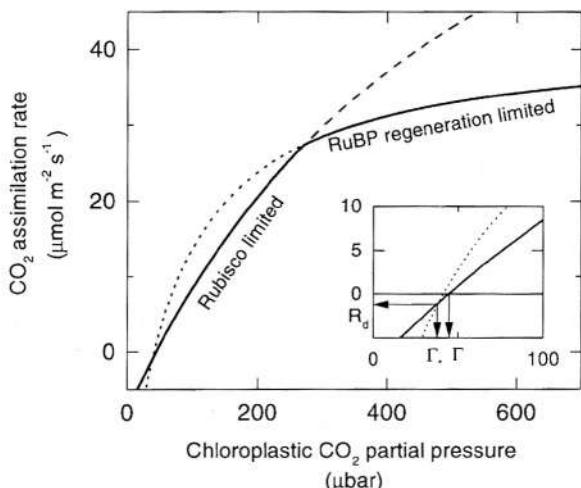


Fig. 1. Modeled rate of CO₂ assimilation as a function of chloroplastic CO₂ partial pressure. The Rubisco limited (RuBP saturated) rate of CO₂ assimilation (Eq. (7)) has a dashed line extension at high CO₂. The electron transport (RuBP regeneration) limited rate (Eq. (9)) has a dotted line extension at low CO₂. The solid curve represents the minimum rate that is the rate of CO₂ assimilation. Model parameters were: $V_{cmax} = 100 \mu\text{mol m}^{-2} \text{s}^{-1}$, $K_c = 259 \mu\text{bar}$, $K_o = 179 \mu\text{bar}$, $\Gamma_* = 38.6 \mu\text{bar}$, $J = 170 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $R_d = 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ (von Caemmerer et al., 1994). The inset depicts the relationship between the CO₂ compensation point Γ , Γ_* and R_d .

and has frequently been measured in vitro on purified Rubisco (Jordan and Ogren, 1981; Parry et al., 1987; Gutteridge, 1990; Kane et al., 1994). $S_{c/o}$ of Rubisco varies between taxa. It is lowest in photosynthetic bacteria (10–20 MM⁻¹, or 268–536 bar bar⁻¹) and intermediate in cyanobacteria (~48 MM⁻¹ or 1286 bar bar⁻¹) and green algae (~60 MM⁻¹, or 1608 bar bar⁻¹), and is greatest in higher plants (MM⁻¹ 80–100, or 2144–2680 bar bar⁻¹) (Jordan and Ogren, 1981). Careful in vitro measurements of $S_{c/o}$ at 25 °C have shown only minor variation amongst C₃ species (Kane et al., 1994). The higher plant numbers state that at equal dissolved concentrations of O₂ and CO₂ the carboxylation rate would be 80 to 100 times the rate of oxygenation (Eq. (2)). At equal partial pressures of O₂ and CO₂ in the gas phase the carboxylation rate is 2000 to 3000 times the oxygenation rate because the solubility of O₂ is much less than that of CO₂ (Table 1).

The CO₂ compensation point, Γ (the CO₂ partial pressure at which there is no net CO₂ assimilation rate) provides the link between the kinetic properties of Rubisco and the gas exchange of leaves. In the absence of any day respiration, R_d , $\Gamma = \Gamma_*$ and is thus directly proportional to the inverse of $S_{c/o}$ (Eq. (2);

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Laing et al., 1974). In the presence of respiratory activity

$$\Gamma = \frac{\Gamma_* + K_c(1 + O/K_o)R_d/V_{cmax}}{I - R_d/V_{cmax}} \quad (10)$$

(Farquhar and von Caemmerer, 1982).

The insert in Fig. 1 illustrates the difference between Γ and Γ_* . Γ has been shown to increase with leaf age and this can be attributed to increases in the ratio R_d/V_{cmax} and provides evidence for the occurrence of mitochondrial respiration not associated with photorespiration in vivo (Azcon-Bieto et al., 1981; Peisker, 1981).

Γ_* and R_d have been estimated from measurements of CO₂ response curves. At low intercellular CO₂ partial pressures and different irradiances the curves intersect at Γ_* (Laisk, 1977). Brooks and Farquhar (1985) used this techniques to determine the temperature dependence of Γ_* in spinach and found that it agreed closely with the temperature dependence of $S_{c/o}$ of spinach made in vitro (Jordan et al., 1984)¹. Laisk and Loreto (1996) made in vivo estimates of $S_{c/o}$ from the slope of the dependence of Γ on oxygen concentration, but the slope is also dependent on the ratio R_d/V_{cmax} (Eq (10)) and gives only approximate values.

Estimates of Γ_* vary between 33 and 46.6 μbar at 25 °C (Evans and Loreto this book) which converts to $S_{c/o}$ in the range of 3181–2253 bar bar⁻¹, or 120–85 MM⁻¹. In vivo estimates of $S_{c/o}$ are consistently 10–20% higher than in vitro measurements and von Caemmerer et al. (1994) discuss possible explanations. Perhaps surprisingly, Γ_* turns out to be a very important parameter in many aspects of mathematical modeling of CO₂ fixation even at the global scale and for that purpose more extensive measurements of temperature dependencies are required, particularly in the lower temperature range.

C. Kinetic Constants of Rubisco

1. In Vitro Estimates

The successful application of Eq. (7) requires accurate knowledge of the Michaelis Menten constants for

¹ Because Rubisco kinetics relate to the CO₂ partial pressure at the site of carboxylation in the chloroplast, which at Γ_* is slightly greater than the measured intercellular CO₂ partial pressure P_{i*} , $\Gamma_* = P_{i*} + R_d/g_p$, where g_p is the conductance to internal diffusion of CO₂ from the intercellular airspace to the chloroplast (von Caemmerer et al., 1994; Chapter 14, Evans and Loreto).

Table 1. In vitro kinetic constants of Rubisco from several C₃ species

Species	K _c (μM)	K _c (μbar)	K _o (μM)	K _o (mbar)	K _c (1+O/K _o) (μbar) at 200mbar O ₂	V _{omax} /V _{cmax}	S _{c/o} ^(c)	Reference
<i>Atriplex glabriuscula</i>	21	629	328	260	1113	0.18	85	Badger and Collatz, 1977
<i>Spinacea oleracea</i>	13.6	407	354	280 ^(a)	698	0.22	120	Badger and Andrews, 1974
	15.2 ^(b)	455	196	156	1038	0.22	60	
<i>Glycine max</i>	9	269	430	341 ^(a)	427	0.58	82	Jordan and Ogren, 1981
<i>Tetragonium expansa</i>	13	389	600	476 ^(a)	552	0.55	81	
<i>S. oleracea</i>	14	419	480	381 ^(a)	639	0.43	80	
<i>Lolium perenne</i>	16	479	500	397 ^(a)	720	0.38	80	
<i>Nicotiana tabacum</i>	11	329	650	516 ^(a)	456	0.77	77	
<i>S. oleracea</i>	11	329	500	397 ^(a)	495	0.52	88	Jordan and Ogren, 1984
<i>Triticum aestivum</i>	11.2	335	383	304	555	0.29	120	Makino et al., 1988
<i>Oryza sativa</i>	8.0	239	335	266	418	0.33	128	
<i>Nicotiana tabacum</i>	11.5	344	222	176	713	0.24	81	Whitney et al., 1998

All values were measured at 25 °C. To convert K_c and K_o values from concentration to partial pressures the solubilities for CO₂ of 0.0334 mol (1 bar)⁻¹ and for O₂ of 0.00126 mol (1 bar)⁻¹ were used. K_c values by Badger and Collatz (1977) and Badger and Andrews (1974) where measured in 100 mM Hepes pH 8.3. The values had been calculated with a pKa = 6.37 and where recalculated here with a pKa = 6.12. Measurements by Jordan and Ogren (1981, 1984) were made with 50 mM Bicine buffer pH 8.3 and a pKa = 6.23 used. Measurements by Makino et al. (1988) were made in 100 mM Bicine pH 8.15 and a pKa = 6.12 was used.

^(a) measured as K_c(O₂); ^(b) measured as K_c(CO₂); ^(c) S_{c/o} values by Jordan and Ogren (1981, 1984) and Whitney et al. (1998) were derived from simultaneous measurements of carboxylation and oxygenation. Other S_{c/o} values where calculated from the individual constants given in the table.

CO₂ and O₂, K_c and K_o. In vitro measurements of K_c vary widely. Yeoh et al. (1981) measured K_c in 28 different C₃ species and reported values from 359 to 778 μbar (12-26 μM). Jordan and Ogren (1981) measured a range from 269 to 479 μbar (9-16 μM) for five C₃ species. Measurements by Seemann et al. (1981) and Seemann and Berry (1982) were 310 μbar (10.4 μM). Makino et al. (1988) measured K_c by a rapid assay with crude leaf extracts in several C₃ species and found values from 203 to 326 μbar (6.8–10.9 μM).

In Table 1, in vitro kinetic constants of Rubisco from C₃ species are collated from experiments where both carboxylase and oxygenase activity have been measured. The oxygenase activity of Rubisco has not been measured as frequently as carboxylation and fewer in vitro values of K_o exist in the literature. The measured values of K_o range from 156 mbar (196 μM) to 516 mbar (650 μM). The K_o of Rubisco is an important parameter determining the CO₂-assimilation rate. In conjunction with K_c, it defines the apparent Michaelis-Menten constant for CO₂, K_c(1 + O/K_o) of Rubisco in the presence of O₂. In vitro measurements of K_c(1 + O/K_o) are about twice

the intercellular CO₂ concentration usually observed in leaves at high light (Table 1).

The greatest uncertainty in the kinetic constants surrounds the ratio of V_{omax}/V_{cmax}. Jordan and Ogren (1981, 1984) calculated this value from their measurements of S_{c/o}, K_c and K_o and arrived at values of 0.54. Lower values of 0.18 to 0.33 have been estimated by Badger and coworkers, Makino et al. (1988) and Whitney et al. (1998) (Table 1).

2. In Vivo Estimates

The study of Rubisco by in vitro methods encounters problems since extraction and purification may cause loss of activity. Furthermore, Rubisco exists in the chloroplast stroma at high concentrations, which are difficult to mimic in vitro. It is therefore attractive to study Rubisco in its natural environment. Previously this has not been possible because other processes, such as the regeneration of RuBP, at high CO₂ partial pressures (Fig. 2) limit CO₂-assimilation rate and carboxylation. However in transgenic tobacco with reduced amounts of Rubisco the capacity for RuBP regeneration is high relative to Rubisco activity such

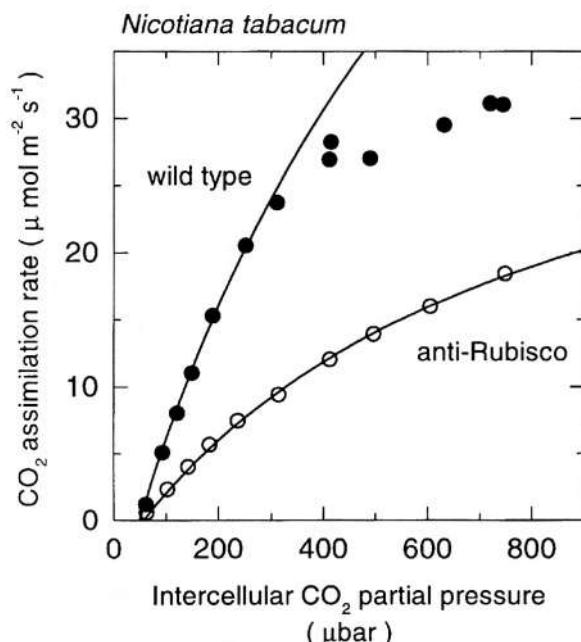


Fig. 2. CO₂ assimilation rate, A , as a function of intercellular CO₂ partial pressure for a leaf of a wild type tobacco (●) and a transgenic tobacco with reduced amount of Rubisco (○). Measurements were made at an irradiance of 1 mmol quanta $\text{m}^{-2} \text{s}^{-1}$ and a leaf temperature of 25 °C. The lines are the predicted A from Eq. (7) with the kinetic constants from Table 2 and the chloroplastic CO₂ partial pressure, C_c , was calculated from $C_c = C_i A/g_i$, where $g_i = 0.3 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$ was the measured CO₂ transfer resistance between intercellular airspace and chloroplasts (von Caemmerer et al., 1994).

that CO₂ assimilation rate is Rubisco limited at all measured CO₂ partial pressures (Fig. 2, Hudson et al., 1992). Measurements of CO₂ response curves at different O₂ concentrations in these plants mimic the expected kinetic behavior of Rubisco in vitro (Figs. 3 and 4). The in vivo estimates of K_c and K_o fall with in the range of in vitro measurements but are at the low end (Table 2). The estimate of $V_{o\max}/V_{c\max}$ from Γ_* , K_c and K_o agree best with the low in vitro estimates of Badger and coworkers (Table 1).

It is possible to quantify Rubisco catalytic site content per unit leaf area with the aid of ¹⁴C labeled carboxy arabinitol-1,5-bisphosphate (CABP). CABP is a reaction intermediate analogue that binds tightly and stoichiometrically to Rubisco catalytic sites. Furthermore since CABP binds significantly more tightly to carbamylated Rubisco sites it is possible to exchange [¹⁴C] CABP from non carbamylated sites with an excess of unlabelled CABP and quantify the number of carbamylated sites as well (Collatz et al., 1979; Butz and Sharkey, 1989). The maximal Rubisco

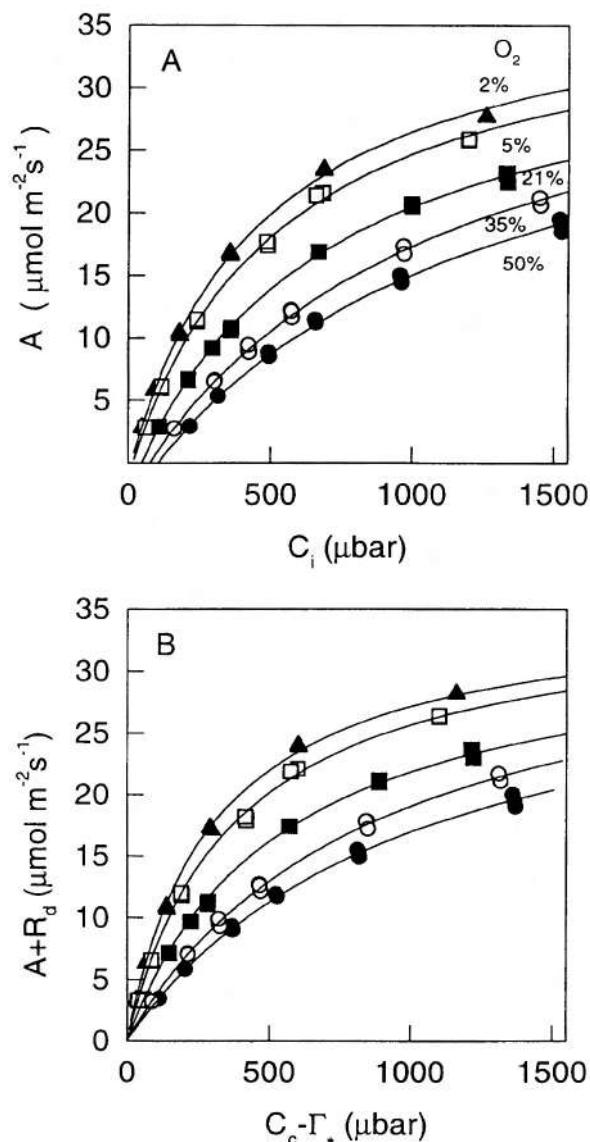


Fig. 3. A CO₂ assimilation rate, A , of a transgenic tobacco leaf with reduced amount of Rubisco as function of intercellular CO₂ partial pressure, C_i , at five different O₂ concentrations. Measurements were made at an irradiance of 1500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ and a leaf temperature of 25 °C. B the data are redrawn as gross CO₂ assimilation ($A + R_d$) versus the CO₂ partial pressure at the site of carboxylation minus Γ_* ($C_i - \Gamma_*$) to show the hyperbolic nature of the curves. The lines in A and B are the predicted A from Eq. (7) with the kinetic constants of Table 2 and C_c was calculated from $C_c = C_i - A/0.3$, where 0.3 was the measured CO₂ transfer resistance between intercellular airspace and chloroplasts (von Caemmerer et al., 1994). 21% O₂ is equivalent to 200 mbar in Canberra.

activity, $V_{c\max}$ is the product of the Rubisco site content multiplied by the catalytic turnover rate k_{cat} (mol CO₂/(mol Rubisco sites)). In vitro maximal Rubisco activity can be measured in leaf extracts from the rate

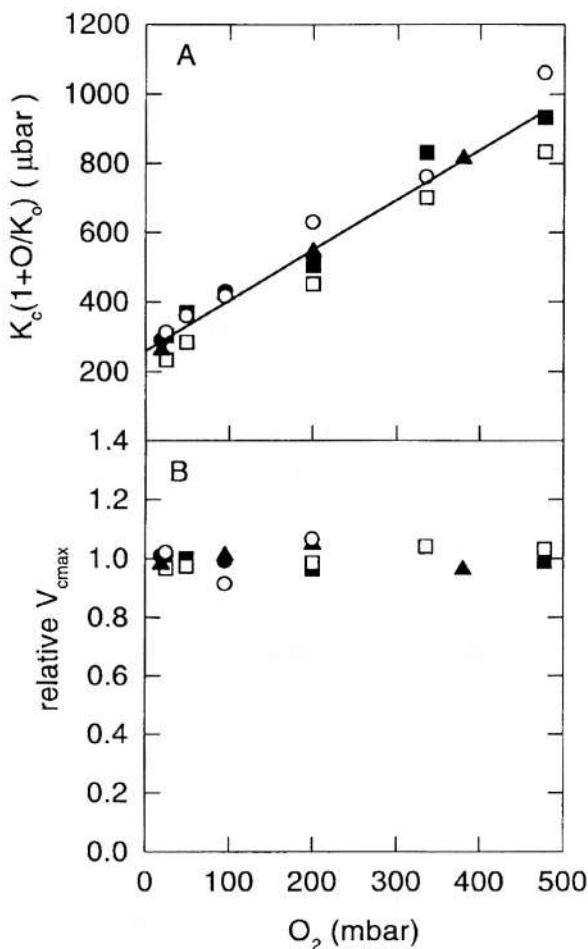


Fig. 4. (A) The apparent K_m (CO_2) as a function of the partial pressure of O_2 . Estimates were derived from Eadie-Hofstee plots of CO_2 response curves similar to those shown in Fig. 3. Different symbols denote different leaves. (B) V_{cmax} as a function of the partial pressure of O_2 . V_{cmax} values were normalized by dividing by the mean values for each leaf (ranging from 35 to 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Estimates were derived from Eadie-Hofstee plots of CO_2 response curves like those shown in Fig. 3. Different symbols denote different leaves.

of $^{14}\text{CO}_2$ incorporation into acid stable products after pre-incubation of the extract with CO_2 and magnesium to achieve full carbamylation. Combined measurements of Rubisco activity and Rubisco site content after rapid extraction of Rubisco have given in vitro k_{cat} values between 2 and 6 mol CO_2 (mol sites) $^{-1} \text{s}^{-1}$ (Table 2, Evans and Seemann, 1984; Makino et al., 1988; Whitney et al., 1999). Transgenic tobacco plants with reduced amounts of Rubisco have provided an opportunity to measure the k_{cat} of Rubisco carboxylase in vivo from combined measurements of CO_2 assimilation rates at high CO_2 partial pressure

Table 2. Rubisco kinetic constants at 25 °C calculated from gas exchange analysis of transgenic tobacco with reduced Rubisco content

K_c	$258 \pm 50 \mu\text{bar}$, (8.6 ± 1.7 μM)
K_o	171 mbar, (215 μM)
$K_c (1 + O/K_o)$ at 200 mbar O_2	560 μbar , (18.7 μM)
Γ^* (a)	38.6 μbar
$S_{c/o}$ (a,b)	97.5
V_{omax}/V_{cmax}	0.255
k_{cat} (mol CO_2 (molsites) $^{-1} \text{s}^{-1}$)	3.53 ± 0.18
k_{cat} (mol CO_2 (molsites) $^{-1} \text{s}^{-1}$) (in vitro)	2.87 ± 0.08

(a) To convert values from partial pressures to concentrations, solubilities for CO_2 of 0.0334 mol (1 bar) $^{-1}$ and for O_2 of 0.00126 mol (1 bar) $^{-1}$ were used (adapted from von Caemmerer et al., 1994).

and measurements of Rubisco site concentrations. The values by von Caemmerer et al. (1994) given in Table 2 are higher than most in vitro values but less than those measured by Whitney et al. (1998).

D. Dependence of Carboxylation Rate on RuBP Concentration

Rubisco has a high affinity for RuBP with an in vitro Michaelis-Menten constant, $K_c \sim 20 \mu\text{M}$ (Badger and Collatz, 1977; Yeoh et al., 1981). However many PCR and PCO cycle intermediates such as FBP, NADPH, 6-phosphogluconate, Pi, and PGA bind to Rubisco's catalytic sites and act as competitive inhibitors which increases the apparent K_c (Badger and Lorimer, 1981). Chloroplasts contain large pools of RuBP and PGA which can both be around 10 mM. This PGA concentrations together with a $K_{i,(\text{PGA})}$ of 0.9 mM is sufficient for a ten-fold increase in K_c (Figs. 5, 6 and 7; Badger et al., 1984; von Caemmerer and Edmonson, 1986; Seemann and Sharkey, 1986; Price et al., 1995). The Rubisco site concentrations in vivo are between 1–4 mM (Evans et al., 1994) and at these concentrations a significant amount of RuBP is bound to Rubisco sites and simple Michaelis-Menten kinetics do not apply. Farquhar (1979) showed that because of the low K_c , the kinetics of Rubisco with respect to free and Rubisco bound RuBP should be such that the carboxylation rate increases linearly with free and bound RuBP until the RuBP pool reaches Rubisco site concentration and then saturates abruptly when the RuBP concentrations exceed the

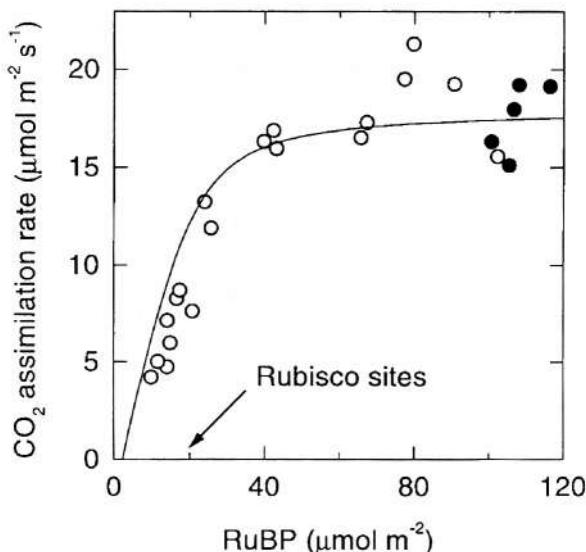


Fig. 5. CO₂ assimilation rate as a function of RuBP pools in wild type (●) and transgenic tobacco with an antisense construct to glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH, ○). Measurements were made at 1.5 mmol quanta m⁻² s⁻¹ an ambient CO₂ partial pressure of 350 μbar and a leaf temperature of 25 °C. The mean Rubisco site concentration of the data set was 20 μmol m⁻² and the mean PGA content = 102 μmol m⁻² (data are redrawn from Price et al., 1995). The solid line depicts relationships between A and free and enzyme bound RuBP pool, R_d , calculated from Eq. (5) together with the following equation, which relates the ratio of RuBP limited to RuBP saturated carboxylation rate, V_r/V_c to R_d (Farquhar, 1979)

$$\frac{V_r}{V_c} = \frac{1}{2} \left\{ \left(1 + \frac{R_d + K_r}{E_t} \right) - \sqrt{\left(1 + \frac{R_d + K_r}{E_t} \right)^2 - 4 \frac{R_d}{E_t}} \right\}.$$

The Rubisco site concentration, $E_t = 2.0 \text{ mM} = 20 \mu\text{mol m}^{-2}$, and $K_r = K_c (1 + [\text{PGA}] / K_{\text{PGA}}) = 0.02 * (1 + 10 / 0.9)$ in the presence of 10 mM PGA (solid line). When $V_r/V_c = 1$, the RuBP saturated CO₂ assimilation rate, $A + R_d$, was taken as 20 μmol m⁻² s⁻¹ and $R_d = 2 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The RuBP limited values of $A + R_d$ were obtained by multiplying V_c in equation (5) by V_r/V_c .

site concentration (Fig. 5). This dependence on total chloroplastic RuBP makes it is possible to describe CO₂ assimilation rate by either the RuBP-saturated or the RuBP-regeneration limited rate of Rubisco (Eqs. (7) and (9)). It has been difficult to test this hypothesis *in vivo*. When varying irradiance varied RuBP regeneration rates, Rubisco carbamylated also changed (Mott et al., 1984; von Caemmerer and Edmondson, 1986). The first confirmation, that the dependence of carboxylation rate on chloroplastic RuBP concentration was similar to that predicted by the model equation shown in Fig. 5 came from an elegant study by Mott et al. (1984). They examined

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the rapid adjustment in CO₂ assimilation rate and RuBP pools after a light transient at time points before a change in Rubisco carbamylated had occurred. Transgenic tobacco plants with an antisense reduction in chloroplastic glyceraldehyde-3-phosphate dehydrogenase activity (anti-GAPDH plants) have reduced rates of RuBP regeneration and RuBP pools without concomitant changes in Rubisco carbamylated. The relationship between assimilation rate and RuBP pools generated from wild type and anti-GAPDH plants is also consistent with the hypothesis of Farquhar (1979) especially when the K_r is increased to take into account the competitive inhibition by PGA (Fig. 5).

There has been some debate over how much RuBP is required to saturate Rubisco sites. Several studies have suggested that RuBP needs to be more than 1.5–2 times the Rubisco site concentration (Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986; Seemann et al., 1987; Price et al., 1995) which is consistent with the data in Fig. 5. The RuBP and PGA pools vary drastically and in opposite direction with CO₂ concentration, with little change in the total esterified phosphate pool. This is probably governed by the strict exchange of triose phosphate and phosphate by the phosphate translocator at the chloroplast envelope (Chapter 6, Flügge). In vivo the RuBP pool is very large at low CO₂ partial pressures and low at high CO₂ partial pressures, and under these conditions PGA pools are also very high (Fig. 6; Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986; Seemann et al., 1987). RuBP and PGA change much less with changing irradiance since several of the PCR cycle enzymes are light regulated (Fig. 7, Stitt, 1996). Curiously, in all species examined, RuBP pool size rarely drops below Rubisco site concentration, which may have something to do with Rubisco's requirement for RuBP to maintain full carbamylated (see below).

E. CO₂ Assimilation Rate Versus Rubisco Activity

In early gas-exchange studies, it was assumed that CO₂ assimilation rate at high light was limited by the physical constraints of CO₂ diffusion (Gaastra 1959). However, it quickly became apparent that there was also a close correlation between CO₂ assimilation rates and maximal extractable Rubisco activity (reviewed by Björkman, 1981). Fig. 8a and b show such correlations for *Phaseolus vulgaris* and

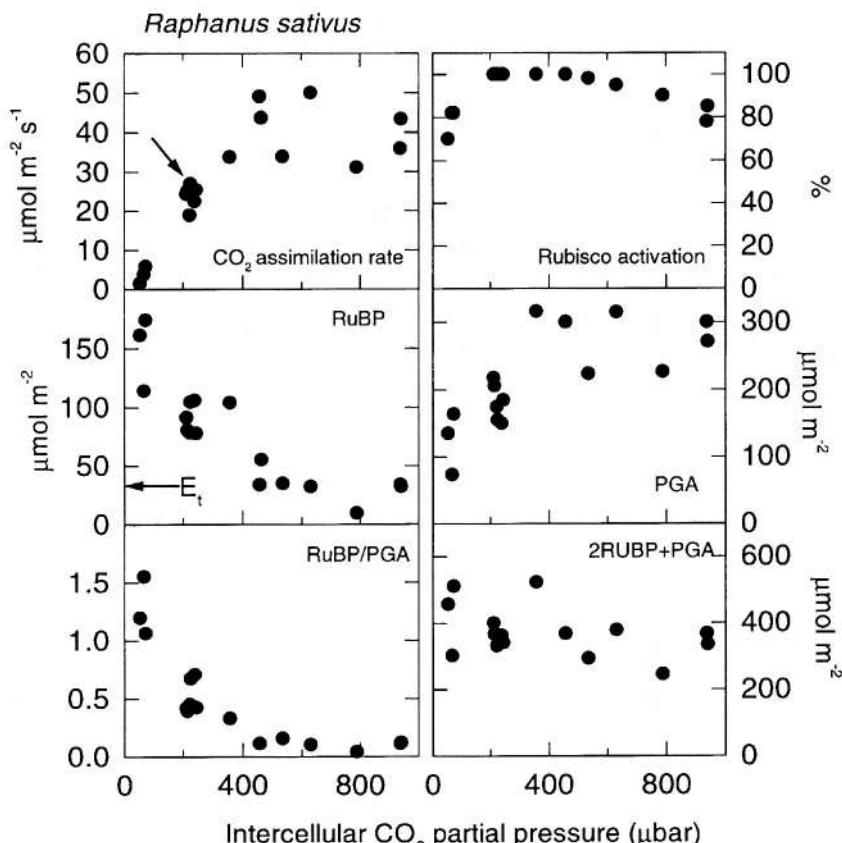


Fig. 6. CO_2 assimilation rate, Rubisco activation and RuBP and PGA contents in *Raphanus sativus* (Radish) at different intercellular CO_2 partial pressures. Measurements were made at a leaf temperature of 25°C and an irradiance of 1.4 mmol quanta $\text{m}^{-2} \text{s}^{-1}$. Each point represents a measurement made on a different leaf. Arrow points to measurements made at an ambient CO_2 partial pressure of 340 μbar . E_t denotes the estimated Rubisco site concentration (redrawn from von Caemmerer and Edmondson, 1986).

Nicotiana tabaccum, but there are many other examples in the literature (Seemann et al., 1981; Makino et al., 1985, 1994b; Evans and Terashima, 1988). Usually measurements are made at a constant ambient CO_2 partial pressure and the relationship tends to be somewhat curvilinear as the chloroplastic CO_2 partial pressure may be less in leaves with greater amounts of Rubisco (Evans et al., 1986; Evans and Terashima, 1988; Makino et al., 1994b). A linear relationship is only expected when measurements are made at a constant chloroplastic CO_2 partial pressure (Eq. (7)). However, when low light measurements of CO_2 assimilation rate are correlated with maximal Rubisco activity, the relationship saturates abruptly as the rate of RuBP regeneration and not Rubisco limits the rate of CO_2 assimilation (Fig. 1; Laurer et al., 1993). It is important to note that the maximal Rubisco activity needs to be at least four times the rate of CO_2 assimilation at ambient

CO_2 and 25 °C to account for subsaturating CO_2 concentrations (Eq. (7), Table 1). In many species it can be difficult to extract sufficient functional Rubisco to account for CO_2 assimilation rates in leaves.

The close correlation between Rubisco and CO_2 assimilation rate occurs because there is little variation in the chloroplastic CO_2 partial pressure at high irradiance (Chapter 14, Evans and Loreto). However some exceptions can be found. For example the obligate CAM plant *Kalanchoe diagremontiana*, which can operate in the C₃ mode during phase IV of the CAM cycle, has a very low internal conductance to CO_2 diffusion. Although *Kalanchoe* leaves have about the same amount of Rubisco as tobacco leaves, CO_2 assimilation rate is only half that of a tobacco leaf at ambient CO_2 partial pressure (Fig. 9, Maxwell et al., 1997). Makino et al. (1994a) noted that when rice was grown at different temperatures the relationship between CO_2 assimilation rate and

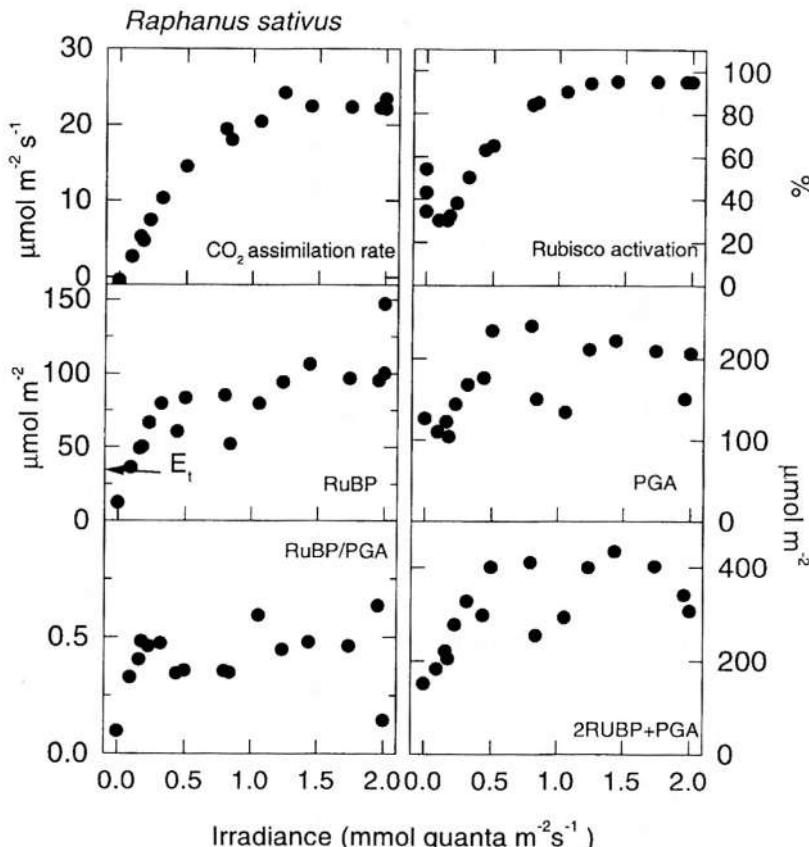


Fig. 7. CO_2 assimilation rate, Rubisco activation and RuBP and PGA contents in *Raphanus sativus* (Radish) at different irradiance. Measurements were made at a leaf temperature of 25°C and at an ambient CO_2 partial pressure of 340 μbar . Each point represents a measurement made on a different leaf. E_t denotes the estimated Rubisco site concentration (redrawn from von Caemmerer and Edmondson, 1986).

extractable Rubisco activity changed and attributed the difference to a possible increase in internal resistance to CO_2 diffusion at low temperatures.

F. Temperature Dependence

The temperature dependencies of Rubisco's carboxylation and oxygenation rates are reflected in the temperature dependency of the CO_2 assimilation rate of leaves (Björkman and Pearcy, 1971; Björkman et al., 1980). The need for accurate estimates of the temperature dependencies of Rubisco kinetic parameters has become apparent as mathematical modelers try to predict the impact of increasing global CO_2 concentrations and temperatures (Bowes, 1991; Long, 1991; Walcroft et al., 1997). The temperature dependence of the kinetic constants can be described by an Arrhenius function of the form given below (Badger and Collatz, 1977).

$$\text{Parameter} = \text{Parameter}(25^\circ\text{C}) \exp[(T - 298)E/(298RT)], \quad (11)$$

where R ($8.314 \text{ JK}^{-1} \text{ mol}^{-1}$) is the universal gas constant and T is temperature in Kelvin (K). Table 3 shows the activation energies (E) of Rubisco kinetic constants determined in *in vitro* experiments. There are only two complete data sets measured on C_3 plants, that of Badger and Collatz (1977) on Rubisco from *Atriplex* and that by Jordan and Ogren (1974) on spinach Rubisco.

The specificity factor of Rubisco decreases with increasing temperature (Eq. (2)). This is because the reaction mechanism of Rubisco involves a 2,3 enediol intermediate and its reaction with O_2 has a higher free energy of activation than the reaction with CO_2 (Andrews and Lorimer, 1987). That is, the oxygenation rate increases more rapidly with temperature than the rate of carboxylation. Fig. 10 shows the

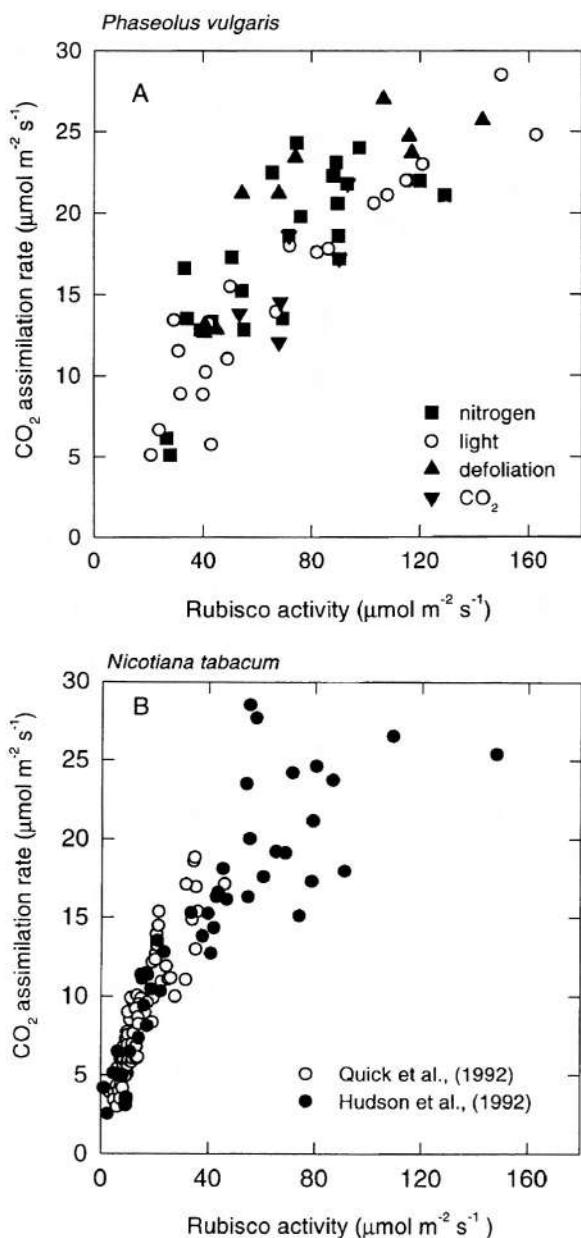


Fig. 8. (A) CO_2 assimilation rate versus in vitro measurements of maximal Rubisco activity in *Phaseolus vulgaris* grown under different nitrogen nutrition, grown at high light and after transfer to low light, during a defoliation treatment and growth at elevated CO_2 . Each point represents a measurement made on a different plant. Measurements were made at 1.5 mmol quanta $\text{m}^{-2} \text{s}^{-1}$, a leaf temperature of 28°C and an ambient CO_2 partial pressure of 330 μbar . (Data redrawn from von Caemmerer and Farquhar, 1984). (B) CO_2 assimilation rate versus in vitro measurements of maximal Rubisco activity in wildtype and transgenic tobacco with reduced amounts of Rubisco grown under different nitrogen nutrition. Measurements were made at saturating light and 25 °C. Data redrawn from Hudson et al., 1992; von Caemmerer et al., 1992 and Quick et al., 1992.

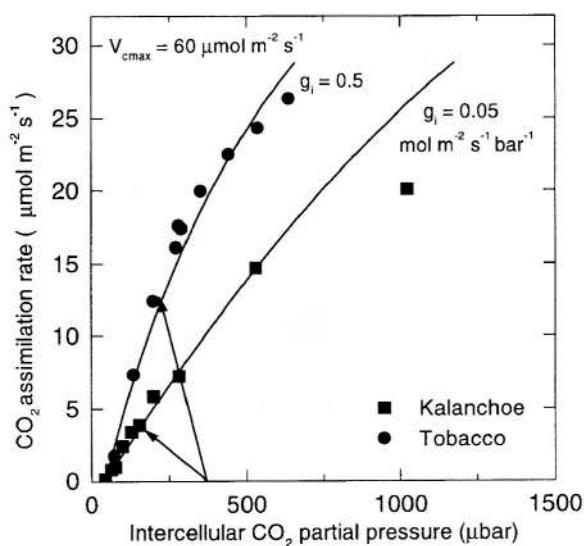


Fig. 9. CO_2 assimilation rate, A , as a function of intercellular CO_2 partial pressure, C_i , for a leaf of tobacco (*Nicotiana tabacum*) (●) and *Kalanchoe diagremontiana* (■) (during C_3 photosynthesis in Phase VI of the CAM cycle) with the same amount of Rubisco sites ($18 \mu\text{mol m}^{-2}$) but different conductances to internal CO_2 diffusion, g_i . The arrows point to assimilation rates at ambient CO_2 partial pressures and the slope of the arrows give the stomatal conductances. Measurements were made at high light and a leaf temperature of 25 °C. The lines are the predicted A from Eq. (7) with the kinetic constants from Table 2 and C_c was calculated from $C_c = C_i - A/g_i$. Data are redrawn from Mate et al. (1996) and Maxwell et al. (1997).

temperature dependence of Rubisco limited CO_2 assimilation rate (Eq. (7)) and Γ_* using the various activation energies given in Table 3. Predictions vary widely at the higher temperatures. However it is likely that CO_2 assimilation rate is limited by the RuBP regeneration capacity at high temperature, in which case the temperature dependency of Γ_* is important. Again predictions vary widely at the higher temperatures. The temperature dependency of Γ_* determined by gas exchange measurements by Brooks and Farquhar (1985) agree moderately well with the estimates of Γ_* from the measurements of Rubisco specificity by Jordan and Ogren (1984).

Comparing the activation energies in Table 3 shows that the ratio of V_{cmax}/K_c does change much with temperature. This is supported by gas exchange measurements that show the initial slope of the CO_2 response curve of CO_2 assimilation to be relatively insensitive to temperature (von Caemmerer and Farquhar, 1981; Kirschbaum and Farquhar, 1984; Sage et al., 1990a). It is perhaps interesting to note that the lower value of S_{d6} of the photosynthetic

Table 3. Activation energies E (J/mol) of Rubisco kinetic constants

Species	K_c	K_o	V_{cmax}	V_{omax}	$S_{c/o}$	Reference
Glycine max (15–35 °C)	53,381	8,279				Laing et al., 1974
Spinacia oleracea (5–35 °C)			54,393	79,497		Badger and Andrews, 1974
Atriplex glabriuscula (above 15 °C)	59,414	35,983	64,853	59,414	-18,000	Badger and Collatz, 1977
(below 15 °C)	10,962	35,983	103,765	103,347	-73,219	
Spinacia oleracea (7–35 °C)	81,655	15,632	74,350	37,566	-29,239	Jordan and Ogren, 1984
model plant	59,356	35,948	58,520	58,520	-23,408	Farquhar et al., 1980

Values of parameters can be calculated at any temperature from the following equation:

Parameter = Parameter (25 °C) $\exp[(T - 298)E/298RT]$, where R (8.314 JK⁻¹ mol⁻¹) is the universal gas constant and T is temperature in degrees Kelvin (K). $Q_{10}(25 °C) = \exp(13.6 \cdot 10^{-6} E)$

bacteria *Rhodospirillum rubrum* is accompanied by a lower activation energy (Chen and Spreitzer, 1992; Lorimer et al., 1993). There have so far been no studies that have looked for species-specific differences in Rubisco's temperature dependency amongst higher plants. Small differences of $S_{c/o}$ at 25 °C could yield more important differences at higher temperatures. The temperature dependence of Γ_* increases the CO₂ compensation point at high temperatures making it difficult for plants to maintain positive CO₂ assimilation rate under conditions where conservation of water is paramount and closure of stomata would be beneficial. The temperature dependence of Γ_* is also reflected in the strong temperature dependency of the quantum yield of C₃ plants (Ehleringer and Björkman, 1977; Farquhar et al., 1980 and Oberhuber and Edwards, 1993) where more electron transport has to be devoted to PCO cycle activity as temperatures increase.

III. In Vivo Regulation of Rubisco Carbamylation and Activity

A. Regulation of Rubisco Activation State by RuBP and Activase

To function, Rubisco's catalytic sites must be activated through the reversible carbamylation of a lysine residue within the site, followed by the rapid binding of an essential Mg²⁺ (Andrews and Lorimer, 1987). It is possible to measure both Rubisco activity and the carbamylation state of Rubisco in leaf extracts (Butz and Sharkey, 1989). Rubisco is usually fully active and carbamylated at ambient CO₂ concentrations and high light (Figs. 5 and 6). By itself, Rubisco would not be fully carbamylated in vivo. The analysis

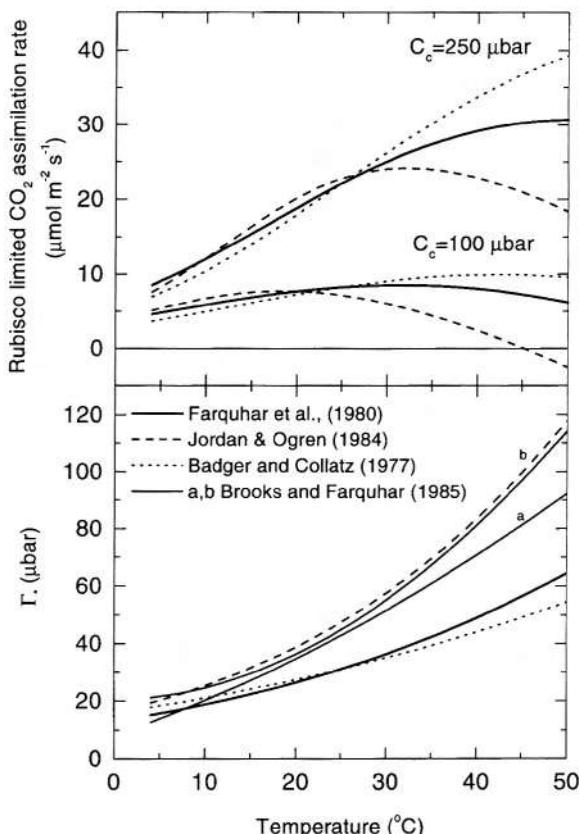


Fig. 10. Modeled rates of Rubisco limited CO₂ assimilation and Γ_* with increasing temperature. CO₂ assimilation rate (Eq. (7) with $R_d = 0$) was calculated with the activation energies given in Table 3 and $V'_{cmax} = 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ at two different chloroplastic CO₂ partial pressures. The following kinetic constants were used: Badger and Collatz (1977) and Farquhar et al. (1980) $K_c = 460 \mu\text{bar}$, $K_o = 330$ and $\Gamma_* = 31 \mu\text{bar}$, Jordan and Ogren (1984); $K_c = 419 \mu\text{bar}$, $K_o = 381 \mu\text{bar}$ and $\Gamma_* = 47 \mu\text{bar}$, Brooks and Farquhar 1985: a $\Gamma^* = 42.7 + 1.68(T-25) + 0.012(T-25)^2$, b $44.7 + 1.88(T-25) + 0.036(T-25)^2$.

of the in vitro kinetics of Rubisco has shown that the equilibrium carbamylation in the absence of RuBP would be less than 25% at the pH, Mg²⁺, and CO₂ concentrations thought to occur in the chloroplast (Lorimer et al., 1976). Besides, when RuBP was present in in vitro assays, it appeared to block carbamylation by binding tightly to non-carbamylated sites (Jordan and Chollet, 1983).

These in vitro difficulties in Rubisco carbamylation are eliminated in vivo by the presence of a second protein, Rubisco activase (Portis, 1990, Salvucci and Ogren, 1996). Activase was first identified in a high CO₂ requiring mutant of *Arabidopsis thaliana* (Sommerville et al., 1982) which had poorly activated Rubisco in the light (Salvucci et al., 1985). Studies with the purified protein have demonstrated that activase requires ATP hydrolysis to function (Streusand and Portis, 1987) and that activase specifically removes sugar phosphates from carbamylated and uncarbamylated Rubisco sites, thereby promoting carbamylation (Portis et al., 1986; Portis, 1990).

1. Activase Mode of Action

Andrews et al. (1995), Mate et al. (1996) and Salvucci and Ogren (1996) proposed models for the mechanism of activase action. They suggested a mechanistic model in which activase functions to open closed polypeptide loops within the protein structure at the catalytic site that normally enclose tight binding ligands. It is hypothesized that when activase is activated by ATP hydrolysis, it can recognize Rubisco with a closed loop protein structure and bind selectively to these catalytic sites. This causes the loops to open, releasing the ligand and returning activase to its inactive form (Fig. 11). The closed loop complexes that activase recognizes can occur with both carbamylated (ECM) and uncarbamylated (E) Rubisco and the unassisted rate at which they release their ligands is very slow (see legend of Fig. 12 for definition of terms). When the ligand is the substrate, RuBP, catalysis provides a rapid means of opening loops for the ECMR complex and which does not require the assistance of activase (Fig. 12). Therefore, activase will induce a much larger increase in the rate of opening of the unproductive ER complexes than it will in the rate of opening of the catalytically-competent, carbamylated complexes. This difference provides the opportunity for Rubisco's carbamylation status to respond to the

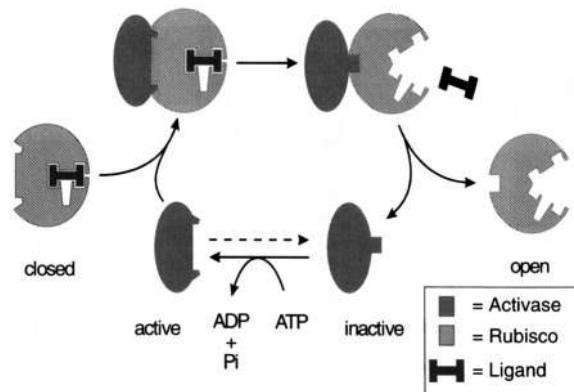


Fig. 11. A schematic model of the function of activase. It is hypothesised that when activase is activated by ATP hydrolysis, it can recognize Rubisco with a closed loop protein structure and bind selectively to these catalytic sites. This causes the loops to open, releasing the ligand and returning activase to its inactive form. The function is the same for all ligands whose binding causes Rubisco's active site loops to close and is not affected by its carbamylation status.

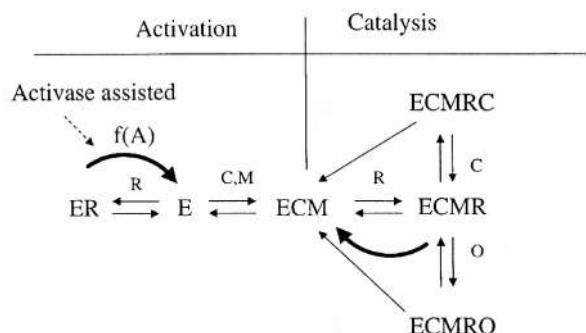


Fig. 12. The sequence of reactions assumed to be involved in the carbamylation and the irreversible catalytic mechanism of Rubisco. E, C, O, M, R stand for Rubisco enzyme site, CO₂, O₂, magnesium, and RuBP respectively. The concentrations are those of the free species. ECMR represents complexes between carbamylated Rubisco and RuBP. ECMRC and ECMRO represent all catalytic intermediates of carboxylation and oxygenation respectively. The thick arrow indicates activase-mediated dissociation of ER and ECMR complexes. f(A) denotes a complex function positively correlated with activase activity, that describes activase mediated dissociation of ER and ECMR. The carbamylation ratio is defined as $\{(ECM + ECMR + ECMRC + ECMRO)/E\}_t$, where E_t is the total concentration of Rubisco sites. $K_f = (k_1 + f(A))/k_2$ defines the ratio (ER)/ER in the steady state. Similarly K_r defines the ratio (ECMR)/(ECMRC + ECMRO) and detailed equations are given by Mate et al. (1996).

concentration of RuBP as well as the activity of activase. Mate et al. (1996) formulated this mechanistic model in simple mathematical terms

using the sequence of carbamylation and catalytic reactions originally proposed by Farquhar (1979). The model suggests that at ambient CO_2 concentration and high RuBP concentrations, carbamylation levels approximating those observed *in vivo* can only occur when the apparent dissociation constant of the ER complex (K_f) is high relative to K_r (Fig. 13). Since, as discussed above, an activase-mediated increase in the rate of ligand dissociation of the ER complex is likely to be larger than the dissociation of the ECMR complex, where catalysis provides an alternative route, activase activity can lead to an increase in the apparent ratio K_f/K_r .

2. The Role of RuBP in Promoting Carbamylation

The suggestion is frequently made that Rubisco carbamylation is modulated to match the rate of RuBP consumption to that of RuBP regeneration (Sage, 1990). The model by Mate et al. (1996) also proposes a role for RuBP concentration in the regulation of carbamylation state (Fig. 13). When activase is active (i.e. K_f/K_r is large) Rubisco's carbamylation status becomes strongly dependent on RuBP concentration when RuBP concentrations fall below the enzyme site concentration. This may provide a very responsive mechanism for ensuring that Rubisco's activity is modulated according to the prevailing rate of RuBP supply, which may vary with environmental conditions, particularly irradiance. For example Mott et al. (1984) showed that a light transient from high to low light resulted in an immediate drop in RuBP concentration followed by deactivation of Rubisco. Deactivation of Rubisco following a transient drop in RuBP pools were also observed by Sharkey et al. (1986) when leaves were transferred to low O_2 concentrations. Portis et al. (1995) reported that Rubisco decarbamylated *in vitro* at subsaturating concentrations of RuBP.

If the ratio K_f/K_r is less than unity, the extent of carbamylation is predicted to be less in the presence of RuBP than that observed in the absence of RuBP (Fig. 13). This highlights the importance of activase, since *in vitro* measurements of K_f (20 nM, Jordan and Chollet, 1983) show it to be two orders of magnitude smaller than K_r .

3. How Much Activase is Required to Keep Rubisco Carbamylated

Tobacco leaves have between 40–100 mg m^{-2} of

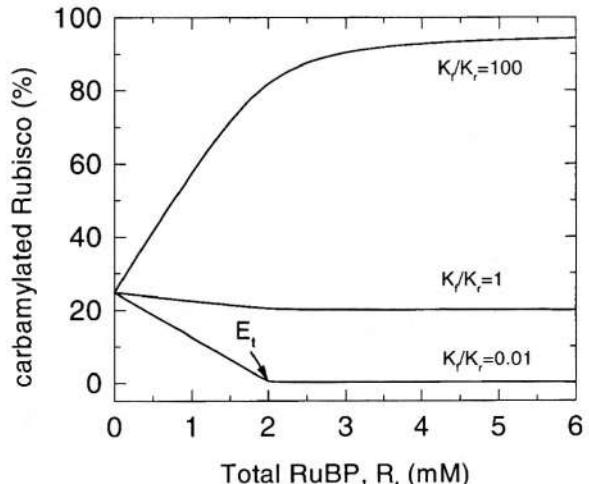


Fig. 13. Predicted carbamylation ratio (defined in Fig. 12) of Rubisco at ambient CO_2 concentration (8 μM) as a function of total RuBP (free and Rubisco bound, R_t) at three different ratios of K_f/K_r , the ratio of the dissociation constant of the ER complex and K_r , the apparent K_m for (RuBP). It is assumed that activase activity increases K_f/K_r . The Fig. is redrawn from Mate et al. (1996) and details of calculations are given there. Symbols are defined in the legend to Fig. 12.

activase, which constitutes approximately 1–2% of leaf soluble protein. If it is assumed that activase is a tetramer with subunits size of 47 kD (Portis, 1990) then the stoichiometry of activase to Rubisco is one activase tetramer to 80 Rubisco catalytic sites. In *Arabidopsis* activase constitutes about 5% of soluble protein and leaves have between 100 to 200 mg m^{-2} , which gives a ratio of one to 20–30 (Eckhard et al., 1997). Activase could be reduced to below 5% of wildtype levels in transgenic tobacco before effects on the steady state levels of Rubisco carbamylation and CO_2 assimilation rate could be seen (Mate et al., 1996, Fig. 14) and similar results were observed in *Arabidopsis* (Eckhard et al., 1997). Thus activase must be acting in the manner of a catalyst, since there is insufficient activase for it to be a permanently bound ligand. Much higher ratios of activase to Rubisco are required *in vitro* to promote carbamylation (Portis et al., 1986; Lan and Mott, 1991).

Although only low levels of activase are required to achieve full carbamylation in the steady state, studies have shown that even small reductions in activase lead to a decrease in the rate of Rubisco activation (Hammond et al., 1995; Jiang et al., 1996; Hammond et al., 1998). Furthermore Andrews et al. (1995) and He et al. (1997) have shown that leaves of transgenic tobacco plants with very low amounts of

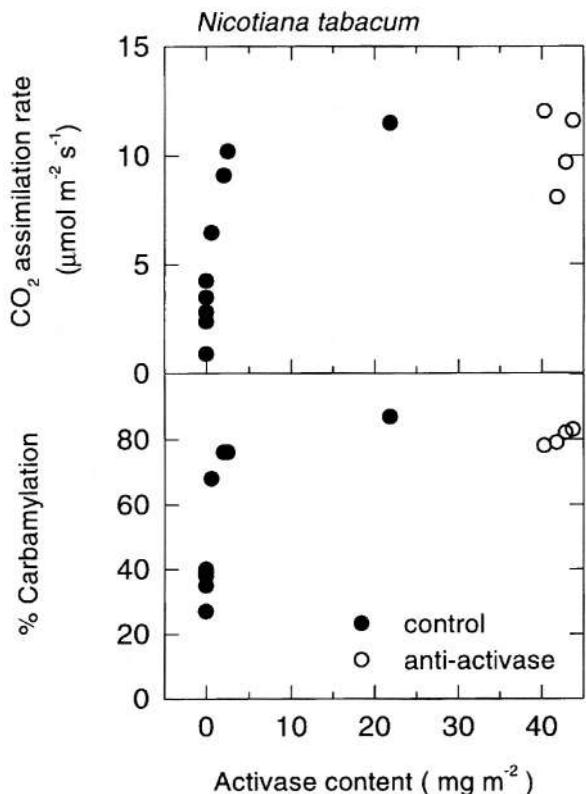


Fig. 14. CO_2 assimilation rate and Rubisco carbamylation for control tobacco leaves and leaves of tobacco with an antisense construct against activase. Measurements were made at 1.5 mmol quanta $\text{m}^{-2} \text{s}^{-1}$ an ambient CO_2 partial pressure of 350 μbar and a leaf temperature of 25 °C. Data are redrawn from Mott et al., 1996.

activase have rates of CO_2 assimilation very much lower than that predicted from the carbamylated Rubisco site content. They were not able to resolve whether this was due to an inhibitor bound to carbamylated sites which was destroyed upon extraction and it leaves the question open to whether activase has another as yet unknown role in Rubisco catalysis *in vivo*.

B. Rubisco Activation State at Different Environmental Conditions

1. Variation in Rubisco Activation State with Irradiance

The activation state (measured by comparison of initial and in vitro activated Rubisco activity) and the carbamylation state of Rubisco (measured by CABP binding as described in Section II.C.2) are low in the

dark and increase with irradiance (Perchorowitz et al., 1981; Mott et al., 1984; von Caemmerer and Edmondson, 1986; Seemann, 1989; Sassenrath-Cole et al., 1994; Krall et al., 1995). Rubisco is never completely decarbamylated in the dark and the carbamylation state is between 30 and 40% depending upon species. The carbamylation level of Rubisco rarely limits steady state rates of CO_2 assimilation and under conditions where RuBP regeneration capacity is low a decrease in carbamylation occurs (Mott et al., 1984; Sage, 1990; Sassenrath-Cole et al., 1994). Changes in stromal pH and magnesium, which saturate at very low irradiances, are insufficient to account for observed changes in Rubisco carbamylation and activity (Woodrow and Berry, 1988). It is likely that both changes in RuBP pool size (and other metabolites such as PGA) and activase activity are involved in the modulation of carbamylation and activity as outlined in section III (Portis, 1992; Salvucci and Ogren, 1996).

At low CO_2 concentrations Rubisco can be almost fully carbamylated at low light (Sage et al., 1990b). This is also evident in gas exchange measurements which show that the initial slope of the CO_2 response curve is frequently independent of irradiance down to quite low irradiance levels (Brooks and Farquhar, 1985; Evans, 1986; Sage et al., 1990b). Under these conditions the capacity for RuBP regeneration exceeds the carboxylation capacity and presumably the thylakoid ΔpH as well as the ATP/ADP ratio are likely to be high which in turn may translate into a greater activase activity. In the same vein, the carbamylation state was found to be high at low irradiance in transgenic tobacco plants with reduced amount of Rubisco (Quick et al., 1991a) whereas there was no difference at high light (von Caemmerer et al., 1992). For shade leaves the irradiance required for full activation of Rubisco appears to be lower than in sun leaves (Seemann, 1989; Krall, 1995), although it is unclear what mechanism underpins this adaptation.

Leaves of some species contain substantial amounts of a nocturnal inhibitor carboxyarabinitol 1-phosphate (CA1P) (Vu et al., 1984; Gutteridge et al., 1986; Servaites et al., 1986; Berry et al., 1987). CA1P binds tightly to the carbamylated (ECM) form of Rubisco and reduces the Rubisco activity that remains after extraction and subsequent *in vitro* activation by CO_2 and magnesium (Seemann and Berry, 1982; Berry et al., 1987; Seemann et al., 1990; Servaites, 1990). Leaves of soybean, *Phaseolus*, tobacco and

Alocasia contain large amounts of CA1P, while other species (e.g. spinach, wheat and *Arabidopsis*) contain very little (Vu et al., 1984; Servaites et al., 1986). The easiest assay for the presence of the nocturnal inhibitor is the combined measurement of Rubisco site concentration and in vitro activated Rubisco activity. A reduction of the catalytic turnover rate is a good indicator for the presence of a CA1P (Seemann, 1989; Seemann et al., 1990). In soybean, CA1P has been shown to form part of the diurnal light regulation of Rubisco (Servaites et al., 1991). High irradiance leads to the rapid removal of CA1P from Rubisco sites. However, in the natural daylight regime there is a more gradual decline in CA1P during the morning which contributes to the observed gradual rise in Rubisco activity (Kobza and Seemann, 1989; Servaites et al., 1991; Sage et al., 1993). Activase has been shown to release CA1P from the ECM complex in vitro (Robinson and Portis, 1988). In transgenic tobacco plants with reduced amounts of activase, CA1P was removed from Rubisco sites more slowly following illumination than in control plants (Mate et al., 1993).

After a period of illumination CA1P is low and requires several hours to increase again when irradiance is decreased and thus CA1P does not seem to play a role in short term modulation of Rubisco activity or of Rubisco regulation at dusk (Sage et al., 1993). Servaites (1990) and Moore and Seemann (1992) have reviewed what is known about the metabolism of CA1 P. The role that CA1P plays in the regulation of Rubisco activity remains unclear. It is obviously not essential for the light regulation of Rubisco in many species.

2. Variation of Rubisco Activation State with CO_2

In vitro, Rubisco carbamylation is strongly dependent on the prevailing CO_2 and magnesium concentration (Laing and Christeller, 1976; Lorimer et al., 1976). The same dependence on CO_2 concentration is however not observed in vivo where RuBP and activase activity are thought to maintain Rubisco activation state (Section III.A; Portis, 1992). However reduced Rubisco activation state has been observed at CO_2 concentrations below the CO_2 compensation point (Fig. 6; von Caemmerer and Edmondson, 1986). A decrease in activation state can also be observed at high CO_2 concentrations (Fig. 6; von Caemmerer and Edmondson, 1986; Sage et al., 1988, 1990b).

Sage et al. (1990b) noted that the deactivation at high CO_2 was more pronounced at low light and suggested that Rubisco decarbamylation occurred to match the rate of Rubisco with the rate of RuBP regeneration to regulate metabolite concentrations. This provides a plausible explanation for the phenomenon but the mechanism of how this is achieved remains unclear. In studies with transgenic tobacco where RuBP regeneration rate has been reduced through a reduction in GAPDH activity, Rubisco shows no concomitant decrease in Rubisco carbamylation (Price et al., 1995). However in this case the ATP/ADP ratios are high and perhaps this is a further indication that Rubisco carbamylation and activity is closely tied to activase activity which in turn is influenced by the ATP/ADP ratio and perhaps the redox state of the electron transport chain (Portis, 1992).

Sharkey et al. (1986a) showed that the extent of Rubisco carbamylation also decreased when CO_2 assimilation rate was limited by the export rate of triose phosphate out of the chloroplast. Under these conditions, which can be induced by a combination of low O_2 concentration and elevated CO_2 concentration, free stromal phosphate and ATP are in short supply (Leegood and Furbank, 1986; Sharkey et al., 1986b; Sharkey and Vanderveer, 1989). Sawada et al. (1990) suggested that the deactivation of Rubisco seen in sink-limited soybean plantlets may also be caused by reduced levels of free phosphate.

3. Time Course of Activation

In vivo, Rubisco activation and deactivation is slow with time constants between four and five min for the activation process and between 20 and 25 min for deactivation process. The light activation and deactivation of other PCR cycle enzymes occurs much more rapidly (for review see, Pearcy et al., 1996). During photosynthetic light induction experiments good correlations have been found between CO_2 assimilation rate and initial Rubisco activity (the maximal Rubisco activity measured in leaf extracts after rapid extraction and without in vitro activation by CO_2 and magnesium) (Pearcy, 1988; Woodrow and Mott, 1989). Both Rubisco's slow activation and the even slower deactivation have a profound effect on the CO_2 fixation capacity of leaves in fluctuating light environments and Pearcy and coworkers have provided detailed analyses (Pearcy, 1990; Sassenrath-Cole et al., 1994; Krall et

al., 1995; Pearcy et al., 1996). No consistent differences in the rate of Rubisco activation and deactivation have been observed between species from different habitats, and not even between species with and without CA1P metabolism (Kobza and Seemann, 1989; Pearcy et al., 1996; Ernstsen et al., 1997). This is surprising since studies with transgenic tobacco and *Ambidopsis* plants with reduced amounts of activase content have shown that the rate of Rubisco activation is almost linearly dependent on the activase content in these plants. There thus appears to be the possibility to modulate the rate of activation. Plants with very low activase content showed very slow rates of photosynthetic induction (Hammond et al., 1995, 1998; Mott et al., 1997). These results also show that whilst activase may have minor control on photosynthetic rate in the steady-state, its importance increases dramatically in plants experiencing fluctuating rates of photosynthesis due to changing environmental conditions (e.g. below a leaf canopy) where rapid changes in irradiance frequently occur (Hammond et al., 1998).

IV. Rubisco Content of Leaves of Plants Grown in Different Environmental Conditions

A. Nitrogen Nutrition

The majority of leaf nitrogen is tied up in the photosynthetic components of the chloroplasts (Chapin et al., 1987; Evans, 1989, 1996; Evans and Seemann, 1989). In C₃ plants approximately 25% of nitrogen is invested in Rubisco alone. The CO₂ concentrating mechanism makes C₄ plants more nitrogen use efficient and Rubisco constitutes only 10 to 15% of leaf nitrogen (Sage et al., 1987). Nitrogen is frequently a scarce commodity and it is not surprising that the Rubisco content of leaves declines with leaf age as nitrogen is remobilized to young developing leaves. There are many studies that have examined the changes in CO₂ assimilation rate with leaf age (Evans, 1983; Makino et al., 1985; Hidema et al., 1991). Rubisco content of leaves increases in expanding leaves and then declines again as leaves senesce and there is usually a strong correlation between CO₂ assimilation rate and Rubisco activity or content and other photosynthetic components (Makino et al., 1985; Hidema et al., 1991; He et al., 1997).

Different nitrogen nutrition also has, among other things, a marked effect on the Rubisco content of leaves (Fig. 8; von Caemmerer and Farquhar, 1981, 1984; Björkman, 1981; Evans, 1983; Sage et al., 1987; Quick et al., 1992; Makino et al., 1994b). However, the differences in Rubisco content are also accompanied by concomitant changes in other chloroplast proteins and chlorophyll suggesting that a similar balance between the capacity for Rubisco carboxylation and RuBP regeneration is maintained. This is evident in measurements of CO₂ response curves where the initial slope and the CO₂ saturated rate change proportionally (von Caemmerer and Farquhar, 1981; Evans, 1983; Evans and Teraschima, 1988; Makino et al., 1994). The relationship between Rubisco and leaf nitrogen is usually linear with a positive intercept and differs between species (Evans, 1989). This means that leaves with a low nitrogen content invest proportionally more nitrogen into Rubisco protein. The relationship between CO₂ assimilation rate and Rubisco activity is curvilinear when measurements made at high light and constant ambient CO₂ concentration are compared (Fig. 8; Evans and Seemann, 1989; Quick et al., 1992; Fichtner et al., 1993). That is, the ratio of CO₂ assimilation rate per Rubisco content declines with increasing Rubisco activity, as other factors such as stomatal and leaf-internal conductance to CO₂ diffusion, the capacity for RuBP regeneration or the activation state of Rubisco exert more control over the rate of CO₂ assimilation (Section II.E). The mechanism by which nitrogen availability controls the abundance of Rubisco protein is largely unknown. It is interesting to note that transgenic tobacco plants with an antisense construct to Rubisco accumulate nitrate in leaves (Quick et al., 1991b; Fichtner et al., 1993; Masle et al., 1993). Recent studies have suggested a direct role of nitrate in the control of plant development and photosynthetic carbohydrate partitioning, acting at the level of gene expression (Scheible et al., 1997a,b). It remains to be identified whether such control operates on genes encoding Rubisco.

B. Irradiance

Plants growing under low light also have lower Rubisco content than high light grown plants (Björkman, 1981; von Caemmerer et al., 1984; Evans, 1987; Seemann, 1989; Lauerer et al., 1994; Krall et al., 1995). Chloroplasts from shade grown plants

show a number of adaptive changes such as increased thylakoid development and increased partitioning of thylakoid protein into light capturing components away from electron transport intermediates such as cytochrome *b/f* complex and ATPase (Björkman, 1981; Evans and Seemann, 1989; Evans, 1996). Seemann et al. (1987) found the relationship between leaf nitrogen and Rubisco content to be independent of growth irradiance in *Phaseolus* and *Alocasia* but the shade plant *Alocasia* had a lower proportion of leaf nitrogen in Rubisco (10% versus 19%). However electron transport capacity, which is strongly correlated with cytochrome *b/f* content, is usually also correlated with Rubisco capacity and both are reduced almost in parallel under low light. This was recently confirmed for rice (Makino et al., 1997a). Although many species reduce the amount of Rubisco on a leaf area basis when transferred to, or grown at low light (von Caemmerer and Farquhar, 1984; Krall et al., 1995) most species do not reduce Rubisco sufficiently for optimal allocation of nitrogen within leaves at low light. For example, when transgenic tobacco with different antisense reductions in Rubisco was grown at 300 μmol quanta $\text{m}^{-2} \text{s}^{-1}$, Rubisco content could be lowered well below wildtype levels before CO_2 assimilation was limited by Rubisco activity at growth light intensities (Quick et al., 1991a, Lauerer et al., 1993).

The Rubisco content of a leaf is dynamic and subject to rapid changes in response to changing environmental conditions, even within fully mature leaves. In an elegant set of experiments Prioul and Reyss (1987, 1988) demonstrated that within a single leaf of tobacco, localized changes in irradiance both up and down brought about parallel changes in the amount of Rubisco protein within a few days. These changes were paralleled by changes in the mRNA encoding the nuclear encoded small subunit of Rubisco but not the plastidic encoded large subunit mRNA. This suggested strong nuclear control of the abundance of Rubisco protein, which was highly sensitive to environmental conditions. Nuclear, rather than plastidic, control was further demonstrated when an antisense construct to the small subunit of Rubisco was shown to be sufficient to reduce the amount of Rubisco small and large subunit protein in transgenic tobacco plants, despite having no influence on the abundance of large subunit mRNA (Rodermel et al., 1988). Much work has focused on the control of Rubisco expression by light intensity and several photoreceptors which detect both light quality and

quantity have been implicated in the regulation of Rubisco abundance including phytochrome, high irradiance responses, a blue light/UV-A photoreceptor and a UV-B photoreceptor. The mechanisms by which these signals affect Rubisco abundance is varied, involving altered rates of gene transcription (Gallagher and Ellis, 1982), mRNA translation (Berry et al., 1990), post-translational mRNA regulation (Peters and Silverthorne, 1995) and protein turnover (Crafts-Brandner et al., 1996). Moreover, a multigene family, the significance of which has yet to be clarified, encodes the small subunit of Rubisco. While the coding sequence of these individual genes is highly conserved, suggesting little difference in the kinetic properties of the proteins they encode, their expression patterns have shown organ specific differences (Dean et al., 1985; Silverthorne and Tobin, 1990). This could imply that expression of individual genes in different tissues could result in altered responses to environmental signals depending on the precise function of the tissue.

C. Elevated CO_2

In an attempt to understand the effects of predicted increases in atmospheric CO_2 on the carbon exchange in the terrestrial biosphere, many studies have examined the effects of long-term growth at elevated CO_2 on CO_2 assimilation and Rubisco function. Several recent reviews have summarized the present knowledge on Rubisco (Bowes, 1991; Sage, 1994; Woodrow, 1994; Drake et al., 1996). Depending on growth conditions and species, both decreases in Rubisco content per leaf area and/or reductions in Rubisco activation states have been observed. However, in some species no change in either parameter occurs (Drake et al., 1996). It has been frequently pointed out that at elevated CO_2 much less Rubisco per electron transport capacity is required to maintain present ambient CO_2 assimilation rates, particularly at higher temperatures, the effect being less marked at low temperature (Woodrow, 1994; Drake et al., 1996). However there is no evidence that plants are able to adjust the balance between Rubisco and electron transport capacity to suit environmental conditions. Experiments with transgenic rice with reduced amounts of Rubisco have demonstrated that plants with 65% of wild-type Rubisco levels were more nitrogen use efficient and may provide a solution for crop growth at elevated CO_2 (Makino et al., 1997b).

In many species gradual decline in assimilation rates are observed after transfer to growth conditions with elevated CO_2 partial pressures. This process has been referred to as a ‘down-regulation’ of the photosynthetic apparatus and is associated with a decline in abundance of photosynthetic proteins including Rubisco (e.g. Besford et al., 1990; Van Oosten and Besford, 1994; Nie et al., 1995; Vu et al., 1997). One explanation for this phenomenon is a feedback regulation of photosynthesis brought about by carbohydrate accumulation within photosynthetic tissues. Several lines of evidence suggest that this is the case. Firstly, carbohydrates frequently accumulate in the leaves of plants grown in elevated CO_2 suggesting that there may be a limitation on the ability of sink tissues in some species to utilize the extra photosynthate produced. Secondly, manipulation of source/sink ratio either via fruit removal (Wittenbach, 1983; Plaut et al., 1987) or increasing sink strength via leaf thinning (Diethelm and Shibles, 1989) altered the rate of leaf photosynthesis and abundance of Rubisco protein. More recently, fruit removal experiments with soybean have identified an insoluble high molecular weight form of Rubisco that is associated with down-regulation of photosynthesis (Crafts-Brandner et al., 1991). This involves the synthesis of a new protein (Rubisco Complex Protein) that is able to complex and insolubilize Rubisco isolated from a variety of sources (Crafts-Brandner and Salvucci, 1994). Thirdly, restriction of carbohydrate export from leaves via cold-girdling of stems (Krapp et al., 1993), ectopic expression of yeast invertase in the cell wall compartment of tobacco (von Schaewen et al., 1990) or reduction of the phloem-specific sucrose transport activity in transgenic potato plants (Kuhn et al., 1996) all brought about photosynthetic down-regulation including decreased amounts of Rubisco protein. Fourthly, similar photosynthetic down-regulation could be induced by feeding sugars to detached leaves (Krapp et al., 1991) and this involved sugar-mediated repression of photosynthetic gene expression (Jang and Sheen, 1994). Since these initial observations, our knowledge of the genes that are controlled by carbohydrates and the mechanism for carbohydrate control have increased dramatically. These topics are outside the scope of this chapter but are thoroughly reviewed by Koch (1996) and Smeekens and Rook (1997).

V. Rubisco and C_4 Photosynthesis

The C_4 photosynthetic pathway is a CO_2 concentrating mechanism that operates between mesophyll and bundle sheath cells of leaves (Hatch and Osmond, 1976; Hatch, 1987). Rubisco is located solely in the bundle sheath and the reactions of the C_4 cycle act to concentrate CO_2 in this cellular compartment. This enhances RuBP carboxylation while inhibiting Rubisco oxygenation, thus reducing the amount of photorespiration. CO_2 concentrations in the bundle sheath have not been measured directly but have been estimated to be between 10- to 100-fold greater than in the ambient air (Furbank and Hatch, 1987; Jenkins et al., 1989). Since Rubisco of C_4 species operates in a very different environment compared to Rubisco of C_3 species the regulation of amount and activity may be different.

A. Rubisco Content and CO_2 Assimilation Rate

Similar to photosynthesis in C_3 species, there is a strong correlation between Rubisco activity (or content) and CO_2 assimilation rate, measured at high irradiance (Fig. 15; Usuda, 1984; Hunt et al., 1985; Sage et al., 1987; Furbank et al., 1996; von Caemmerer et al., 1997). Because Rubisco operates close to CO_2 saturation, the relationship between maximal Rubisco activity and CO_2 assimilation rate should be close to 1:1. In transgenic *Flaveria bidentis* where amounts of Rubisco were reduced via antisense without concomitant changes in the amounts of other photosynthetic enzymes, the decline in CO_2 assimilation rate was not as great as the decline in Rubisco content. This may indicate that in wildtype plants other enzymes like pyruvate Pi dikinase may be limiting the rate or that there may be a small increase in bundle-sheath CO_2 concentrations, as a result of low Rubisco content, and that this enhances Rubisco activity in the antisense plants. Figure 16 shows CO_2 response curves of CO_2 assimilation rates characteristic of C_4 species. Unlike in C_3 species, Rubisco determines the saturated rate of CO_2 assimilation rate whereas the rate of PEP carboxylation determines the initial slope of the response curve and the CO_2 compensation point is very low and largely independent of oxygen concentration.

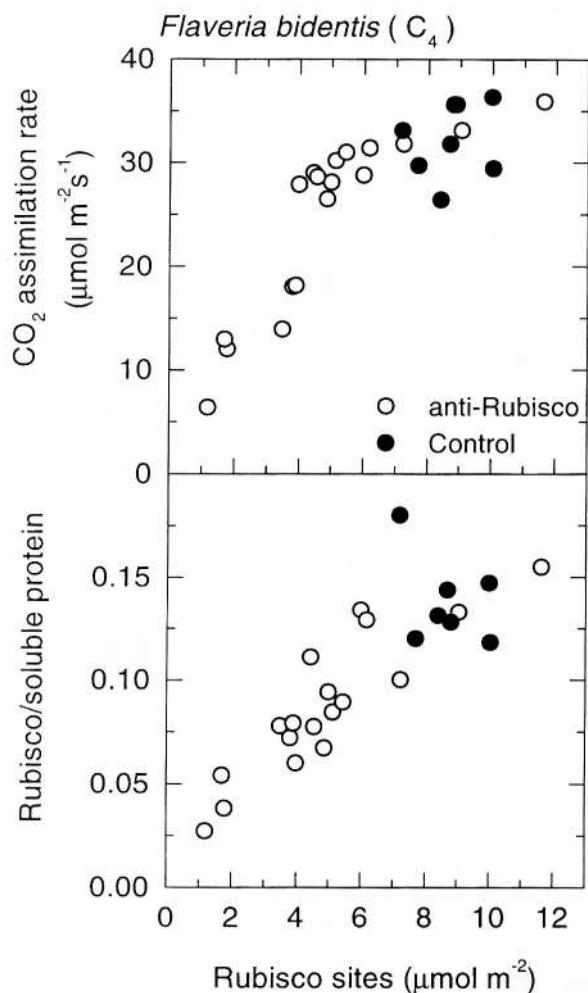


Fig. 15. CO₂ assimilation rate and the ratio of Rubisco to soluble protein versus Rubisco site content in the C₄ species *Flaveria bidentis* (●, wild-type and ○ transgenic *F. bidentis* with reduced amounts of Rubisco). Measurements were made at an ambient CO₂ concentration of 350 μbar, an irradiance of 2 mmol quanta m⁻² s⁻¹ and a leaf temperature of 25 °C. Data are redrawn from von Caemmerer et al., 1997.

B. Rubisco Kinetic Constants

The kinetic constants of Rubisco from C₄ species differ from those of C₃ species (Fig. 17; Yeoh et al., 1980, 1981; Jordan and Ogren, 1983; Seemann et al., 1984; Wessinger et al., 1989; Hudson et al., 1990). In particular, the K_c can be several folds higher than in C₃ species. Yeoh et al. (1980) measured the K_c in 60 grass species including 24 C₄ species. They found the K_c for C₃ species to range between 13–26 μM (389–778 μbar) and that of C₄ species between 28–64 μM (838–1916 μbar). In C₄ species that have

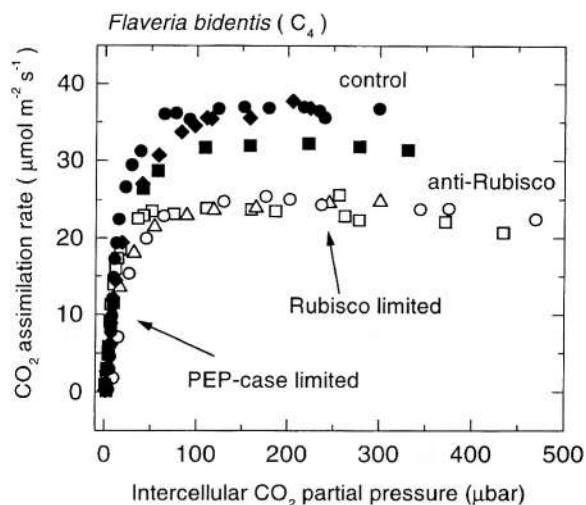


Fig. 16. CO₂ response curves of CO₂ assimilation rate in the C₄ species *Flaveria bidentis* (closed symbols, wild-type and open symbols, transgenic with a 40 % reduction in Rubisco content). Measurements were made at an irradiance of 2 mmol quanta m⁻² s⁻¹ and a leaf temperature of 25 °C. Data are redrawn from von Caemmerer et al., 1997.

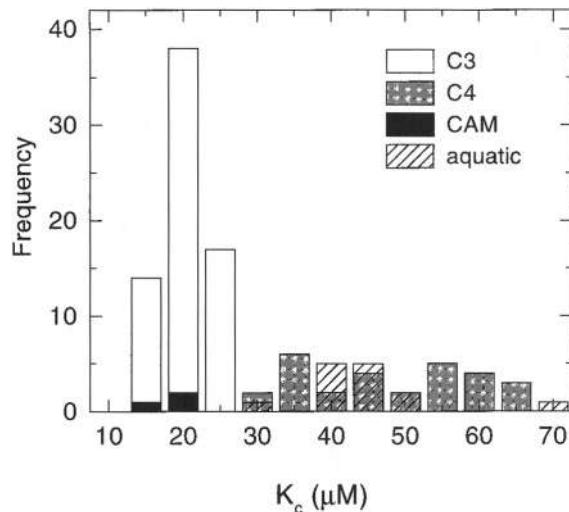


Fig. 17. Distribution of Michaelis-Menten constant for CO₂, K_c , for different species. Data are replotted from Yeoh et al. (1980, 1981).

PS II activity in the bundle sheath, bundle sheath O₂ concentrations can be greater than ambient concentrations (Raven, 1977; Berry and Farquhar, 1978) and the apparent K_m (CO₂) in the presence of O₂ could be more than double the above values. If C₄ species evolved from C₃ ancestors with Rubisco with a high affinity for CO₂, this trait was apparently

readily lost with the development of a CO_2 concentrating mechanism. Seemann et al. (1984) also showed that the high K_c is associated with a higher catalytic turnover rate (~1.2 times that of C_3 Rubisco). The extensive measurements of the K_c in C_4 species are not matched by extensive measurements of the relative specificity, $S_{c/o}$, or the K_o . In the few C_4 species examined, $S_{c/o}$ is very similar to that of C_3 species (Jordan and Ogren, 1981; Kane et al., 1994). Where measured, the K_o is also greater than the C_3 values, however more detailed measurements of Rubisco kinetic constants of C_4 species are required (Table 4; Badger et al., 1974; Jordan and Ogren, 1981).

C. Rubisco Activity and Carbamylation

One might expect that the high CO_2 partial pressures occurring in the bundle sheath may themselves promote full carbamylation, however C_4 species are also known to have activase (Salvucci et al., 1987) and the requirement for the removal of RuBP and other phosphorylated inhibitors apparently remains. The nocturnal inhibitor CA1P has been detected in a number of C_4 species (Vu et al., 1984; Servaites et al., 1986; Moore et al., 1991). However, interesting differences to C_3 species exist. For example in some C_4 -species (e.g. *Zea mays*) the degree of dark inactivation is very much less than that of C_3 species

(Usuda, 1985, 1990; Sage and Seemann, 1993) and not all C_4 species show light modulated variations in Rubisco carbamylation (Sage and Seemann, 1993).

VI. Rubisco and CAM Photosynthesis

In plants with crassulacean acid metabolism (CAM) photosynthesis is compartmented into four distinct phases over a diel course (Osmond, 1978). The CAM photosynthetic pathway is a temporal CO_2 concentrating mechanism with atmospheric CO_2 fixed nocturnally through PEP carboxylase and the reduced product, malic acid, stored in the vacuole overnight (phase I). A second phase of atmospheric CO_2 uptake (phase II) may occur at dawn, which represents the transition from PEP carboxylase (C_4) to Rubisco (C_3) carboxylation (Osmond and Björkman, 1975; Osmond, 1978; Griffiths et al., 1990). During phase III, stomata close and malic acid efflux from the vacuole and decarboxylation of malate generates a high intercellular partial pressure of CO_2 , which may approach 40 mbar (Cockburn et al., 1979; Spalding et al., 1979), while CO_2 is re-fixed through Rubisco. Following decarboxylation the stomata may re-open in the afternoon, and phase IV reflects the shift from C_4 to C_3 atmospheric CO_2 fixation.

The CAM pathway permits flexibility in response to environmental stimuli. For example, the transitional

Table 4. In vitro kinetic constants of Rubisco from several C_4 species and a CAM plant

Species	K_c (μM)	K_c (μbar)	K_o (μM)	K_o (mbar)	$K_c(1+O/K_o)$ μbar at 200mbar O_2	V_{omax}/V_{cmax}	$S_{c/o}$ ^(c)	Reference
<i>Atriplex spongiosa</i>	27	814	710 ^a	563	1103	0.14		Badger et al., 1974
	26 ^b	769	160	127	1980			
<i>Zea mays</i> (exp1)	33	978	550	436	1427			
<i>Zea mays</i> (exp2)	28	838	610	484	1184			
<i>Zea mays</i>	34	1018	810	643 b	1062	0.3	78	Jordan and Ogren, 1981
<i>Amaranthus hybridus</i>	16	480	640	508 b	669	0.38	82	
<i>Kalanchoe diagremontiana</i> (CAM)	14	420	490	389	591	0.24		

All values were measured at 25 °C. To convert K_c and K_o values from concentration to partial pressures the solubilities for CO_2 of 0.0334 mol (1 bar)⁻¹ and for O_2 of 0.00126 mol (1 bar)⁻¹ were used. K_c values by Badger et al. (1974) where measured in 100 mM Hepes pH 8.3. The values had been calculated with a pKa = 6.37 and where recalculated here with a pKa = 6.12. Measurements by Jordan and Ogren (1981), were made with 50 mM Bicine buffer pH 8.3 and a pKa = 6.23 was used.

^a measured as $K_i(\text{O}_2)$

^b measured as $K_i(\text{CO}_2)$

^(c) $S_{c/o}$ values by Jordan and Ogren (1981,1984) were derived from simultaneous measurements of carboxylation and oxygenation.

phases of daytime CO_2 uptake (II and IV) may be lost in response to drought stress and following prolonged drought, the stomata may remain closed over the entire 24 h period (Kluge and Ting, 1978; Martin, 1996). Under well watered conditions, on the other hand, additional atmospheric CO_2 fixation can occur via C_3 photosynthesis during the transitional phases (II and IV), and this may constitute a significant proportion of carbon gain, in addition to that fixed at night (Borland and Griffiths, 1996). CO_2 fixation via Rubisco is associated with a larger discrimination against $^{13}\text{CO}_2$ compared to $^{12}\text{CO}_2$ than is CO_2 fixation via PEP carboxylase. Borland and Griffiths (1996, 1997) have used combined measurements of carbon isotope discrimination and CO_2 assimilation to assess the amount of PEP carboxylation versus Rubisco carboxylation occurring during phase II and IV.

Rubisco is a substantial component of the proteins in CAM plants exceeding PEP carboxylase in amount and in maximal activity (Kluge and Ting, 1978; Israel and Nobel, 1994, 1995). In *Opuntia fiscus-indica* PEP carboxylase and Rubisco correspond to 10 and 30% of extractable soluble protein (Israel and Nobel, 1995), the Rubisco value being typical of that found in C_3 species. The ratios of extractable maximal PEP carboxylase to maximal Rubisco activity are usually one to one or less (Israel and Nobel, 1994, 1995,) which contrasts with the C_4 photosynthetic pathway where an excess of PEP carboxylase activity is essential for the generation of elevated bundle sheath CO_2 concentrations.

There are only very few measurements of Rubisco kinetics constants for CAM plants. Although like C_4 species CAM species have evolved from C_3 species and have developed a CO_2 concentrating mechanism, the kinetic constants measured are C_3 like (Table 4; Badger et al., 1975; Yeoh et al., 1981). The CAM pathway requires no special regulation of Rubisco activity. Vu et al. (1984) and Servaites et al. (1986) identified several CAM plants with very low nocturnal Rubisco activity indicative of the presence of nocturnal inhibitors. Nocturnal inhibition of Rubisco is however not a general feature of CAM metabolism (Moore et al., 1992; Osmond et al., 1996).

VII. The Role of Rubisco in $\text{C}_3\text{-C}_4$ Intermediates

Over 20 species of plants exhibit photosynthetic characteristics that are intermediate between C_3 and

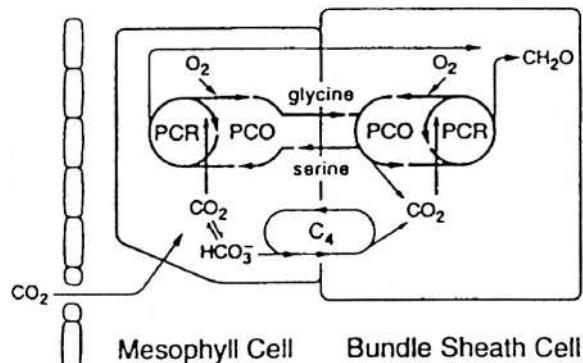


Fig. 18. Schematic representation of carbon metabolism in $\text{C}_3\text{-C}_4$ intermediates. CO_2 assimilation by the PCR cycle occurs in both mesophyll and bundle sheath cells. CO_2 is supplied to the bundle sheath via decarboxylation of glycine from both mesophyll and bundle sheath PCO cycles and in some species, additional CO_2 is supplied via a C_4 cycle.

C_4 plants in that they show reduced photorespiratory CO_2 release and CO_2 compensation points in the range of 7–30 μbar (Edwards and Ku, 1987; Rawsthorne, 1992). All of these plants show a degree of Kranz anatomy, but interveinal distances are much greater than in C_4 species. Fig. 18 shows a schematic summary of $\text{C}_3\text{-C}_4$ pathways but it is important to keep in mind that the biochemistry of CO_2 fixation varies among $\text{C}_3\text{-C}_4$ species. In all cases Rubisco is present in both mesophyll and bundle-sheath cells, but activities of C_4 pathway enzymes is generally low (Edwards and Ku, 1987). In several types of intermediates (e.g. *Panicum milioides*, *Neurachne minor* and *Moricandia arvensis*) there is no evidence of C_4 cycle activity, the intermediate character with respect to the gas exchange characteristics appears to be entirely due to efficient refixation of photorespiratory CO_2 in the bundle sheath. The mesophyll mitochondria of *M. arvensis* and many other intermediates (like true C_4 plants) have only low activities of glycine decarboxylase (Rawsthorne, 1992). Thus photosynthesis in leaves of these intermediates might involve shuttling of photorespiratory intermediates such as glycine from mesophyll to bundle sheath and the return of serine to the mesophyll (Hylton et al., 1988).

The quantitative feasibility of such localized photorespiratory CO_2 release accounting for the observed CO_2 exchange characteristics of $\text{C}_3\text{-C}_4$ intermediates was demonstrated by the model of von Caemmerer (1989). The recycling of photorespiratory CO_2 cannot result in a large increase in photosynthetic

rate at ambient CO_2 concentration and at most 15% of leaf Rubisco might be allocated to the bundle sheath for such recycling purposes. However it may be advantageous when stomata restrict CO_2 supply or at high temperatures when photorespiratory rates increase (Monson, 1989; von Caemmerer, 1992).

Some $\text{C}_3\text{-C}_4$ intermediates of *Flaveria* show appreciable C_4 cycle activity (Monson et al., 1986; Dai et al., 1996). However, all of the Rubisco's of $\text{C}_3\text{-C}_4$ intermediates that have been examined have C_3 -like kinetic constants (Jordan and Ogren, 1981; Yeoh et al., 1980; Hudson et al., 1990; Wessinger et al., 1989). Little is known about regulation of Rubisco activity in these species.

VIII. Conclusion

There have been considerable advances during the last ten years in our knowledge of Rubisco physiology; we now have a working model of Rubisco regulation by Rubisco activase and how this is integrated with the variety of tight binding inhibitors. Transgenic plants have allowed direct quantification of the control that Rubisco exerts on photosynthesis in a variety of environmental conditions confirming and improving our understanding of the major role played by this enzyme in photosynthetic metabolism.

Many signals (e.g. light quality and quantity, carbohydrates, nitrate and cytokinins) that alter the abundance of Rubisco have been identified. However more information is required to understand how these processes are integrated. Increasing advances in plant molecular biology will allow progress in our knowledge of the spatial and temporal signal transduction pathways that coordinate the amount and activity of Rubisco with the growth and development of the plant.

References

- Andrews TJ and Lorimer GH (1987) Rubisco: Structure, mechanisms and prospects for improvement. In: Hatch MD, Boardman NK (eds) The Biochemistry of Plants, Vol 10, pp 132–219. Academic Press, New York
- Andrews TJ, Hudson GS, Mate CJ, von Caemmerer S, Evans JR and Arvidsson YBC (1995) Rubisco: The consequences of altering its expression and activation in transgenic plants. *J Exp Bot* 46: 1293–1300
- Azcon-Bieto J, Farquhar GD and Caballero A (1981) Effects of temperature, oxygen concentration, leaf age and seasonal

variations on the CO_2 compensation point of *Lolium perenne* L: Comparison with a mathematical model including non photorespiratory CO_2 production in the light. *Planta* 152: 497–504

Badger MR and Andrews TJ (1974) Effects of CO_2 , O_2 and temperature on a high-affinity form of ribulose diphosphate carboxylase-oxygenase from spinach. *Biochem Biophys Res Commun* 60: 204–210

Badger MR and Collatz GJ (1977) Studies on the kinetic mechanism of ribulose-1,5-bisphosphate carboxylase and oxygenase reactions, with particular reference to the effect of temperature on kinetic parameters. *Carnegie Inst Washington Yearbook* 76: 355–361

Badger MR and Lorimer GH (1981) Interaction of sugar phosphates with the catalytic site of ribulose-1,5 bisphosphate carboxylase. *Biochemistry* 20: 2219–2225

Badger MR, Andrews TJ and Osmond CB (1974) Detection in C_3 , C_4 and CAM plant leaves of a low $K_m(\text{CO}_2)$ form RuDP carboxylase having a high RuDP oxygenase activity at physiological pH. In: Avron M (ed) Proceedings of the Third International Congress on Photosynthesis, pp 1421–1429. Elsevier, Amsterdam

Badger MR, Sharkey TD and von Caemmerer S (1984) The relationship between steady-state gas exchange of bean leaves and the levels of carbon-reduction cycle intermediates. *Planta* 160: 305–313

Berry JA and Farquhar GD (1978) The CO_2 concentration function of C_4 photosynthesis: a biochemical model. In: Hall D, Coombs J and Goodwin T (eds), Proceedings of the 4th International Congress on Photosynthesis, pp 119–131. Biochemical Society, London

Berry JA, Lorimer GH, Pierce J, Seemann JR, Meek J and Freas S (1987) Isolation, identification and synthesis of 2-carboxy-arabinitiol 1-phosphate, a diurnal regulator of ribulose-bisphosphate carboxylase activity. *Proc Natl Acad Sci USA* 84: 734–738

Berry JO, Breiding DE and Klessig DF (1990) Light mediated control of translation initiation of ribulose 1,5 bisphosphate carboxylase in amaranth cotyledens. *Plant Cell* 2: 795–803

Besford RT, Ludwig LJ, Withers AC (1990) The greenhouse effect: Acclimation of tomato plants growing in high carbon dioxide, photosynthesis and ribulose-1,5-bisphosphate carboxylase protein. *J Exp Bot* 41: 925–932

Björkman O (1981) Responses to different quantum flux densities. In: Lange OL, Nobel PS, Osmond CB and Ziegler H (eds) Encyclopedia of Plant Physiol., New Series, Vol 12A, pp 57–107. Springer-Verlag, Berlin

Björkman O and Pearcy RW (1971) Effect of growth temperature on the temperature dependence of photosynthesis in vivo and on CO_2 fixation by carboxydismutase in vitro in C_3 and C_4 species. *Carnegie Inst Washington Yearb* 70: 520–526

Björkman O, Badger MR and Armond PA (1980) Response and adaptation of photosynthesis at high temperature. In: Turner NC and Kramer PJ (eds) Adaptation of Plants to Water and High Temperature Stress. John Wiley and Sons, New York

Borland AM and Griffiths H (1996) Variations in the phases of crassulacean acid metabolism and regulation of carboxylation patterns determined by carbon-isotope-discrimination techniques. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 230–249. Springer-Verlag, Berlin

- Borland AM and Griffiths H (1997) A comparative study on the regulation of C₃ and C₄ carboxylation processes in the constitutive crassulacean acid metabolism plant *Kalanchoe daigremontiana* and the C₃-CAM intermediate *Clusia minor*. *Planta* 201: 368–378
- Bowes G (1991) Growth at elevated CO₂: Photosynthetic responses mediated through Rubisco. *Plant Cell Environ* 14: 795–806
- Bowes G, Ogren WL and Hageman RH (1971) Phosphoglycolate production catalysed by ribulose diphosphate carboxylase. *Biochem Biophys Res Commun* 45: 71116–71122
- Brooks A, and Farquhar GD (1985) Effect of temperature on the CO₂/O₂ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* 165: 397–406
- Brooks A, Portis AR Jr and Sharkey TD (1988) Effects of irradiance and methylvioletogen treatment on ATP, ADP, and activation of ribulose bisphosphate carboxylase in spinach leaves. *Plant Physiol* 88: 850–853
- Butz ND and Sharkey TD (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylating ratios. *Plant Physiol* 89: 735–739
- Chapin FS III, Bloom CB, Field RH and Waring RH (1987) Plant responses to multiple environmental factors. *Bioscience* 37: 49–57
- Chen Z and Spreitzer RJ (1992) How various factors influence the CO₂/O₂ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Photosynth Res* 31: 157–164
- Cockburn W, Ting IP and Sternberg L (1979) Relationships between stomatal behaviour and internal carbon dioxide concentration in crassulacean acid metabolism plants. *Plant Physiol* 63: 1029–1032
- Collatz GJ, Badger MR, Smith C and Berry JA (1979) A radioimmune assay for RuBP carboxylase protein. *Carnegie Inst Wash Yearb* 78: 171–174
- Crafts-Brandner SJ, Salvucci ME and Egli DB (1991) Fruit removal in soybean induces the formation of an insoluble form of ribulose-1,5-bisphosphate carboxylase/oxygenase in leaf extracts. *Planta* 183: 300–306
- Crafts-Brandner SJ and Salvucci ME (1994) The Rubisco complex protein: A protein induced by fruit removal that forms a complex with ribulose-1,5-bisphosphate carboxylase/oxygenase. *Planta* 194: 110–116
- Dai Z, Ku MSB and Edwards GE (1996) Oxygen sensitivity of photosynthesis and photorespiration in different photosynthetic types in the genus *Flaveria*. *Planta*, 198: 563–571
- Dean C, van den Elzen P, Tamaki S, Dunsmuir P and Bedbrook J (1985) Differential expression of the eight genes of petunia ribulose bisphosphate carboxylase small subunit multi-gene family. *EMBO J* 4: 3055–3061.
- Diethelm R. and Shibles R. (1989) Relationship of enhanced sink demand with photosynthesis and amount and activity of ribulose 1,5-bisphosphate carboxylase in soybean leaves. *J Plant Physiol* 134 70–74.
- Drake BG, Gonzalez-Meler MA and Long SP (1997) More efficient plants: A consequence of rising atmospheric CO₂? *Annu Rev Plant Physiol Plant Mol Biol* 48: 609–639
- Eckhard NA, Snyder GW, Portis AR Jr and Ogren WL (1997) Growth and photosynthesis under high and low irradiance of *Arabidopsis thaliana* antisense mutants with reduced ribulose-1,5-bisphosphate carboxylase/oxygenase activase content. *Plant Physiol* 113: 575–586
- Edwards GE and Ku MSB (1987) Biochemistry of C₃-C₄ intermediates. In: Hatch MD and Boardman NK (eds) *The Biochemistry of Plants*, Vol 10, pp 275–325. Academic Press, New York
- Ehleringer J and Björkman O (1977) Quantum yields for CO₂ uptake in C₃ and C₄ plants. *Plant Physiol* 59: 86–90
- Ernstsen J, Woodrow IE and Mott KA (1997) Responses of Rubisco activation and deactivation rates to variations in growth-light conditions. *Photosynth Res* 52: 117–125
- Evans JR (1983) Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum*). *Plant Physiol* 72: 297–302.
- Evans JR (1986) The relationship between carbon-dioxide-limited photosynthetic rate and ribulose-1,5-bisphosphate carboxylase content in two nuclear-cytoplasm substitution lines of wheat, and the coordination of ribulose-1,5-bisphosphate carboxylase and electron transport capacities. *Planta* 167: 351–358
- Evans JR (1989) Photosynthesis and nitrogen relationships in leaves of C₃ plants. *Oecologia* 78: 9–19
- Evans JR (1996) Developmental constraints on photosynthesis: Effects of light and nutrition. In: Baker NR (ed) *Photosynthesis and the Environment*, pp 281–300. Kluwer Academic Publishers, Dordrecht
- Evans JR and Seemann JR (1984) Differences between wheat genotypes in specific activity of RuBP carboxylase and the relationship to photosynthesis. *Plant Physiol* 74: 759–765
- Evans JR and Seemann JR (1989) The allocation of protein nitrogen in the photosynthetic apparatus: Costs, consequences and control. In: Briggs WR (ed) *Photosynthesis*, pp 183–205. Alan R Liss, New York
- Evans JR and Terashima I (1988) Photosynthetic characteristics of spinach leaves grown with different nitrogen treatments. *Plant Cell Physiol* 29: 157–165
- Evans JR and von Caemmerer S (1996) CO₂ diffusion inside leaves. *Plant Physiol* 110: 339–346
- Evans JR, Sharkey TD, Berry JA and Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO₂ diffusion in leaves of higher plants. *Austr J Plant Physiol* 13: 281–292
- Evans JR, von Caemmerer S, Setchell BA and Hudson GS (1994) The relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with reduced content of Rubisco. *Aust J Plant Physiol* 21: 475–495
- Farquhar GD (1979) Models describing the kinetics of ribulose bisphosphate carboxylase-oxygenase. *Arch Biochem Biophys* 193: 456–468
- Farquhar GD and von Caemmerer S (1982) Modelling of photosynthetic responses to environmental conditions. In: Lange OL, Nobel PS, Osmond CB, Ziegler H (eds) *Physiological Plant Ecology II. Encyclopedia of Plant Physiology*, New Series, Vol. 12B. Springer Verlag, Heidelberg
- Farquhar GD, von Caemmerer S and Berry JA (1980) A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. *Planta* 149: 78–90
- Fersht A (1984) *Enzyme Structure and Mechanism*. Second Edition. WH Freeman and Company, New York,
- Fichtner K, Quick WP, Schulze ED, Mooney HA, Rodemerl SR, Bogorad L and Stitt M (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ rbcS. V Relationship between photosynthetic rate, storage strategy, biomass allocation and

- vegetative plant growth at three different nitrogen supplies. *Planta* 190: 1–9
- Furbank RT and Hatch MD (1987). Mechanism of C₄ photosynthesis. The size and composition of the inorganic carbon pool in bundle-sheath cells. *Plant Physiol* 85: 958–964
- Furbank RT, Chitty JA, von Caemmerer S and Jenkins CL (1996) Antisense RNA inhibition of RbcS gene expression in the C₄ plant *Flaveria bidentis*. *Plant Physiol* 111: 725–734
- Gaastra P (1959) Photosynthesis of crop plants as influenced by light, carbon dioxide, temperature and stomatal diffusion resistance. *Meded Landbouwhogeschool Wageningen* 59: 1–68
- Gallagher TF and Ellis RJ (1982) Light-stimulated transcription of genes for two chloroplast polypeptides in isolated pea nuclei. *EMBO J* 1: 1493–1498.
- Griffiths H, Broadmeadow MSJ, Borland AM and Hetherington CS (1990) Short-term changes in carbon-isotope discrimination identify transitions between C₃ and C₄ carboxylation during crassulacean acid metabolism. *Planta* 181: 604–610
- Gutteridge S (1990) Limitations of the primary events of CO₂ fixation in photosynthetic organisms: The structure and mechanism of Rubisco. *Biochim Biophys Acta* 1015: 1–14
- Gutteridge S, Parry MAJ, Burton S, Keys AJ, Mudd A, Feeney J, Servaite JC and Pierce C (1986) A nocturnal inhibition of carboxylation in leaves. *Nature* 324: 274–276
- Hammond ET, Hudson GS, Andrews TJ and Woodrow IE (1995) Analysis of Rubisco activation using tobacco with antisense RNA to Rubisco activase. In: Mathias P(ed) *Photosynthesis: From Light to Biosphere*, Vol V, pp 293–296. Kluwer Academic Publishers, Dordrecht
- Hammond ET, Andrews TJ, Mott, KA and Woodrow IE (1998) Regulation of Rubisco activation in antisense plants of tobacco containing reduced levels of Rubisco activase. *Plant Journal* 14: 101–110
- Hatch MD (1987) C₄ photosynthesis a unique blend of modified biochemistry, anatomy and ultra structure. *Biochim Biophys Acta* 895: 81–106
- Hatch MD and Osmond CB (1976) Compartmentation and transport in C₄ photosynthesis. In: Stocking CR and Heber U (eds) *Transport in Plants III. Intracellular Interactions and Transport Processes*. Encyclopedia of Plant Physiology New Series, Vol 3, pp 144–184. Springer-Verlag, Berlin
- He Z, von Caemmerer S, Hudson G, Price GD, Badger MR and Andrews TJ (1997) Rubisco activase deficiency delays senescence of Rubisco but progressively impairs its catalysis during tobacco leaf development. *Plant Physiol* 115: 1569–1580
- Hidema J, Makino A, Mae T and Ojima K (1991) Photosynthesis characteristics of rice leaves aged under different irradiances from full expansion through senescence. *Plant Physiol* 97: 1287–1293
- Hudson GS, Mahon JD, Anderson PA, Gibbs MJ, Badger MR, Andrews TJ and Whitefeld PR (1990) Comparison of rbcL genes for the large subunit of ribulose bisphosphate carboxylase from closely related C₃ and C₄ species. *J Biol Chem* 265: 808–814
- Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC and Andrews TJ (1992) Reduction of ribulose-bisphosphate carboxylase/oxygenase content by antisense RNA reduced photosynthesis in tobacco plants. *Plant Physiol* 98: 294–302
- Hunt ER, Weber JA and Gates DM (1985) Effects of nitrate application on *Amaranthus powellii* Wats. III. Optimal allocation of leaf nitrogen for photosynthesis and stomatal conductance. *Plant Physiol* 79: 619–624
- Hylton CM, Rawsthorne S, Smith AM, Jones DA and Woolhouse HW (1988) Glycine decarboxylase is confined to the bundle sheath cells of C₃-C₄ intermediate species. *Planta* 175: 452–459
- Israel AA and Nobel PS (1994) Activities of carboxylating enzymes in the CAM species *Opuntia fiscus-indica* grown under current and elevated CO₂ concentration. *Photosynth Res* 40: 223–229
- Israel AA and Nobel PS (1995) Growth temperature versus CO₂ uptake, Rubisco and PEPcase activities, and enzyme high-temperature sensitivities for a CAM plant. *Plant Physiol Biochem* 33: 345–351
- Jang J and Sheen J (1994) Sugar sensing in higher plants. *Plant Cell* 6: 1665–1679
- Jenkins CLD, Furbank RT and Hatch MD (1989) Inorganic carbon diffusion between C₄ mesophyll and bundle sheath cells. *Plant Physiol* 91: 1356–1363
- Jiang CZ, Quick WP, Alfred R, Kleibenstein D and Rodermel RS (1994) Antisense RNA inhibition of rubisco activase expression. *Plant J* 5: 787–789
- Jordan DB and Chollet R (1983) Inhibition of ribulose bisphosphate carboxylase by substrate ribulose-1,5-bisphosphate. *J Biol Chem* 258: 13752–13758
- Jordan DB and Ogren WL (1981) Species variation in the specificity of ribulose carboxylase/oxygenase. *Nature* 291: 513–515
- Jordan DB and Ogren WL (1983) Species variation in kinetic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Planta* 161: 308–313
- Jordan DB and Ogren WL (1984) The CO₂/O₂ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Planta* 161: 308–313
- Kane HJ, Viil J, Entsch B, Paul K, Morell MK and Andrews TJ (1994) An improved method for measuring the CO₂/O₂ specificity of ribulosebisphosphate carboxylase-oxygenase. *Aust J Plant Physiol* 21: 449–461
- Kirschbaum UF and Farquhar GD (1984) Temperature dependence of whole leaf photosynthesis in *Eucalyptus pauciflora* Sieb.ex.Spreng. *Aust J Plant Physiol*, 11, 519–538
- Kluge M and Ting IP (1978) Crassulacean Acid Metabolism: Analysis of an Ecological Adaptation. Springer-Verlag, Berlin
- Kobza J and Seemann JR (1989) Light dependant kinetics of 2-carboxyarabinitol-1-phosphate metabolism and ribulose-1,5-bisphosphate carboxylase activity in vivo. *Plant Physiol* 89: 174–179
- Koch KE (1996) Carbohydrate modulated gene expression in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 509–540
- Krall JP, Shevelena EV and Pearcy RW (1995) Regulation of photosynthetic induction state in high and low light grown soybean and *Alocasia macrorrhiza* (L.) G.Don. *Plant Physiol* 109: 307–317
- Krapp A and Stitt M (1995) An evaluation of direct and indirect mechanisms for the ‘sink-regulation’ of photosynthesis in spinach: Changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source leaves. *Planta* 195: 313–323
- Krapp, A. Quick, W. P. and Stitt, M. (1991) Ribulose-1,5-bisphosphate carboxylase-oxygenase, other Calvin-cycle enzymes, and chlorophyll decrease when glucose is supplied

- to mature spinach leaves via the transpiration stream. *Planta* 186: 58–69
- Krapp A, Chaves MM, David MM, Rodrigues ML, Pereira JS and Stitt M (1994) Decreased ribulose 1,5-bisphosphate carboxylase/oxygenase in transgenic tobacco transformed with antisense rbcS. VII. Impact on photosynthesis and growth in tobacco growing under extreme high irradiance and high temperatures. *Plant Cell Environ* 17: 945–953
- Kuehn C, Quick WP, Schulz A, Riesmeier JW, Sonnewald U and Frommer WB (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Environ* 19: 1115–1123
- Laing WA and Christeller JT (1976) A model for the kinetics of activation and catalysis of ribulose 1,5-bisphosphate carboxylase. *Biochemistry J* 159: 563–570
- Laing WA, Ogren W and Hageman R (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2 , O_2 and ribulose-1,5 diphosphate carboxylase. *Plant Physiol* 54: 678–685
- Laisk A (1977) Kinetics of Photosynthesis and Photorespiration in C_3 Plants. Nauka, Moscow
- Laisk A and Loreto F (1996) Determining photosynthetic parameters from leaf CO_2 exchange and chlorophyll fluorescence. *Plant Physiol* 110: 903–912
- Lan Y and Mott KA (1991) Determination of apparent K_m values for RuBP carboxylase/oxygenase activase using the spectrophotometric assay of Rubisco activity. *Plant Physiol* 95: 604–609
- Lan Y, Woodrow IE and Mott KA (1992) Light-dependent changes in ribulose-1,5-bisphosphate carboxylase activity in leaves. *Plant Physiol* 99: 305–309
- Lauerer M, Saftic D, Quick WP, Labate C, Fichtner K, Schulze E-D, Rodermel SR, Bogorad L and Stitt M (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ rbcS. VI. Effect on photosynthesis in plants grown at different irradiance. *Planta* 190: 332–345.
- Leegood RC and Furbank RT (1986) Stimulation of photosynthesis by 2% oxygen at low temperature is restored by phosphate. *Planta* 168: 84–93
- Long SP (1991) Modification of the response of photosynthetic productivity to rising temperature by atmospheric CO_2 concentration: Has its importance been underestimated? Opinion. *Plant Cell Environ* 14: 729–740
- Lorimer GH, Badger MR and Andrews TJ (1976) The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications. *Biochemistry* 15: 529–536
- Lorimer GH, Chen YR and Hartman FC (1993). A role for the ε -amino group of lysine-334 of ribulose-1,5-bisphosphate carboxylase in the addition of carbon dioxide to the 2,3-enediol(ate) of ribulose 1,5-bisphosphate. *Biochemistry* 32: 9018–9024
- Makino A, Mae T and Ohira K (1985) Relation between nitrogen and ribulose-1,5-bisphosphate carboxylase/oxygenase in rice leaves from emergence through senescence. Quantitative analysis by carboxylation/oxygenation and regeneration of ribulose 1,5-bisphosphate. *Planta* 166: 414–420
- Makino A, Mae T and Ohira K (1988) Differences between wheat and rice in the enzymic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase and the relationship to photosynthetic gas exchange. *Planta* 174: 30–38
- Makino A, Nakano H, and Mae T (1994a) Effects of growth temperature on the responses of ribulose-1,5-bisphosphate carboxylase, electron transport components and sucrose synthesis enzymes to leaf nitrogen in rice and their relationships to photosynthesis. *Plant Physiol* 105: 1231–1238
- Makino A, Nakano H, and Mae T (1994b) Responses of ribulose-1,5-bisphosphate carboxylase, cytochrome *f* and sucrose synthesis enzymes in rice leaves to leaf nitrogen and their relationships to photosynthesis. *Plant Physiol* 105: 173–179
- Makino A, Sato T, Nakano H and Mae T (1997a) Leaf photosynthesis, plant growth and nitrogen allocation in rice under different irradiances. *Planta* 203: 390–398
- Makino A, Shimada T, Takumi S, Kaneko K, Matsuoka M, Shimamoto K, Nakano H, Miyao Tokutomi M, Mae T and Yamamoto N (1997b) Does a decrease in ribulose-1,5-bisphosphate carboxylase by antisense rbsc lead to higher N-use efficiency of photosynthesis under conditions of saturating CO_2 and light in rice plants. *Plant Physiology* 114: 483–491
- Martin CE (1996) Putative causes and consequences of recycling CO_2 via crassulacean acid metabolism. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 192–203. Springer-Verlag, Berlin
- Masle J, Hudson GS and Badger MR (1993) Effects of atmospheric CO_2 concentration on growth and nitrogen use in tobacco plants transformed with an antisense gene to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol* 103: 1075–1088
- Mate CJ, Hudson GS, von Caemmerer S, Evans JR and Andrews TJ (1993) Reduction of ribulose bisphosphate carboxylase activase levels in tobacco (*Nicotiana tabacum*) by antisense RNA reduces ribulose bisphosphate carboxylase carbamylation and impairs photosynthesis. *Plant Physiol* 102: 1119–1128
- Mate CJ, von Caemmerer S, Evans JR, Hudson GS and Andrews TJ (1996) The relationship between CO_2 assimilation rate, Rubisco carbamylation and Rubisco activase content in activase-deficient transgenic tobacco suggests a simple model of activase action. *Planta* 198: 604–613
- Maxwell K, von Caemmerer S and Evans JR (1997) Is a low internal conductance to CO_2 diffusion a consequence of crassulacean acid metabolism. *Aust J of Plant Physiol* 24: 777–786
- Monson KR (1989) The relative contributions of reduced photorespiration and improved wateruse efficiencies to the advantages of C_3 - C_4 photosynthesis in Flaveria. *Oecologia* 80: 215–221
- Monson KR, Ku MSB and Edwards GE (1986) Co-function of C_3 and C_4 photosynthetic pathways in C_3 , C_4 and C_3 - C_4 intermediate Flaveria species. *Planta* 168: 493–502
- Moore Bd and Seemann JR (1992) Metabolism of 2-carboxyarabinitol 1-phosphate in leaves. *Plant Physiol* 99: 1551–1555
- Moore Bd, Kobza J and Seemann JR (1991) Measurements of 2-carboxyarabinitol 1-phosphate in plant leaves by isotope dilution. *Plant Physiol* 96: 208–213
- Moore Bd, Sharkey TD, Kobza J and Seemann JR (1992) Identification and levels of 2'-carboxyarabinitol in leaves. *Plant Physiol* 99: 1546–1550
- Mott KA, Jensen RG, O'Leary JW and Berry JA (1984)

- Photosynthesis and ribulose 1,5-bisphosphate concentrations in intact leaves of *Xanthium strumarium* L. *Plant Physiol* 76: 968–971
- Mott KA, Snyder GW and Woodrow IE (1997) Kinetics of Rubisco activation as determined from gas exchange measurements in antisense plants of *Arabidopsis thaliana* containing reduced levels of Rubisco activase. *Austr J Plant Physiol* 24: 811–818
- Nie G, Hendrix DL, Webber AN, Kimball BA and Long SP (1995) Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated CO_2 concentration in the field. *Plant Physiology* 108, 975–983
- Oberhuber W and Edwards G (1993) Temperature dependence of the linkage of quantum yield of photosystem II to CO_2 fixation in C_4 and C_3 plants. *Plant Physiol* 101: 507–512
- Osmond CB (1978) Crassulacean acid metabolism: A curiosity in context. *Ann Rev of Plant Physiol* 29: 379–114
- Osmond CB and Björkman O (1975) Pathways of CO_2 fixation in the CAM plant *Kalanchoe daigremontiana*. II Effects of O_2 and CO_2 concentration on light and dark fixation. *Aust J Plant Physiol* 2: 155–162
- Osmond CB, Popp M and Robinson SA (1996) Stoichiometric nightmares: Studies of photosynthetic O_2 and CO_2 exchanges in CAM plants. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, pp 230–249. Springer-Verlag, Berlin
- Parry MAJ, Schmidt CNG, Cornelius MJ, Millard BN, Burton S, Gutteridge S, Dyer TA and Keys AJ (1987) Variations in properties of ribulose-1,5-bisphosphate carboxylase from various species related to different amino acid sequences. *J Exp Bot* 38: 1260–1271
- Pearcy RW (1988) Photosynthetic utilisation of light flecks by understorey plants. *Austr J Plant Physiol* 15: 223–238
- Pearcy RW (1990) Sunflecks and photosynthesis in plant canopies *Annu Rev Plant Physiol Mol Biol* 41: 421–453
- Pearcy RW, Krall JP and Sassenrath-Cole GF (1996) Photosynthesis in fluctuating light environments. In: Baker NR (ed) *Photosynthesis and the Environment*, pp 321–346. Kluwer Academic Publishers, Dordrecht
- Peisker M, Ticha I and Catsky J (1981) Ontogenetic changes in the internal limitations to bean leaf photosynthesis. 7. Interpretations of the linear correlation between CO_2 compensation concentration and CO_2 evolution in darkness. *Photosynthetica* 15: 161–168
- Perchorowicz TJ, Raynes DA and Jensen RG (1981) Light limitation of photosynthesis and activation of ribulose-1,5-bisphosphate carboxylase in wheat seedlings. *Proc Natl Acad Sci USA* 78: 5, 2985–2989
- Peters JL and Silverthorne J (1995) Organ-specific stability of two *Lemna* rbcS mRNAs is determined primarily in the nuclear compartment. *The Plant Cell* 7: 131–140
- Plaut Z, Mayoral ML and Reinhold L (1987) Effect of altered sink: Source ratio on photosynthetic metabolism of source leaves. *Plant Physiol.* 85: 786–791
- Portis AR Jr (1990) Rubisco activase. *Biochem Biophys Acta* 1015: 15–28
- Portis AR Jr (1992) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity. *Annu Rev Plant Physiol Plant Mol Biol* 43: 415–37
- Portis AR Jr, Salvucci ME and Ogren WL (1986) Activation of ribulosebisphosphate carboxylase/oxygenase at physiological CO_2 and ribulose bisphosphate concentrations by rubisco activase. *Plant Physiol* 82: 967–971
- Portis AR Jr, Lilley RMCC and Andrews TJ (1995) Subsaturating ribulose-1,5-bisphosphate concentration promotes inactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Studies using continuous substrate addition in the presence and absence of Rubisco activase. *Plant Physiol* 109: 1441–1451
- Price GD, Evans JR, von Caemmerer S, Kell P, Yu J-W and Badger MR (1995) Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO_2 assimilation via a reduction in ribulose bisphosphate regeneration in transgenic tobacco plants. *Planta* 195: 369–378
- Prioul JL and Reyss A (1987) Acclimation of Rubisco and mRNAs to changing irradiance in adult tobacco leaves. *Plant Physiol* 84: 1238–1243
- Prioul JL and Reyss A (1988) Rapid variations in the content of the RNA of the small subunit of ribulose-1,5-carboxylase of mature tobacco leaves in response to localised changes in light quantity. Relationships between the activity and quantity of the enzyme. *Planta* 174: 488–494
- Quick WP, Schurr U, Scheibe R, Schulze E-D, Rodermel SR, Bogorad L and Stitt M (1991a) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' rbcS. I Impact on photosynthesis in ambient growth conditions. *Planta* 183: 542–554
- Quick WP, Schurr U, Fichtner K, Schulze E-D, Rodermel SR, Bogorad L, and Stitt M (1991b) The impact of decreased Rubisco on photosynthesis growth, allocation and storage in tobacco plants which have been transformed with antisense rbcS. *Plant J* 1: 51–58
- Quick WP, Fichtner K, Schulze E-D, Wendler R, Leegood RC, Mooney H, Rodermel SR, Bogorad L and Stitt M (1992). Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' rbcS. IV Impact on photosynthesis in conditions of altered nitrogen supply. *Planta* 188: 522–531
- Raven JA (1977) Ribulose bisphosphate carboxylase activity in terrestrial plants: Significance of O_2 and CO_2 diffusion. *Curr Adv Plant Sci* 9: 579–794
- Rawsthorne S (1992) $\text{C}_3\text{-C}_4$ intermediate photosynthesis: Linking physiology to gene regulation. *Plant J* 2: 267–274
- Robinson SP and Portis JR (1988) Release of the nocturnal inhibitor, carboxyarabitol 1-phosphate from Rubisco by Rubisco activase. *FEBS Lett* 233: 413–416
- Rodermel SR, Abbott MS and Bogorad L (1988) Nuclear-organelle interactions: Nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell* 55: 673–681
- Sage RF (1990) A model describing the regulation of ribulose-1,5-bisphosphate carboxylase, electron transport and triosephosphate use in response to light and CO_2 in C_3 plants. *Plant Physiol* 94: 1728–1734
- Sage RF (1994) Acclimation of photosynthesis to increasing atmospheric CO_2 : The gas exchange perspective. *Photosynth Res* 39: 351–368
- Sage RF and Seemann JR (1993) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in response to reduced light intensity in C_4 plants. *Plant Physiol.* 102: 21–28

- Sage RF, Pearcy WR and Seemann JR (1987) The nitrogen use efficiency of C₃ and C₄ plants. III Leaf nitrogen effects on the activity of carboxylating enzymes in *Chenopodium album* (L.) and *Amaranthus retroflexus* (L.). *Plant Physiol* 85: 355–359
- Sage RF, Sharkey TD and Seemann JR (1988) The response of ribulose-1,5-bisphosphate carboxylase/oxygenase activation state and the pool sizes of photosynthetic intermediates to elevated CO₂ in *Phaseolus vulgaris*. *Plant Physiol* 174: 407–416
- Sage RF, Sharkey TD and Pearcy RW (1990a) The effect of leaf nitrogen and temperature on the CO₂ response of photosynthesis in the C₃ dicot *Chenopodium album* L. *Aust J Plant Physiol* 17: 135–148
- Sage RF, Sharkey TD and Seemann JR (1990b) Regulation of ribulose-1,5-bisphosphate carboxylase activity in response to light intensity and CO₂ in the C₃ annuals *Chenopodium album* L and *Phaseolus vulgaris* L. *Plant Physiol* 94: 1735–1742
- Sage RF, Reid CD, Moore Bd and Seemann JR (1993) Long-term kinetics of the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in plants with and without 2-carboxyarabinitol 1-phosphate. *Planta* 191: 220–230
- Salvucci ME (1989) Regulation of Rubisco activity in vivo. *Physiol Plant* 77: 164–171
- Salvucci ME and Ogren WL (1996) The mechanism of rubisco activase: Insights from studies of the properties and structure of the enzyme. *Photosynth Res* 47: 1–11
- Salvucci ME, Portis AR Jr and Ogren WL (1985) A soluble chloroplast protein catalyzes ribulose-bisphosphate carboxylase/oxygenase activation in vivo. *Photosynth Res* 7: 193–201
- Salvucci ME, Werneke JM, Ogren WL and Portis AR Jr (1987) Purification and species distribution of rubisco activase. *Plant Physiol* 84: 930–936
- Sassenrath-Cole GF, Pearcy RW and Steinmaus S (1994) The role of enzyme activation state in limiting carbon assimilation under variable light conditions. *Photosynth Res* 41: 295–304
- Sawada S, Enomoto S, Tozu T and Kasai M (1995) Regulation of the activity of ribulose-1,5-bisphosphate carboxylase in response to changes in the photosynthetic source-sink balance in intact soybean plants. *Plant Cell Physiol* 36: 551–556
- Scheible W-R, Lauerer M, Schulze E-D, Caboche M and Stitt M (1997a) Accumulation of nitrate in the shoot acts as a signal to regulate root-shoot allocation in tobacco. *Plant J* 11: 671–691.
- Scheible W-R, Gonzalez-Fontes A, Lauerer M, Muller-Röber B, Caboche M and Stitt M. (1997b) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* 9: 783–798
- Seemann, JR (1989) Light adaptation of photosynthesis and the regulation of ribulose-1,5-bisphosphate activity in sun and shade plants. *Plant Physiol* 91: 379–386
- Seemann JR and Berry JA (1982) Interspecific differences in the kinetic properties of RuBP carboxylase protein. *Carnegie Inst Washington Yearb* 81: 78–83
- Seemann JR and Sharkey TD (1986) Salinity and nitrogen effects on photosynthesis, ribulose-1,5-bisphosphate carboxylase and metabolite pool sizes in *Phaseolus vulgaris*. *Plant Physiol* 81: 788–791
- Seemann JR, Tepperman JM and Berry JA (1981). The relationship between photosynthetic performance and the levels and kinetic properties of RuBP carboxylase-oxygenase from desert winter annuals. *Carnegie Inst. Washington Yearb* 80: 67–72
- Seemann JR, Badger, MR and Berry JA (1984) Variations in the specific activity of ribulose-1,5-bisphosphate carboxylase between species utilizing differing photosynthetic pathways. *Plant Physiol* 74: 791–794
- Seemann JR, Berry JA, Freas SM and Krump MA (1985) Regulation of ribulose-1,5-bisphosphate carboxylase activity in vivo by a light modulated inhibitor of catalysis. *Proc Natl Acad Sci USA* 82: 8024–8028
- Seemann JR, Sharkey TD, Wang JL and Osmond CB (1987) Environmental effects on photosynthesis, nitrogen-use efficiency, and metabolite pools in leaves of sun and shade plants. *Plant Physiol* 84: 796–802
- Seemann JR, Kirschbaum MUF , Sharkey TD and Pearcy RW (1988) Regulation of ribulose-1,5-bisphosphate carboxylase activity in *Alocasia macrorrhiza* in response to step changes in irradiance. *Plant Physiol* 99: 227–231
- Seemann JR, Kobza J and Moore BD (1990) Metabolism of 2-carboxyarabinitol 1-phosphate and the regulation of ribulose-1,5-bisphosphate carboxylase activity. *Photosynth Res* 23: 119–130
- Servaites JC (1990) Inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase by 2-carboxyarabinitol-1-phosphate. *Plant Physiol* 92: 867–870
- Servaites JC and Geiger DR (1995) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase by metabolites. *J Exp Bot* 46 1277–1283
- Servaites JC, Perry MAJ, Gutteridge S and Keys AJ (1986) Species variation in the predawn inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol* 82: 1161–1163
- Servaites JC, Shieh WJ and Geiger DR (1991) Regulation of photosynthetic carbon reduction cycle by ribulose bisphosphate and phosphoglyceric acid. *Planta Physiol* 97: 1115–1121
- Sharkey TD (1989) Evaluating the role of Rubisoe regulation in photosynthesis in C₃ plants. *Phil Trans R Soc Lond B* 323: 435–448
- Sharkey TD (1990) Feedback limitation of photosynthesis and the physiological role of ribulose 1,5 bisphosphate carbamylation. *Bot Mag Tokyo* 2: 87–105
- Sharkey TD and Vanderveer PJ (1989) Stromal phosphate concentration is low during feedback limited photosynthesis. *Plant Physiology* 91: 679–684
- Sharkey TD, Seemann JR, and Berry JA (1986a). Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase carboxylase activity in response to changing partial pressure of O₂ and light in *Phaseolus vulgaris*. *Plant Physiol* 81: 788–791
- Sharkey TD, Stitt M, Heineke, D, Gerhardt R, Raschke K, and Heldt HW (1986b) Limitation of photosynthesis by carbon metabolism. II O₂insensitive CO₂ uptake results from limitation of triosephosphate utilisation. *Plant Physiol* 81: 1123–1129
- Silverthorne J and Tobin EM (1990) Post-transcriptional regulation of organ-specific expression of individual rbcS mRNAs in *Lemna gibba*. *Plant Cell*, 2: 1181–1190
- Smeekens S and Rook F (1997) Sweet sensations: Sugar sensing and sugar mediated signal transduction in plants. *Plant Physiol* 115: 7–13
- Somerville CR, Portis AR Jr, Ogren WL (1982) A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase in vivo. *Plant Physiol* 70: 381–387
- Spalding MH, Stumpf DK, Ku MSB, Burris RH and Edwards GE

- (1979) Crassulacean acid metabolism and diurnal variations of CO_2 and O_2 concentrations in *Sedum pectinatum* DC. Aust J of Plant Physiol 6: 557–567
- Stitt M (1996) Metabolic regulation of photosynthesis. In: Baker NR (ed) Photosynthesis and the Environment, pp 151–190. Kluwer Academic Publishers, Dordrecht
- Stitt M and Schulze D (1994) Does rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. Plant Cell Envir 17: 465–488
- Stitt M, Quick WP, Schurr U, Schulze E-D, Rodermel SR and Bogard L (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with ‘antisense’ *rbcS*. II Flux control coefficients for photosynthesis in varying light, CO_2 , and air humidity. Planta 183: 555–566
- Streusand VJ and Portis AR Jr (1987) Rubisco activase mediates ATP-dependent activation of ribulose bisphosphate carboxylase. Plant Physiol 85: 152–154
- Usuda H (1984) Variations in the photosynthesis rate and activity of photosynthetic enzymes in maize leaf tissue of different ages. Plant Cell Physiol 25: 1297–1301
- Usuda H (1985) The activation of ribulose-1,5-bisphosphate carboxylase in maize leaves in dark and light. Plant Cell Physiol 26: 1455–1463
- Usuda H (1990) Light and C_4 photosynthesis: How can the stromal system sense differences in light intensity to adjust its activities to overall flux? Bot Mag Tokyo Special Issue 2: 159–173
- Van-Oosten JJ, Besford RT (1994) Sugar feeding mimics effect of acclimation to high CO_2 -rapid down regulation of RuBisCO small subunit transcripts but not of the large subunit transcripts. J Plant Physiol 143: 306–312
- von Caemmerer S (1989) Biochemical models of photosynthetic CO_2 -assimilation in leaves of C_3 - C_4 intermediates and the associated carbon isotope discrimination. I. A model based on a glycine shuttle between mesophyll and bundle-sheath cells. Planta 178: 463–474
- von Caemmerer S (1992) Carbon isotope discrimination in C_3 - C_4 intermediates. Plant Cell Environ 15: 1063–1072
- von Caemmerer S and Edmondson DL (1986) The relationship between steady state gas exchange in vivo RuP₂ carboxylase activity and some carbon cycle intermediates in *Raphanus sativus*. Aust J Plant Physiol 13: 669–688
- von Caemmerer S and Evans JR (1991) Determination of the average partial pressure of CO_2 in chloroplasts from leaves of several C_3 plants. Aust J Plant Physiol 18: 287–305
- von Caemmerer S and Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153: 376–387
- von Caemmerer S and Farquhar GD (1984) Effects of partial defoliation, changes of irradiance during growth, short-term waterstress and growth at enhanced $p(\text{CO}_2)$ on the photosynthetic capacity of leaves of *Phaseolus vulgaris* L. Planta 160: 320–329
- von Caemmerer S, Evans JR, Hudson GS, Arvidsson YBC, Setchell BA and Andrews TJ (1992) Photosynthesis in transgenic tobacco plants with reduced amount of Rubisco contents. In: Murata N (ed) Research in Photosynthesis, Vol IV, pp 595–602. Kluwer Academic Publishers
- von Caemmerer S, Evans JR, Hudson GS and Andrews TJ (1994) The kinetics of ribulose bisphosphate carboxylase-oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. Planta 195: 88–97
- von Caemmerer S, Millgate A, Farquhar GD and Furbank RT (1997) Reduction of Rubisco by antisense RNA in the C_4 plant *Flaveria bidentis* leads to reduced assimilation rates and increased carbon isotope discrimination. Plant Physiol 113: 469–77
- von Schaewen A, Stitt M, Schmidt R, Sonnewald U and Willmitzer L (1990) Expression of a yeast derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to an accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J 9: 3033–3044
- Vu JCV, Allen LH and Bowes G (1984) Dark/light modulation of ribulose-1,5-bisphosphate carboxylase activity in plants from different photosynthetic categories. Plant Physiol 76: 843–845
- Vu JCV, Allen LH, Boote KJ and Bowes G (1997) Effects of elevated CO_2 and temperature on photosynthesis and Rubisco in rice and soybean. Plant Cell Environ 20: 68–76
- Walcroft AS, Whitehead D, Silvester WB and Kelliher FM (1997) The response of photosynthetic model parameters to temperature and nitrogen concentration in *Pinus radiata* D.Don. Plant Cell Environ 20: 1338–1348
- Wessinger ME, Edwards GE and Ku MSB (1989) Quantity and kinetic properties of ribulose 1,5-bisphosphate carboxylase in C_3 , C_4 , and $\text{C}_3\text{-C}_4$ intermediate species of *Flaveria* (Asteraceae). Plant Cell Physiol 30: 665–677
- Whitney SM, von Caemmerer S, Hudson GS, Andrews TJ (1999) Directed mutagenesis of the large subunit of tobacco Rubisco assessed in vivo. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, Vol 5, pp 3359–3362. Kluwer Academic Publishers, Dordrecht
- Wittenbach, VA (1983) Effect of pod removal on leaf photosynthesis and soluble protein composition of field-grown soybeans. Plant Physiol 73: 121–124
- Woodrow IE (1994) Optimal acclimation of C_3 photosynthetic system under enhanced CO_2 . Photosynth Res 39: 401–412
- Woodrow IE and Berry JA (1988) Enzymatic regulation of photosynthetic carbon dioxide fixation. Ann Rev Plant Physiol Mol Biol 39: 533–594
- Woodrow IE and Mott KA (1989) Rate limitation non steady state photosynthesis by ribulose-1,5-bisphosphate carboxylase in spinach. Aust J Plant Physiol 16: 487–500
- Yeoh H-H, Badger MR and Watson L (1980) Variations in $K_m(\text{CO}_2)$ of ribulose-1,5-bisphosphate carboxylase among grasses. Plant Physiol 66: 1110–1112
- Yeoh H-H, Badger MR and Watson L (1981). Variations in kinetic properties of ribulose-1,5-bisphosphate carboxylases among plants. Plant Physiol 67: 1151–1155

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Chapter 5

Photorespiration

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Summary

Photorespiration results in the light-dependent uptake of O_2 and release of CO_2 . Oxygenation of Ribulose-1,5-bisphosphate, an unavoidable process, leads to the production of glycolate-2-P. The recycling of glycolate-2-P into glycerate-3-P via the photorespiratory cycle (C₂ cycle) requires a large machinery, consisting of more than 15 enzymes and translocators, distributed over three different organelles, i.e. the chloroplast, peroxisome and mitochondrion. Complex compartmentation is an essential trait of photorespiration. The most fascinating reaction in the C₂ cycle occurs in the mitochondria when glycine molecules formed in the peroxisomes are broken down by a complex of proteins (H-, P-, T- and L-proteins) which byconcerting their activities, catalyze the oxidative decarboxylation (glycine decarboxylase) and deamination of glycine with the formation of CO_2 , NH_3 and the concomitant reduction of NAD⁺ to NADH. The remaining carbon of glycine is transferred to tetrahydropteroylpolyglutamate ($H_4PteGlu_n$ or folate) to form $CH_2H_4PteGlu_n$. The H-protein plays a pivotal role in the complete sequence of reactions since its prosthetic group (lipoic acid) interacts successively with the three other components of the complex and undergoes a cycle of reductive methylamination, methylamine transfer and electron transfer. The availability of folate to glycine decarboxylase and its recycling through serine hydroxymethyltransferase (SHMT) reaction is a critical step for glycine oxidation during photorespiration. Numerous shuttles exist to support transamination, ammonia refixation and the supply or export of reductants

generated or consumed (via malate-oxaloacetate shuttles) in the photorespiratory pathway. A porin-like channel which is anion selective, represents the major permeability pathway of the peroxisomal membrane. It is tempting to parallel the accumulation of Rubisco in the stroma of the chloroplast with the accumulation of glycine decarboxylase in the matrix of mitochondria because both enzymes reach millimolar concentrations.

I. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is present at a tremendous concentration in the stroma of the chloroplasts (0.2 g.ml^{-1} ; Rubisco accounts for up to 30% of total nitrogen in a typical C_3 leaf), is a bifunctional enzyme, catalyzing both the carboxylation and the oxygenation of ribulose-1,5-bisphosphate (RuBP) (Lorimer, 1981). The two reactions involve the competition of molecular CO_2 with O_2 for the enediol form of RuBP which is first generated at the active site of the enzyme. Once the enediol has reacted with either of the gases, the enzyme is committed to form products (Pierce et al., 1986; Bainbridge et al., 1995; Gutteridge and Gatenby, 1995; Cleland et al., 1998). The partitioning of RuBP between carboxylation and oxygenation is dependent on the kinetic parameters of Rubisco. The carboxylation of RuBP results solely in the formation of glycerate-3-P. While five-sixths of glycerate-3-P molecules thus formed are used for regeneration of RuBP, the remaining one-sixth is either exported from the chloroplasts as triose phosphate for synthesis of sucrose and other products in the cytosolic compartment or metabolized to form starch within the chloroplasts. Oxygenation of RuBP leads to the production of one molecule of glycerate-3-P and one molecule of glycolate-2-P (a two carbon compound). Glycolate-2-P, which is formed under ambient conditions at very high rates, is salvaged in the photorespiratory pathway (C_2 cycle) (Fig. 1). In the course of this pathway two molecules of glycolate-2-P are metabolized to form one molecule of glycerate-3-P (PGA) and CO_2 and these carbon compounds are used immediately for the regeneration of RuBP via the reductive pentose phosphate pathway

(C_3 cycle) without the net synthesis of triose phosphate. C_3 cycle and the C_2 cycle operate, therefore, in imperfect synchronization. In leaves under ambient conditions the rate of oxygenation to carboxylation has been estimated to be as high as 0.4 (Sharkey, 1988). Low intercellular concentrations of CO_2 , as may occur, for example, under water stress (e.g. whenever stomata are closed) can result in even higher ratios. Conversely, doubling the CO_2 concentration increases the ratio of Rubisco carboxylation/oxygenation activities by approximately two-fold, reducing photorespiration by about 50% (Sharkey, 1988).

The reaction sequence of the C_2 cycle, has been primarily resolved by Edward Tolbert and collaborators (Tolbert, 1980). Very detailed reviews of the photorespiratory pathway have been published by Chollet and Ogren (1975), Lorimer and Andrews (1981), Huang et al. (1983), Ogren (1984) and Husic et al. (1987). Some of the key regulatory properties of the photorespiratory cycle have been carefully reviewed together with the regulatory interactions that might occur between photorespiration and the Benson-Calvin cycle by Leegood et al. (1995). The value of numerous mutant plants (*Hordeum*, pea, and *Arabidopsis thaliana*) in the exquisite elucidation of the mechanism of photorespiration and its relationships with CO_2 fixation and amino acid metabolism has been highlighted by Somerville (1982), Somerville and Ogren (1982) and Blackwell et al. (1988). The mutants were unable to survive in air, but could thrive in atmospheres containing a high concentration of CO_2 (or low $[\text{O}_2]$).

II. The Photorespiratory Pathway

Glycolate-2-P formed in the chloroplasts is hydrolyzed to glycolate, as catalyzed by phosphoglycolate phosphatase present in the chloroplast stroma (Fig. 1). In tobacco this enzyme was found to be a dimer with a subunit size of about 32 kDa (Hardy and Baldy, 1986; Belanger and Ogren, 1987).

Abbreviations: FMN – flavin mononucleotide; GGAT – glutamate:glyoxylate aminotransferase; $H_4\text{PteGlu}_n$ – tetrahydropteroylpolyglutamate; PGA – glycerate-3-P; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP – ribulose-1,5-bisphosphate; SGAT – serine:glyoxylate aminotransferase; SHMT – serine hydroxymethyltransferase

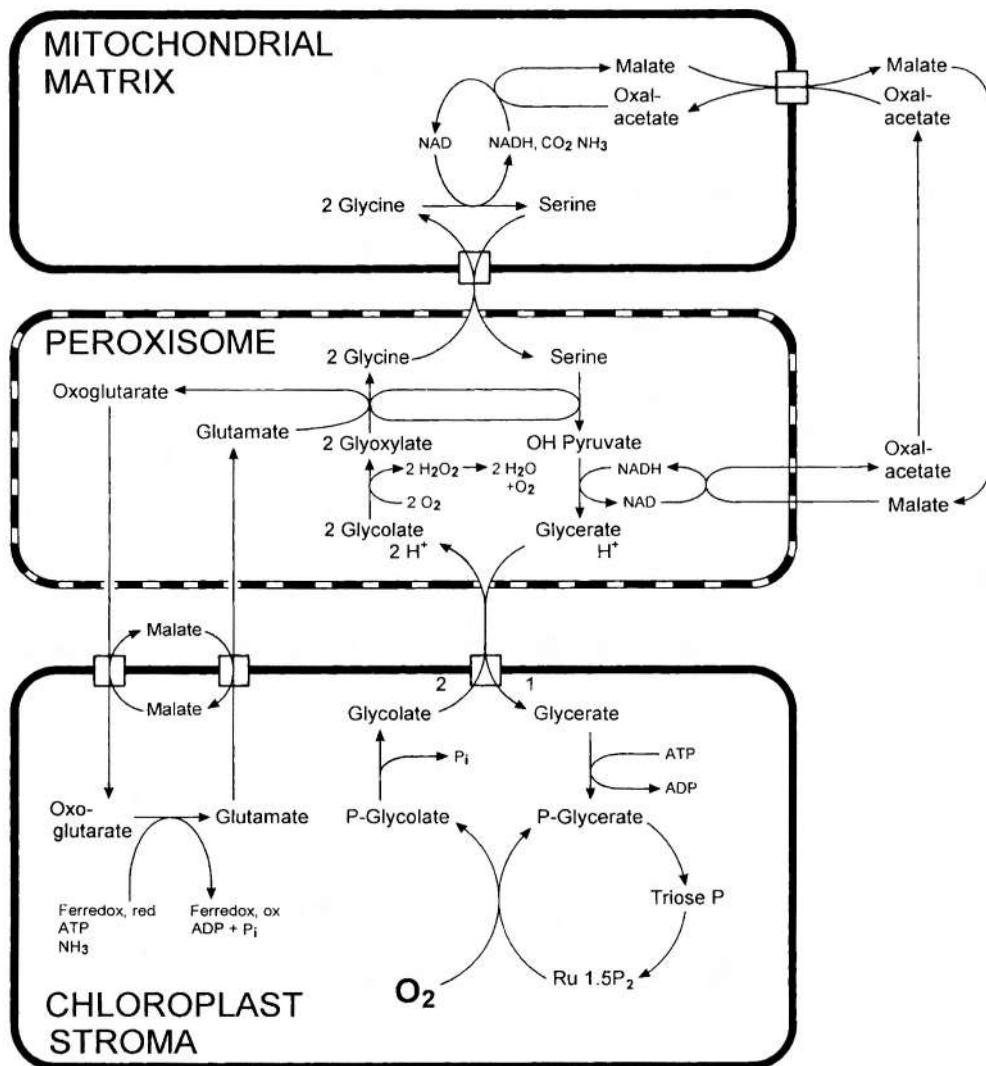


Fig 1. Scheme of the photorespiratory pathway. The recycling of glycolate-2-P into glycerate-3-P via the photorespiratory cycle (C₂ cycle) requires more than 15 enzymes and translocators distributed over three different organelles i.e. the chloroplast, peroxisome and mitochondrion. Numerous shuttles exist to support transamination, ammonia refixation and the supply or export of reductants generated or consumed (via malate-oxaloacetate shuttles) in the photorespiratory pathway

The enzyme is saturated at very low glycolate-2-P concentrations ($K_m < 100 \mu\text{M}$), which enables a very efficient hydrolysis of the glycolate-2-P formed by the oxygenase reaction (Husic et al., 1987). In mutants lacking phosphoglycolate phosphatase, characterized by Somerville and Ogren (1979), photosynthetic CO₂ assimilation was very rapidly inhibited upon exposure to air.

The glycolate is exported from the chloroplasts in exchange for glycerate (Fig. 1). This exchange is catalyzed by a translocator having similar affinities for glycolate, glyoxylate, D-glycerate and D-lactate

(Howitz and McCarty, 1985a,b). By this translocator, glycolate and glycerate can be either transported together by counter exchange for each other, or individually by H⁺ symport (or OH⁻ antiport) (Howitz and McCarty, 1991). This flexibility allows that the amount of glycerate returning to the chloroplasts to be only half of the amount of glycolate released from the chloroplasts. In addition, the activity of the glycolate-glycerate translocator appears to be high enough to account for in vivo photorespiratory carbon fluxes (Howitz and McCarty, 1985a,b).

The glycolate has to enter the peroxisomal

compartment (peroxisomes are bounded by only a single membrane) to be further metabolized (Fig. 1). The uptake of metabolites into the peroxisomes proceeds via pores (see below), probably formed by a porin present in the peroxisomal membrane. In the peroxisomal matrix the glycolate is oxidized by O_2 to glyoxylate, as catalyzed by glycolate oxidase, with the concomitant reduction of the flavin mononucleotide (FMN) prosthetic group. This enzyme with a K_m for glycolate of 0.25–0.4 mM (Husic et al., 1987) catalyzes an irreversible reaction. FMN is reoxidized by oxygen to produce hydrogen peroxide which is, in turn, decomposed by catalase, a heme-containing enzyme. Most of the hydrogen peroxide is degraded by matrix catalase, but the high K_m (millimolar range) for the enzyme could result in low residual concentrations diffusing into contact with the inner surface of the limiting peroxisomal membrane which contains an ascorbate peroxidase (Bunkelmann and Trelease, 1996) functioning as a peroxide scavenger (Asada, 1992). Glycolate oxidase [a tetramer or other oligomer with subunits of 37–40 kDa (Hall et al., 1985; Volokita and Somerville, 1987)] is one of the very few peroxisomal proteins for which a high resolution crystal structure is available (Lindqvist et al., 1991). The enzyme crystallizes in an octameric form and the subunit contains an eight-fold beta/alpha-barrel motif corresponding to the FMN domain which is also found in other FMN-dependent enzymes. The apoprotein has a different structure from the holoprotein (Sandalova and Lindqvist, 1993) indicating that the cofactor plays a determinant structural role. The amino acids involved in the structure of the active site have been studied (Stenberg et al., 1995). In addition, the signal for targeting glycolate oxidase into the plant peroxisomes resides on the mature polypeptide and its N-terminal 59 amino acids are dispensable for protein import in an ATP-dependent and temperature-dependent manner (Hornig et al., 1995; Volokita, 1991). Indeed peroxisomes do not possess DNA or a protein-synthesizing machinery and acquire proteins by import from the cytosol, usually without proteolytic processing (Lazarow and Fujiki, 1985).

The structure of catalase is far from being elucidated. Generally catalase from plant tissues is composed of four heme-containing subunits with a MW between 54 and 59 kDa. In many plants, catalase exists in multiple forms (Scandalios, 1994; Havar et al., 1996) and little is known about the molecular

basis of catalase heterogeneity. In addition, assembly of catalase holoenzymes has not been widely studied (Kunce and Trelease, 1986; Redinbaugh et al., 1990; Banjoko and Trelease, 1995). Transgenic tobacco with 0.05 to 0.15 times the catalase activity of wild type has been reported (Chamnongpol et al., 1996), and it was shown that under high photorespiratory conditions necrotic lesions were produced in leaves owing to dramatic accumulation of H_2O_2 .

The glyoxylate is converted in the peroxisomes by transamination to glycine in two alternative ways, as catalyzed by serine:glyoxylate amino transferase (SGAT) and glutamate:glyoxylate amino transferase (GGAT), normally operating in a 1:1 ratio (Fig. 1). A detailed examination of the transaminases in spinach leaf peroxisomes has been made (Rehfeld and Tolbert, 1972). Both amino transferases contain pyridoxal 5'-phosphate (PLP) as cofactor. In contrast with the animal proteins, which usually release their cofactor during the purification procedure, the PLP in plant amino transferases is generally very tightly bound to the enzyme (Givan, 1980). SGAT probably occurs as a dimer of different subunits of 45 and 47 kDa (Hondred et al., 1987). However, despite the fact that the enzyme has been partially purified from several plants, its quaternary structure is not well defined (Noguchi and Hayashi, 1980; Ireland and Joy, 1983; Hondred et al., 1987). Serine is the preferred amino donor (with K_m values between 0.6 and 2.7 mM) and glyoxylate is the preferred amino acceptor (with K_m values between 0.15 and 4.6 mM) (Nakamura and Tolbert, 1983). In contrast to GGAT, which like other amino transferases catalyzes a reversible reaction, the conversion of glyoxylate to glycine catalyzed by SGAT is virtually irreversible. GGAT [molecular weight of the native enzyme in spinach 98 kDalton (Noguchi and Hayashi, 1981)] probably also consists of two subunits. Again, the quaternary structure of the enzyme is not well defined. While SGAT is relatively specific for serine as the amino donor, GGAT is less specific. It also reacts with alanine as amino donor and with 2-oxoglutarate as acceptor (Noguchi and Hayashi, 1981; Ta and Joy, 1986). The K_m values for glyoxylate and the amino donor (glutamate or alanine) are 0.15 and 2–3 mM respectively. Interestingly decarboxylation of glyoxylate readily occurs in *Arabidopsis* and barley mutants lacking key enzymes involved in photorespiration such as serine hydroxymethyltransferase (Somerville and Ogren, 1981) and SGAT (Murray et al., 1987). Likewise decarboxylation of glyoxylate

also occurs in leaves treated with phosphinothrinicin, a potent inhibitor of glutamine synthetase (Wendler et al., 1992). Glyoxylate can be metabolized, therefore, by reactions other than transamination. However, as demonstrated by Somerville and Somerville (1983), the preferred route of glyoxylate metabolism during the course of photorespiration is amination rather than conversion to formate and CO₂.

The glycine thus formed is released from the peroxysome via pores and taken up into the mitochondria to be oxidized (Fig. 1). The most fascinating reaction in the C₂ cycle occurs in the mitochondria when glycine molecules formed in the peroxisomes are broken down by a complex of proteins (glycine decarboxylase or glycine cleavage) which by concerted their activities, catalyse the oxidative decarboxylation and deamination of gly-

cine with the formation of CO₂, NH₃ and the concomitant reduction of NAD⁺ to NADH (Douce and Neuburger, 1989; Rawsthorne et al., 1995). The remaining carbon, the methylene carbon of glycine, is then transferred to 5,6,7,8-tetrahydropteroylpolyglutamate (H₄PteGlu_n; oligo- (Imeson et al., 1990) to form N^{5,N¹⁰}-methylene-5,6,7,8-tetrahydropteroylpolyglutamate (CH₂H₄PteGlu_n) (Fig. 2). The latter compound reacts with a second molecule of glycine in a reaction catalysed by serine hydroxymethyltransferase (SHMT) to form serine (Douce and Neuburger, 1989; Rawsthorne et al., 1995) (Fig. 2). In the course of glycine decarboxylation and deamination one molecule of serine leaves the mitochondrion, and two molecules of glycine are taken up. Despite the evidence that glycine and serine transporters are common in a number of

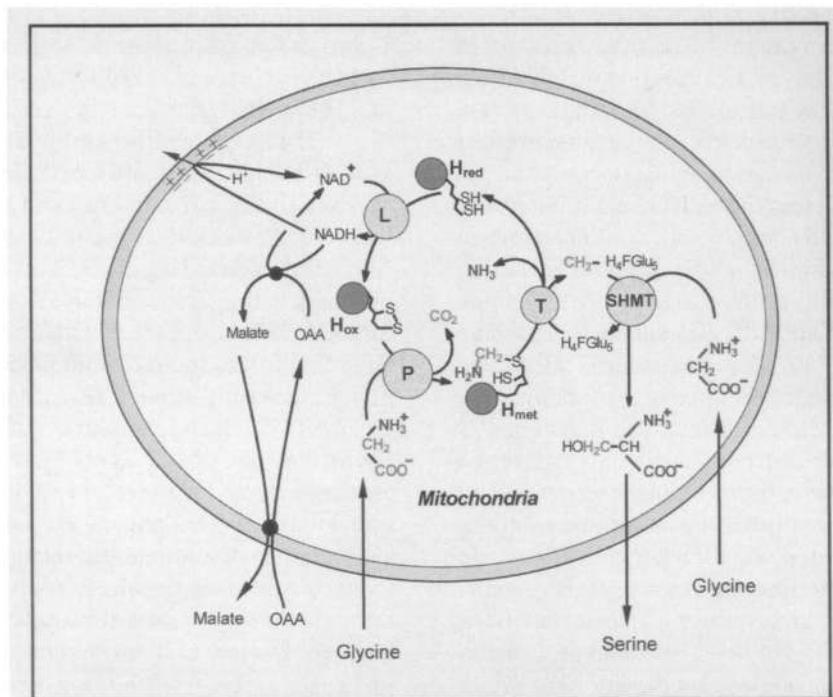


Fig. 2. Schematic representation of the glycine oxidation in green leaf mitochondria. During photorespiration glycine is cleaved in the matrix space by the glycine cleavage system containing four protein components (named P-protein, H-protein, T-protein and L-protein) to CO₂, NH₃ and N^{5,N¹⁰}-methylene-5,6,7,8-tetrahydropteroylpolyglutamate(CH₂H₄PGlu_n). The latter compound reacts with a second mole of glycine to form serine and 5,6,7,8-tetrahydropteroylpolyglutamate (H₄PGlu_n) in a reaction catalyzed by serine hydroxymethyltransferase (SHMT). H-protein plays a pivotal role in the complete sequence of reactions since its prosthetic group, 5[3-(1,2-dithiolanyl]pentanoic acid (lipoic acid), interacts successively with the three other components of the complex and undergoes a cycle of reductive methylamination, methylamine transfer and electron transfer. NADH thus produced is oxidized either by the respiratory chain tightly coupled to the synthesis of ATP or by oxaloacetate, owing to the malate dehydrogenase located in the matrix space working in the reverse direction. A rapid malate-oxaloacetate transport shuttle appears to play an important role in the photorespiratory cycle in catalyzing the transfer of reducing equivalents generated in the mitochondria during glycine oxidation to the peroxisomal compartment for the reduction of hydroxypyruvate. Note the unusual stoichiometry of two glycine molecules entering the mitochondrial matrix in exchange for one serine leaving.

biological systems, there is no consensus that the much larger flux of glycine across the inner membrane is carrier-mediated. Indeed, according to several groups influx of glycine is not related to the energy status of the mitochondrion and appears diffusional because transport rates increase linearly up to very high glycine concentrations (for a review, see Douce and Neuburger, 1989). This would allow a flexible stoichiometry for the exchange reaction that would accomodate the 2:1 ratio needed for the reaction by which glycine is metabolized. Nevertheless, because of the very high flux through the photorespiratory pathway one would expect that the transfer of glycine and serine is translocator-mediated, and there are indeed experimental results which suggest that such translocators may exist in plant mitochondria (Walker et al., 1982; Yu et al., 1983; Oliver 1987; Douce and Neuburger, 1989). For the present, the details of glycine and serine transport in green leaf mitochondria remain a mystery, and more work is needed to confirm a mechanism. The question whether both glycine and serine are transported by a single protein or by two different ones cannot be answered at present.

Serine released from the mitochondria enters the peroxisomal matrix, where it is converted to hydroxypyruvate by the above mentioned serine:glyoxylate amino transferase (Fig. 1). Since mitochondrial oxidation of two molecules of glycine yields one molecule of serine, only half of the glyoxylate can be transaminated by serine, and therefore glutamate:glyoxylate amino transferase is required for transamination of the remaining second half of the glyoxylate. Since only serine:glyoxylate amino transferase catalyzes an irreversible reaction, serine is a preferential amino donor over glutamate for glyoxylate transamination, ensuring that the serine released from the mitochondria is further metabolized in the peroxisomes. Thus the conversion of serine to hydroxypyruvate is almost unidirectional, which strongly suggests that photorespiratory serine does not exert a feed-back control of its own synthesis. Indeed the mitochondrial concentration of serine is a key regulatory point of the glycine cleavage system [serine binds to the P-protein in a manner that is competitive with the binding of glycine (K_m glycine = 6 mM; K_i serine = 4 mM)](see below) and that the rate of serine export is a critical step for glycine oxidation. Interestingly, Somerville and Ogren (1980), Murray et al. (1987) and McHale and Zelitch (1988) demonstrated that mutations blocking

serine:glyoxylate aminotransferase in various plants are conditionally lethal, permitting normal growth in a CO₂-enriched atmosphere, but converting photorespiration into a lethal process in air.

In the peroxisomes, hydroxypyruvate is reduced by NADH to glycerate in a reaction catalyzed by hydroxypyruvate reductase (Fig. 1). The enzyme has been purified to homogeneity from greening cucumber cotyledons and spinach leaves and was found to occur as a dimer consisting of subunits of 41–47 kDalton (Kohn et al., 1970; Titus et al., 1983; Greenler et al., 1989; Givan and Kleczkowski, 1992). The enzyme displays a high preference for NADH (K_m = 6 μM) rather than NADPH. The peroxisomal hydroxypyruvate reductase has also been named glyoxylate reductase, since this enzyme also catalyzes the reduction of glyoxylate. This probably does not play any role under physiological conditions, however, since the K_m of the enzyme for glyoxylate (5–15 mM) is two orders of magnitude higher than the K_m for hydroxypyruvate (62–120 μM) (for ref. see Husic et al., 1987). Estimation of the equilibrium constant ($K_{eq} = [D\text{-glyceric acid}] \text{NAD}/[\text{hydroxypyruvic acid}] \text{NADH}[\text{H}^+] = 1.6 \times 10^{12} \text{ M}^{-1}$) indicates that the reaction strongly favors glycerate production that is the forward motion of the photorespiratory cycle. NADH is generated in peroxisomes through a specific malate dehydrogenase activity. The work of Kim and Smith (1994) suggests that there is a single gene encoding glyoxysomal and peroxisomal malate dehydrogenase. Leaves also contain a powerful cytosolic hydroxypyruvate reductase activity preferring NADPH to NADH as a cofactor (Kleczkowski and Randall, 1988; Givan and Kleczkowski, 1992). This reductase could use glyoxylate as an alternative substrate. This enzyme is not believed to be involved in the main route of carbon flow through the glycolate pathway. It could serve as backup reaction utilizing hydroxypyruvate and/or glyoxylate leaked from the peroxisomes to the cytosol (Kleczkowski et al., 1990). Presumably, NADPH is generated in the cytosol through the cytosolic oxidative pentose-phosphate pathway. Convincing evidence that cytosolic hydroxypyruvate reductase can play a role in photorespiration come from Barley mutants lacking the peroxisomal NADH-dependent hydroxypyruvate reductase: in these plants hydroxypyruvate reduction proceeds, very likely, in the cytosolic compartment (Murray et al., 1989).

The glycerate formed from hydroxypyruvate reduction leaves the peroxisomes and is transported

to the chloroplast stroma, where it is phosphorylated in an almost irreversible reaction to 3-phosphoglycerate ($K_{eq} = 300$) by glycerate kinase (Fig. 1) (Kleczkowski et al., 1985). The enzyme purified from leaves is a monomer of about 40 kDa (Schmidt and Edwards, 1983) and displays K_m values of 0.25 mM and 0.21 mM for glycerate and ATP respectively (Kleczkowski et al., 1985). Its kinetic properties are consistent with a random sequential binding of the substrates. Glycerate kinase activity exceeds the maximal rate of glycerate transport and is, therefore, probably not a limiting step of the photorespiratory cycle (Husic et al., 1987).

In a plant cell, chloroplasts and mitochondria are mostly in close proximity to each other. The NH_4^+ (and or NH_3) released by mitochondrial oxidation of glycine passes through the inner membrane of mitochondria and chloroplasts. Whether this passage occurs by simple diffusion, or is brought about by specific ion channels or translocators [a gene from *Arabidopsis* has recently been identified for a high affinity ammonia transporter which is expressed as a transcript in roots (Ninnemann et al., 1994)] is still a matter of debate. Likewise one of the major unresolved aspects of the inner membranes of mitochondria and chloroplasts in all eukaryotes concerns the CO_2 permeability of the membranes. In other words it is not known which Carbon inorganic species (CO_2 , HCO_3^-) is transported in cell organelles. In this connection Rolland *et al.* (1997) using a mutant of *Chlamydomonas reinhardtii* have suggested the existence of a specific system within the plastid envelope which promotes efficient inorganic carbon uptake into chloroplasts.

The refixation of NH_4^+ in the chloroplasts proceeds in the same way as in nitrate assimilation (Lea et al., 1978; Givan et al., 1988). In mesophyll cells NH_4^+ reacts with glutamate and ATP to form glutamine and ADP, as catalyzed by glutamine synthetase, followed by glutamate synthase catalyzing the reaction of glutamine with 2-oxoglutarate with reduction by ferredoxin to two molecules of glutamate. One molecule of glutamate thus formed is exported to the peroxisomes as amino donor for glutamate:glyoxylate amino transferase in exchange for 2-oxoglutarate. The chloroplast is the sole location for glutamine synthetase activity in mesophyll cells. Likewise the ferredoxin-dependent glutamate synthase is exclusively located in the chloroplasts of C_3 and C_4 leaves (Becker et al., 1993; Lea and Forde, 1994). Mutants of Barley deficient in glutamine

synthetase (Blackwell et al., 1987; Wallsgrove et al., 1987) accumulate high amounts of ammonia in the leaves when placed in air owing to the rapid conversion of glycine to serine during the process of photorespiration in the mitochondria. On the other hand, mutants of ferredoxin-dependent glutamate synthase of various plants including *Arabidopsis*, barley and pea (for a review see Lea and Forde, 1994) accumulate glutamine instead of ammonia. The careful analysis of all these mutants has confirmed the pathway of ammonia assimilation by the glutamate synthase cycle (Lea et al., 1978) and not via glutamate dehydrogenase. It is clear, therefore, that during the course of photorespiration, 2-oxoglutarate is massively imported into the chloroplast, and glutamate, deriving from the glutamine synthetase/glutamate synthase cycle, is exported towards the peroxisome. Two different dicarboxylate antiport systems with overlapping substrate specificities are involved in this process. The 2-oxoglutarate/malate translocator imports 2-oxoglutarate in exchange for stromal malate whereas export of glutamate from the chloroplast in exchange for malate is catalyzed by the glutamate/malate translocator. Malate is the counterion for both translocators, resulting in 2-oxoglutarate /glutamate exchange without net malate import (Woo et al., 1987; Flügge et al., 1996) (Fig. 1). Weber et al. (1995) have determined the nucleotide sequence of a cDNA clone encoding the spinach chloroplast 2-oxoglutarate/malate translocator and its deduced amino acid sequence. The translocator contains a 12-helix motif and probably functions as a monomer. Surprisingly all other organellar translocators known so far, including those from mitochondria, belong to a distinct group of transporters that have 5–7 transmembrane helices functioning as dimers. The transit peptide of this translocator is extremely long and differs from those of other inner envelope membrane proteins but its import characteristics closely resemble those described for other inner envelope membrane proteins. Expression of the coding region of this translocator in yeast cells and subsequent reconstitution of the recombinant protein in liposomes revealed that this translocator mediates the exchange of 2-oxoglutarate with malate.

III. Glycine Oxidation

Rapid glycine oxidation which requires the

functioning of two enzymatic complexes (glycine decarboxylase and serine hydroxymethyltransferase) working in concert has been shown to be exclusively present in mitochondria from leaf tissues. It is a key step of the photorespiratory cycle because it results in the conversion of a two-carbon molecule into a three-carbon molecule that, thereafter, could be reintroduced in the Benson-Calvin cycle (Oliver et al., 1990; Douce et al., 1994; Oliver, 1994; Oliver and Raman, 1995; Rawsthorne et al., 1995).

A. Reaction Catalysed by the Glycine Decarboxylase Multienzyme Complex

The glycine decarboxylase multienzyme complex has been purified from plant mitochondria (Walker and Oliver, 1986; Bourguignon et al., 1988). Like its mammalian counterpart (Kikuchi and Hiraga, 1982) it contains four different components enzymes designated as the P-protein (a homodimer containing pyridoxal phosphate, 105 kDa), H-protein (a monomeric lipoamide-containing protein, 14 kDa), T-protein (a monomer catalyzing the $\text{H}_4\text{PteGlu}_n$ -dependent step of the reaction, 41 kDa), and L-protein (a homodimer containing flavin adenine dinucleotide [FAD] and a redox active cystine residue, 59 kDa). Likewise the glycine decarboxylase complex from plant leaf mitochondria is closely related to similar enzyme complexes found in bacteria such as *Ptetococcus glycinophilus* and *Athrobacter globiformis* (Klein and Sagers, 1966; Kochi and Kikuchi, 1969). The primary sequence of all these proteins from different sources were determined by cDNA and genomic cloning. The range of organisms includes *Pisum sativum* as the first plant species for which sequence data for all the subunits are available (Bourguignon et al., 1993; Bourguignon et al., 1996; Macherel et al., 1990; Kim and Oliver, 1990; Turner et al., 1992a). Together these proteins form a loose multimeric complex. Indeed all the protein components of the glycine cleavage system dissociate very easily and behave as non-associated proteins following membrane rupture after several cycles of freezing and thawing (Bourguignon et al., 1988) or acetone extraction (Walker and Oliver, 1986). Although instability of the complex has precluded isolation of the intact structure (the complex falls apart upon chromatography on gel filtration) (Bourguignon et al., 1988) it has been possible to isolate the individual component proteins and to reconstitute the functional complex that catalyses

the conversion of glycine into serine and NADH in the presence of NAD^+ , $\text{H}_4\text{PteGlu}_n$ and SHMT (Bourguignon et al., 1988). Measurements of the amount of each component protein within the matrix and analysis of the optimal subunit ratios for reconstitution suggest a subunit stoichiometry of four P-protein/27 H-protein/9 T-protein/2 L-protein.

The 13.3 kDa lipoamide-containing H-protein plays a pivotal role in the complete sequence of reactions since its prosthetic group, 5[3-(1,2-dithiolanyl]pentanoic acid (lipoic acid), interacts successively with the three other components of the complex and undergoes a cycle of reductive methylation, methylamine transfer and electron transfer (Fig. 2). The lipoyl moiety in the H-protein is attached by an amide linkage to the ϵ -amino group of a lysine residue (Lysine 63 in the 131 amino acid pea H-protein; Mérand et al., 1993) which is located in the loop of a hairpin configuration (Parès et al., 1994; Cohen-Addad et al., 1995) (Fig. 3). When the complex is diluted it tends to dissociate into its component enzymes and the H-protein acts as a mobile co-substrate that commutes between the other three proteins. The reaction begins with the amino group of glycine forming a Schiff base with the pyridoxal phosphate of the P-protein. The carboxyl group of glycine is lost as CO_2 and the remaining methylamine moiety is passed to the lipoamide cofactor of the H-protein (the methylamine intermediate, which is a rather stable structure, can be separated easily from the oxidized H-protein on ion-exchange chromatography; Neuburger et al., 1991). During the course of this reaction the lipoamide-methylamine arm formed rotates to interact readily with several specific amino acid residues located within a cleft at the surface of the H-protein (Fig. 3). Such a situation locks the methylamine group into a very stable configuration within a hydrophobic pocket preventing the non enzymatic release of NH_3 and formaldehyde due to nucleophilic attack by water molecules. In other words the H-protein not only picks up the methylamine group in one active site and delivers it to another active site, but the protein also protects the methylamine group while doing so. The lipoamide-bound methylamine group is shuttled to the T-protein where the methylene carbon is transferred to $\text{H}_4\text{PteGlu}_n$ to produce $\text{CH}_2\text{H}_4\text{PteGlu}_n$ and the amino group is released as NH_3 . During the course of this reaction the methylamine group undergoes a nucleophilic attack by the N-5 atom of the pterin ring

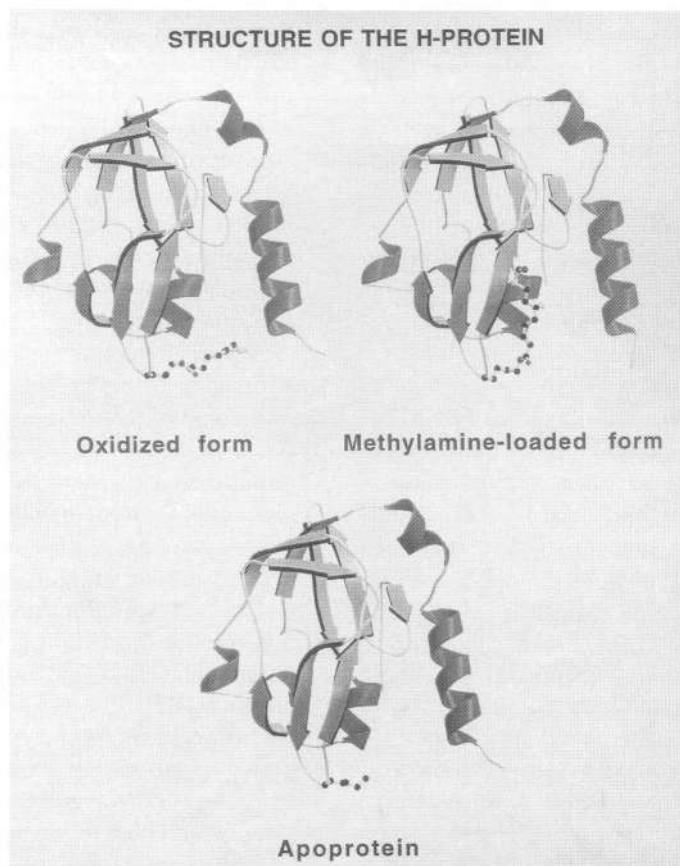


Fig. 3. Structure of the H-protein from the pea leaf glycine decarboxylase complex. Schematic ribbon representation of the overall folding of the proteins (oxidized form, methylamine-loaded form and apoprotein). The conformations of all these forms are virtually identical, consisting of seven β -strands in a sandwich structure made of two antiparallel β -sheets. Note that the lipoate cofactor attached to a specific lysine side chain is located in the loop of a hairpin configuration but following methylamine transfer it is pivoted to bind into a cleft at the surface of the H-protein. The apoprotein exhibits the same structure than the holoprotein indicating that the cofactor plays no determinant structural role. See also Color Plate 3.

of $H_4PteGlu_{\text{II}}$. Only the 6S stereoisomer of $H_4PteGlu_{\text{II}}$, the natural occurring form, is the substrate for this reaction *in vivo* (Besson et al., 1993). Finally, the L-protein (lipoamide dehydrogenase) catalyzes the regeneration of the oxidized form of lipoamide with the sequential reduction of FAD and NAD^+ . In pea leaf mitochondria, the pyruvate dehydrogenase and glycine decarboxylase complexes share the same dihydrolipoamide dehydrogenase (E3 component of pyruvate dehydrogenase, L-protein of glycine decarboxylase) and this raises some interesting questions about the regulation of synthesis and control of the distribution of this unique enzyme associated with different complexes (Bourguignon et al., 1996). All of the reactions catalysed by the glycine cleavage system are fully reversible. For example, the H- and L-proteins together catalyse the reversible exchange

of electrons between NADH and lipoamide bound to the H-protein (Neuburger et al., 1991).

Compounds that react with either the lipoamide cofactor of the H-protein (such as arsenite) or with the pyridoxal phosphate of the P-protein (such as carboxymethoxyamine, methoxylamine and acethydrazide), strongly inhibit the glycine cleavage system (Sarojini and Oliver, 1985). We believe that molecules acting at the level of the protein components of the glycine decarboxylase complex would exhibit herbicidal potency. Indeed experiments using *Arabidopsis* showed that in plants deficient in glycine decarboxylase activity, photosynthesis was not impaired in non-photorespiratory conditions, but was irreversibly inhibited in atmospheres that allowed the rapid production of glycolate by chloroplasts (Somerville and Ogren, 1982; Somerville and

Somerville, 1983). Likewise the host-selective toxin victorin [a group of closely related cyclized pentapeptides produced by the fungus *Cochliobolus victoriae*] (Macko et al., 1985)], which severely inhibits glycine decarboxylase activity [it binds strongly to the P- and H-proteins (Wolpert et al., 1994; Navarre and Wolpert, 1995)] causes symptoms typical of victoria blight disease on susceptible plants.

B. Reaction Catalysed by Serine Hydroxymethyltransferase

In leaf mitochondria, the major function of SHMT is to recycle $\text{CH}_2\text{H}_4\text{PteGlu}_n$, produced by the T-protein activity, to $\text{H}_4\text{PteGlu}_n$, to allow the continuous operation of the glycine-oxidation reaction (Bourguignon et al., 1988) (Fig. 2). SHMT has been purified from pea leaf mitochondria and is a 220 kDa homotetramer with a subunit molecular weight of 53 kDa (Bourguignon et al., 1988; Turner et al., 1992b; Besson et al., 1995). Each subunit has a single pyridoxal-phosphate bound as a Schiff base to an ϵ -amino group of a lysine residue (SHMT is yellow, exhibiting an absorption maximum at 428 nm due to the pyridoxal-P bound as a Schiff base) (Schirch, 1984). In plants, the rate constant of serine to glycine conversion by SHMT is about 15 times higher than the rate constant of the reverse direction and this shows that the reaction favors serine to glycine conversion (Besson et al. 1993). It is clear, therefore, that the SHMT reaction must be permanently pushed out of equilibrium, towards the production of serine and $\text{H}_4\text{PteGlu}_n$ to allow the whole process to take place. This was apparently the case because Rébeillé et al. (1994) have observed high $\text{CH}_2\text{H}_4\text{PteGlu}_n/\text{H}_4\text{PteGlu}_n$ ratios in the medium during the steady-state course of glycine oxidation. These very high ratios were presumably the result of the highest glycine decarboxylase activity compared with SHMT activity, that they measured in the matrix space. If the glycolate pathway is strictly cyclic as suggested by Lorimer and Andrews (1981), then all $\text{CH}_2\text{H}_4\text{PteGlu}_n$ formed upon glycine oxidation must be used for serine synthesis. In other words, $\text{CH}_2\text{H}_4\text{PteGlu}_n$ arising from glycine during operation of the glycolate pathway does not gain access to the general C_1 pool. Somerville and Ogren (1982) were among the first to show that glycine decarboxylase is not essential under nonphotorespiratory conditions.

The glutamate chain length (one to six glutamate residues) influences the affinity constant for

$\text{H}_4\text{PteGlu}_n$ and the maximal velocities displayed by SHMT and T-protein. Thus, the affinity of tetrahydrofolate for the T-protein of the glycine decarboxylase system and SHMT increases considerably with the number of glutamate residues (Besson et al., 1993). These results are consistent with analyses of plant mitochondrial folates which revealed a pool of polyglutamates (approx. 1 nmol/mg protein) dominated by tetra and pentaglutamates which accounted for approx. 25 and 55% respectively of the total pool (Besson et al., 1993). This chain may have a key role in controlling the velocity and the co-ordination of the two reactions catalysed by glycine decarboxylase and SHMT. Indeed folylpolyglutamates are known to increase the efficiency of sequential folate-dependent enzymes by enhancing the 'channeling' of intermediates between the active sites of protein complexes. The polyglutamates of leaf mitochondria are probably largely bound in a non-tight fashion to the active sites of T-protein and tetrameric SHMT, the two major folate-dependent enzymes (Rébeillé et al., 1994; Prabhu et al., 1996).

On the basis of the molecular masses of these proteins, and taking into account the fact that the tetrameric SHMT bound one folate molecule per subunit and the monomeric T-protein bound one folate molecule, Besson et al. (1993) have calculated that these two proteins were able to bind all the folate of the mitochondria (approx. 1 nmol/mg protein). There is some strong evidence that the negatively charged α -carboxyl groups of the poly- γ -glutamate chain could bind at specific points, such as basic groups of the proteins. In this context, it is notable that regions of the amino acid sequence of the pea T-protein are characterized by the alignment of basic residues (Bourguignon et al., 1993), a favorable condition for ionic interaction with the negatively charged polyglutamate chain. However, a close comparison of the known primary amino acid sequence of the T-protein (Bourguignon et al., 1993) with that of SHMT (Turner et al., 1992b), both enzymes originating from pea leaf mitochondria, did not reveal conserved folylpolyglutamate-binding consensus sequences (Bourguignon et al., 1993). Each folate-dependent enzyme might have its own specific folate-binding site, or the binding sites might rely on secondary or tertiary structures. The results of Rébeillé et al. (1994) also indicated that $\text{H}_4\text{PteGlu}_n$ binding to folate-dependent proteins significantly contributed to the protection of this readily oxidizable compound [for in vitro manipulation this molecule requires strict

anaerobic conditions (Bourguignon et al., 1988)].

C. Metabolic Control of Glycine Oxidation

The rate of glycine release during the course of photorespiration is as much as 50% of the photosynthetic rates of about $3 \mu\text{mol CO}_2 \text{ fixed mg}^{-1}$ chlorophyll min^{-1} and some five times the rate of normal tricarboxylic acid cycle activity. In order to accomplish rapid rates of glycine oxidation to cope with all the glycine molecules flooding out of the peroxisomes, the glycine decarboxylase system linked to SHMT is present at high concentrations within the mitochondrial matrix (where it comprises about half of the soluble proteins in mitochondria from fully expanded green leaves; 0.2 g per ml of matrix). Such a situation readily explains the relatively lower lipid-to-protein and cytochrome-to-protein ratios in leaf mitochondria compared to those from non-photosynthetic tissues (Gardestrom et al., 1983; Day et al., 1985a). This is in contrast with the situation observed in mammalian mitochondria where glycine decarboxylase represents a minute fraction of the total matrix protein (in humans, its absence due to genetic deficiency leads to a dramatic accumulation of glycine in the blood and to severe neurological diseases).

Glycine is rapidly oxidized by mitochondria from C₃ plant leaves, the rate of glycine oxidation being between 200–400 nmol O₂ consumed $\text{min}^{-1} \text{ mg}^{-1}$ of protein (Douce et al., 1977, Day et al., 1985a, b). The concentration of NAD⁺ within the mitochondrial matrix influences strongly the activity of NAD⁺-linked glycine decarboxylase, with higher concentrations stimulating. This can be altered by adding NAD⁺ to isolated mitochondria owing to the presence of a specific NAD⁺ carrier on the inner mitochondrial membrane (Neuburger et al., 1985). In addition glycine decarboxylase activity is not affected by light, by reversible covalent modification, by control proteins or by proteolytic activation. Its catalytic activity is only regulated by the NADH/NAD⁺ molar ratio which affects the L-protein directly and hence regeneration of the oxidized lipoyl moiety which is bound to the H-protein. Neuburger et al. (1986) have shown that NADH is a competitive inhibitor with respect to NAD⁺ ($K_m \text{ NAD}^+ = 75 \mu\text{M}$; $K_i \text{ NADH} = 15 \mu\text{M}$). This means that increasing the ratio of NADH to NAD⁺ in the matrix space will result in a logarithmic increase in inhibition of enzyme activity. Parenthetically, the activity of the pyruvate dehydrogenase complex containing lipoamide

dehydrogenase is also regulated by NADH, which is competitive with respect to NAD⁺. Consequently, NADH generated at extremely high rates during glycine oxidation must be reoxidized at equally high rates if photorespiration is to continue (see below).

The glycine metabolism of C₄ plants is almost unknown. This, at least in part, is due to the difficulties in isolating sufficient amount of mitochondria from bundle-sheath cells. However, in plants which use the C₄ dicarboxylic acid pathway, it seems that bundle-sheath cells, in contrast with mesophyll cells (Gardestrom and Edwards, 1985; Ohnishi and Kanai, 1983), have the capacity to decarboxylate glycine at high rates. Indeed, mesophyll cells of C₄ plants also lack the ability to produce glycine via photorespiration because they lack Rubisco (Edwards and Walker, 1983). This unique distribution of photorespiratory enzymes in leaves of C₄ plants has profound implications for leaf metabolism (Cheng et al., 1988).

The distribution of glycine decarboxylase in leaves of *Moricandia arvensis* (C₃-C₄ intermediate photosynthesis) resembles that in leaves of C₄ species (Rawsthorne, 1992). However the mesophyll cells of *Moricandia arvensis*, in contrast with the mesophyll cells of C₄ plants, have the capacity to catalyze the photorespiratory pathway at least as far as glycine. Consequently the major site of release of photorespiratory CO₂ in leaves of *Moricandia arvensis* is in the mitochondria of the inner wall of the bundle-sheath cells. Such a localization greatly facilitates the light-dependent recapture of photorespiratory CO₂ (The bundle-sheath cell mitochondria are in close association with overlying chloroplasts through which the CO₂ must pass).

D. Developmental Biology

Northern and western blot analyses of the expression of the glycine decarboxylase in pea revealed that the P-, H-, and T-proteins were expressed almost exclusively in the leaf tissue and a light-dependent transcriptional control of the genes encoding these proteins was suggested (Kim and Oliver, 1990; Macherel et al. 1990; Kim et al., 1991; Turner et al., 1992a; Bourguignon et al. 1992). From run-on transcription measurements, nuclei isolated from green pea leaves make about eight times more H-protein and P-protein mRNA than do nuclei isolated from etiolated plants (Srinivasan et al., 1992). However, in contrast to these proteins, the expression of the L-protein occurs in all of the tissue examined

so far because it is also the component of other mitochondrial complexes, in particular the pyruvate dehydrogenase complex (Bourguignon et al., 1996).

All the component proteins of the glycine decarboxylase complex were nuclear-encoded, synthesized on 80S cytosolic ribosomes, and imported into the mitochondria (Srinivasan et al., 1992). The accumulation of glycine decarboxylase proteins in mitochondria from green leaves could be attributed to a light-dependent transcriptional control of the genes encoding these proteins (Srinivasan et al., 1992). The analysis of steady-state levels of *rbcS* mRNA during pea leaf development revealed a pattern of expression similar to that of the P-, H-, and T-proteins (Vauclare et al., 1996). These results suggest that the genes encoding specific glycine decarboxylase proteins and photosynthetic genes such as *rbcS* follow a similar transcriptional regulation scheme, as proposed earlier by Kim and Oliver (1990). In fact several light-responsive elements (GT boxes with the GGTAA consensus core sequence) have been characterized in the promoter region of the *gdcH* gene from *Arabidopsis* (Srinivasan and Oliver, 1995). These authors also suggest that more control elements may be responsible for the constitutive low levels of gene expression noted in all non-photosynthetic tissues. In support of this suggestion, two main transcription sites were detected in the gene encoding H-protein (Macherel et al., 1992). However, such a basal expression may occur, but is certainly negligible compared with the very high level of expression in leaves required for photorespiration.

It is tempting, therefore, to parallel the accumulation of glycine decarboxylase in the matrix of mitochondria with the accumulation of Rubisco in the stroma of the chloroplast because both enzymes reach millimolar concentrations when plants are grown in light conditions. Likewise, mitochondrial SHMT (Turner et al., 1992b), peroxysomal hydroxypyruvate reductase (Berthoni and Becker, 1996), glycolate oxidase (Tsugeki et al., 1993), Ser:glyoxylate aminotransferase (Hondred et al., 1987), and chloroplastic glutamine synthetase (Edwards and Coruzzi, 1989) mRNA accumulations are strongly stimulated by white light. For example, the GUS reporter gene was used to compare the expression patterns of the pea plastidic glutamine synthetase genes in transgenic tobacco plants (Edwards et al., 1990). It was found that the promoter of glutamine synthetase gene conferred light-regulated expression

on the GUS gene in photosynthetic cells of leaves, stems and cotyledons. Interestingly, elevated CO₂ was also shown to inhibit the white-light induced accumulation of mRNA for several photorespiratory enzymes including chloroplastic glutamine synthetase (Edwards and Coruzzi, 1989) and peroxisomal hydroxypyruvate reductase (Berthoni and Becker, 1996). Does elevated CO₂ also affect the white-light-induced appearance of P-, T-, and H-mRNAs in dark-adapted leaves to allow fine tuning of the mRNA levels to provide amounts of active glycine decarboxylase appropriate for the current metabolic state?

In monocotyledonous leaves, the P-, H-, and T-proteins of glycine decarboxylase accumulate along the developmental gradient from the young tissue at the leaf base to the older tissue at the tip (Rogers et al., 1991; Tobin and Rogers, 1992). It has been found that photosynthetic and photorespiratory enzymes including glycine decarboxylase proteins, are both located in the matured region of the leaf and follow the same gradient of differentiation and a similar spatial expression. However during the course of pea leaf development, the appearance of glycine oxidation capacity seems to be closely related to the opening of the leaflets, which occurs after the 7-day stage, an event that allows the leaf to function as a morphologically efficient solar captor. This was correlated with the dramatic accumulation of the glycine decarboxylase complex proteins, which was shown to occur in preexisting mitochondria, producing an increase in their density. Thus in the case of young green leaves (7-d-old plants) or etiolated leaves, the bulk of the mitochondria remained in the top part of the self-generated Percoll gradient, whereas the mitochondria isolated from mature leaves (12-d-old plants) were found in the bottom part of the gradient (Vauclare et al., 1996). In this case, and in contrast with monocotyledonous leaves, there was a pronounced lag time between the appearance of Rubisco and glycine decarboxylase that is possibly due to a translational control that is lifted when the leaflets open. Indeed although the mRNAs for both enzymes follow similar patterns during the development of pea leaves, the biosynthesis of Rubisco starts several days before that of glycine decarboxylase. The underlying physiological meaning is that during development, photorespiration appears only after photosynthesis has reached a level of activity that requires high amounts of photorespiratory enzymes to cope with the recycling of two-carbon metabolites.

This implies the existence of post-transcriptional control of gene expression. It may result from some as yet unidentified metabolic signals from fully mature chloroplasts or peroxisomes (glycolate? glycine?). Interestingly Sinclair et al. (1996) have recently shown that glycine induces the expression of the gene encoding the P-protein together with glycine decarboxylase activity in *Saccharomyces cerevisiae*.

The synthesis of various cofactors including lipoic acid and $\text{H}_4\text{PteGlu}_n$ should keep pace with the rapid accumulation of glycine decarboxylase and SHMT within the mitochondrial matrix occurring during the course of green leaf development. In this connection, Spronk and Cossins (1972) observed that green cotyledons have much higher folate levels than etiolated leaves of similar ages. Likewise, the biosynthesis of folate compounds in pea seedlings during germination increased more rapidly in light than in dark (Okinaka and Iwai, 1970). Neuburger et al. (1996) calculated that approx. 50% of the total folate pool in pea leaves was associated with the mitochondria whereas only 6–7% was associated with chloroplasts, the remaining 40–45% of the folate pool being presumably associated with the cytosol and/or the nuclei. Considering the small size of the mitochondrial compartment, these organelles have very likely the highest folate concentration. The results of Neuburger et al. (1996) also indicated that higher plant mitochondria are a major site for tetrahydrofolate synthesis. Indeed they observed that all the enzymes required for $\text{H}_4\text{PteGlu}_n$ synthesis including dihydropterin pyrophosphokinase, dihydropteroate synthase, dihydrofolate synthase, dihydrofolate reductase and a folylpolyglutamate synthetase were located in this compartment. In other words plant mitochondria are able to synthesize $\text{H}_4\text{PteGlu}_n$ from 6-hydroxymethyl-7,8-dihydropterin, *p*-aminobenzoic acid and glutamate de novo (*p*-aminobenzoic acid is synthesized from chorismate, a branch point in the aromatic acid pathway which has been localized in plastids and cytosolic compartment. On the other hand there are actually no data available concerning the subcellular localization of 6-hydroxymethyl-7,8-dihydropterin in higher plants). Surprisingly Neuburger et al. (1996) were not able to detect these enzymes in chloroplasts, nuclei or cytosol. This observation, together with the fact that the bifunctional 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase enzyme was synthesized with a putative mitochondrial transit peptide and that a

single copy gene was observed in nuclear DNA, strongly suggests that plant mitochondria are, indeed, the unique site of $\text{H}_4\text{PteGlu}_n$ synthesis in plants (Rebeillé et al., 1997). This raises the question as to whether the metabolic flux through this biosynthetic pathway responds to the dramatic accumulation of folate-dependent enzyme (T-protein and SHMT) observed during the course of leaf development. This is an intriguing question that will need to be addressed. Such a localization also raises the problem of folate transport through the inner mitochondrial membrane and its distribution in the cytosol and plastids.

Although many organisms including plants are capable of synthesizing lipoate, the biosynthetic pathway is not fully described in any system (Parry, 1983). Very likely octanoic acid serves as a specific precursor of lipoic acid. One problem is the nature of the sulfur donor. The second problem associated with the conversion of octanoic acid into lipoic acid concerns the mechanism of the sulfur introduction process. Obviously a considerable work remains to be done in order to clarify lipoic acid biosynthesis in higher plant cells. We do believe that the synthesis of this important cofactor occurs in the mitochondrial matrix space because lipoic acid functions as the coenzyme in those mitochondrial multienzyme complexes that catalyze the oxidation of α -ketoacids and glycine. In support of this suggestion Wada et al. (1997) have demonstrated that pea leaf mitochondria contain not only acyl carrier protein (ACP), but all enzymes required de novo fatty acid synthesis. Their data suggested that a major part of the de novo synthesized fatty acids is used for biosynthesis of octanoic acid the putative precursor of lipoic acid. This last result could define the subcellular organization of lipoic acid biosynthesis in eukaryotes.

Highly specific lipoyl protein ligases are required to attach lipoate to the appropriate lysine residues of the lipoate-dependent enzymes. This raises the question as to whether plant H-protein is lipoylated within the cytosol and then translocated into its final site of accumulation, or is targeted into mitochondria as apoprotein and subsequently lipoylated. Two major arguments support the mitochondrial lipoylation hypothesis. First, bovine mitochondria contain a lipoyl-protein ligase requiring lipoyl-AMP as a substrate (Fujiwara et al., 1994). Second, Macherel et al. (1996) have constructed an expression system for mature pea H-protein in *E. coli* that allows production of large amounts of recombinant enzyme

as the apoform or the lipoylated form depending on the addition of lipoic acid to the growth medium. Very high rates of lipoylation of the H-apoprotein thus formed were measured *in vivo* and *in vitro* using intact bacteria or bacterial extract. Since the apoprotein was shown to have the same overall conformation as the native pea H-protein (Fig. 3) these results indicate that the lipoyl-protein ligase recognizes a three-dimensional structure in order to lipoylate the target lysine residue found at the extremity of the hairpin loop exactly as it is in the active protein. The lysine residue which become lipoylated is part of a conserved sequence motif V-K-A in which the two aminoacid residues V, A might be able to interact with lipoyl-protein ligase. Mutagenesis studies with the lipoyl domain from plant H-protein, however, indicate that these two aminoacid residues are not required for lipoylation (Macherel, Bourguignon and Douce, unpublished data). In conclusion the work of Macherel et al. (1996) strongly suggests that the attachment of lipoate to the appropriate lysine residue occurs once the H-protein is fully folded that is after its transport in the form of an uncoiled precursor through the mitochondrial inner membrane.

IV. Transfer of Reducing Equivalents from the Mitochondria and the Chloroplasts to the Peroxisomes

The conversion of hydroxypyruvate to glycerate in the peroxisomal matrix requires NADH as reductant. β -oxidation of fatty acids, the only pathway known in leaf peroxisomes to generate NADH (Beavers, 1979; Gerhardt, 1981), is too low in its activity to support the very high rates of hydroxypyruvate reduction required in an illuminated leaf. Peroxisomes are, therefore, dependent on the supply of reducing equivalents from outside. The question arises, whether the required NADH is delivered directly from the cytosol, or by redox shuttle, e.g. a malate-oxaloacetate shuttle.

In isolated peroxisomes, high rates of glycerate production from serine have been observed when NADH was added as reductant (Schmitt and Edwards, 1983; Reumann et al., 1994). A closer investigation of this reaction, however, revealed that under such conditions, due to lack of reductant (malate) in the peroxisomal matrix, hydroxypyruvate leaks out of the peroxisomes, and is reduced by hydroxypyruvate

Table 1. Kinetic constants malate or NADH added as reductant of glycerate formation from glycolate, serine and glutamate by isolated peroxisomes. Data of Reumann et al., (1994).

Reducant	K_M [μM]	V_{max} [$\mu\text{mol mg}^{-1} \text{protein} \cdot \text{min}^{-1}$]
Malate	990	1.48
NADH	25	0.15

reductase present in the medium. Because of the very high activity of hydroxypyruvate reductase in the peroxisomes, the artificial release of a minor proportion of this enzyme to the medium is sufficient to catalyze the observed glycerate formation in the presence of added NADH. After correcting for this external reduction, the maximal velocity of glycerate production with external NADH as reductant was found to be one order of magnitude lower than with malate as reductant (Table 1). Moreover, the K_M for NADH is approx. six times higher than the NADH concentration in the cytosol of a mesophyll cell, which is in the range of $1 \mu\text{M}$ (Heineke et al., 1991). With malate added as reductant, isolated peroxisomes produce glycerate at physiological rates. These results demonstrate that peroxisomes are supplied with reducing equivalents not by direct uptake of NADH but by indirect transfer via a malate-oxaloacetate shuttle.

Reducing equivalents can be delivered to the peroxisomes by malate-oxaloacetate shuttle from the mitochondria (Fig. 4), where an equimolar amount of the NADH required for hydroxypyruvate reduction is generated by the oxidation of glycine (Fig. 1). The NADH to NAD ratio in the mitochondria is more than 50 times higher than in the cytosol (Heineke et al., 1991) and in the peroxisomes (Raghavendra and Heldt, unpublished). NADH produced in the mitochondria can be re-oxidized rapidly by oxaloacetate (OAA), owing to the very high activity of malate dehydrogenase located in the matrix space. A very powerful phthalonate-sensitive oxaloacetate carrier has been characterized in all the plant mitochondria isolated so far (Douce and Neuburger, 1989). The rapid phthalonate-sensitive uptake of oxaloacetate is half-saturated at micromolar concentrations of oxaloacetate ($K_m_{OAA} = 5 \mu\text{M}$; $V_{max} = 2 \mu\text{mol mg}^{-1} \text{protein min}^{-1}$; the initial rates of oxaloacetate were measured using a rapid filtration technique within the first 150 ms and at 10°C (Neuburger and Douce, unpublished)). This rapid malate-oxaloacetate transport shuttle, the equivalent

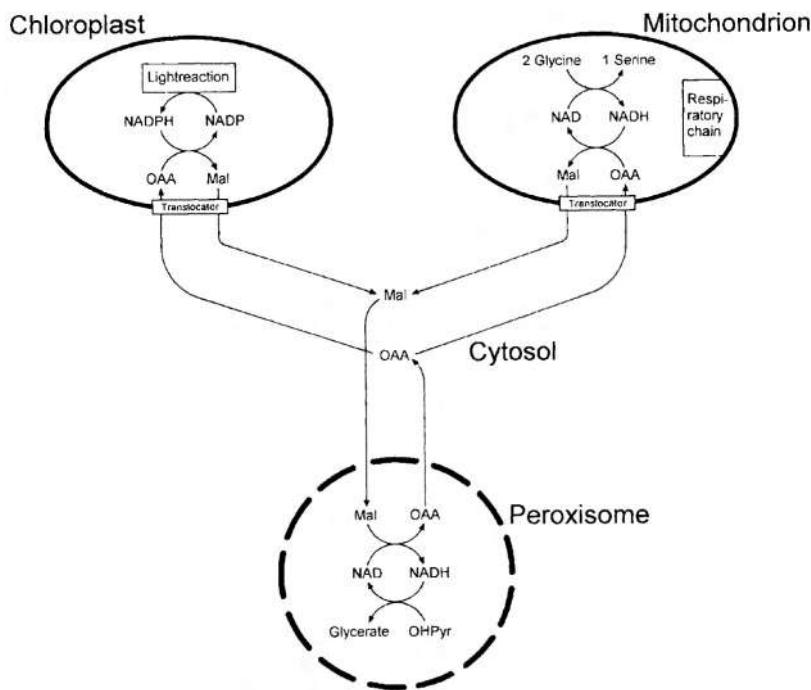


Fig 4. Scheme of redox transfer between the chloroplasts, the mitochondria and peroxisomes. Note that the conversion of hydroxypyruvate (OHPr) in the peroxisomal matrix requires NADH as reductant. Reducing equivalents are delivered to the peroxisomes by malate-oxaloacetate shuttles from mitochondria and chloroplasts

of which is not found in mammalian mitochondria, appears to play an important role in the photorespiratory cycle, in catalyzing a substantial amount of reducing equivalents generated in the mitochondria during glycine oxidation to be transferred to the peroxisomal compartment for the reduction of β -hydroxypyruvate (Ebbighausen et al., 1985). This export of reducing equivalents from the mitochondria is readily visualized when small amounts of oxaloacetate are added to leafmitochondria oxidizing glycine. At an extramitochondrial oxaloacetate concentration of $50 \mu\text{M}$ the influx of oxaloacetate is so severe that NAD^+ -linked glycine dependent O_2 consumption comes to a stop because NADH is totally sequestered by soluble matrix malate dehydrogenase (the equilibrium of the malate dehydrogenase reaction lies far towards malate formation). Alleviation of respiratory inhibition subsequently occurs when the oxaloacetate is reduced. Interestingly, in the presence of oxaloacetate, the glycine decarboxylase reaction in isolated mitochondria is able to operate faster than when the NADH is reoxidized exclusively via respiratory complex I under state 3 conditions, i.e. during the coupling of electron transport to ATP production

(Lilley et al., 1987). Because of the high activity of the mitochondrial malate-oxaloacetate shuttle (Manning and Heldt, 1993), the peroxisomal hydroxypyruvate reduction would act as a drain. The NADH generated from glycine oxidation would be totally consumed for the formation of malate and would be no longer available to support ATP synthesis by the respiratory chain. Mitochondrial ATP synthesis, however, is required during photosynthesis to supply energy to the cytosol of mesophyll cells (Krömer and Heldt, 1991b). In fact, mitochondria were found to deliver only about half of the reducing equivalents required for peroxisomal hydroxypyruvate reduction in a leaf. Chloroplasts provide the remaining portion (Krömer and Heldt, 1991; Hanning and Heldt, 1993a).

Also chloroplasts export reducing equivalents at a high rate by malate-oxaloacetate shuttle (Hatch et al., 1984) (Fig. 4). In this case the source is NADPH generated by non-cyclic electron transport, forming malate via NADP -malate dehydrogenase present in the stroma. Because of the high redox gradient between the NADPH/NADP system in the stroma and the NADH/NAD system in the cytosol, the transfer of redox equivalents from the stroma to the

cytosol has to be regulated. The site of regulation is the stromal NADP-malate dehydrogenase. The enzyme is activated by thioredoxin and therefore only active in the light (Rébeillé and Hatch, 1986a,b; Scheibe, 1987). In addition to this, increasing concentrations of NADP inhibit the reductive activation of the enzyme by thioredoxin. Thus a decrease in stromal NADP concentration, which corresponds to an increase of the chloroplastic NADPH to NADP ratio, switches on chloroplastic malate dehydrogenase. This allows the enzyme to function as a valve (Scheibe, 1990), through which excessive reducing equivalents can be exported from the chloroplast via a malate-oxaloacetate shuttle to the peroxisomes. In other words the NADPH to NADP ratio as well as the thioredoxin redox state may be critical in determining the level of NADP-malate dehydrogenase activity *in vivo* (Rébeillé and Hatch, 1986a,b).

V. The Compartmentation of Peroxisomal Metabolism

Glyoxylate and H_2O_2 , formed as intermediates of the photorespiratory pathway are toxic to the plant cell. Photosynthesis of isolated chloroplasts is completely inhibited by the addition of low concentrations of glyoxylate and H_2O_2 (Flügge et al., 1980). Both substances react with SH-groups of thioredoxin-activated enzymes. The compartmentation of the conversion of glycolate to glycine in the peroxisomes allows the interconversion of these toxic products at the site of their formation so that they do not invade other cell compartments. Such compartmentation cannot be achieved by a membrane, since membranes are normally quite permeable for H_2O_2 and monocarboxylates (undissociated form) such as glyoxylate. In fact, the compartmentation of the reactions of the photorespiratory pathway in the peroxisomes is due to specific properties of the peroxisomal matrix.

When mitochondria or chloroplasts are exposed to an osmotic shock by suspending the organelles for a short time in distilled water, the boundary membranes of these organelles disrupt and the 'soluble' proteins (stroma, matrix), are released from the disrupted organelles, and metabolic compartmentation is abolished. When peroxisomes, however, are suspended for 10 min at 20 °C in distilled water, the boundary membrane is ruptured, but the matrix

proteins remain aggregated in the form of particles in the size of peroxisomes, and the compartmentation of peroxisomal reactions is maintained (Heupel et al., 1991). Intermediates of peroxisomal metabolism, i.e. glyoxylate, H_2O_2 and hydroxypyruvate, are not released from these particles during glycolate oxidation. This finding allows the conclusion that the compartmentation of peroxisomal metabolism is not caused by the boundary membrane. Apparently the enzymes of the photorespiratory pathway are arranged in the peroxisomal matrix in the form of a multi-enzyme complex, by which the product of a reaction binds immediately to the enzyme of the following reaction and is therefore not released, in a process referred to as metabolite channeling (Heupel and Heldt, 1994).

As even in life nothing is perfect, one might assume that in spite of metabolite channeling, it may happen that a minor portion of glyoxylate or hydroxypyruvate escapes from the peroxisomes. For such a case there are rescue enzymes present in the cytosol which use NADPH to convert glyoxylate to glycolate (NADPH-glyoxylate dehydrogenase) and hydroxypyruvate into glyceral (Givan and Kleckowski, 1992).

VI. Transfer of Metabolites Across the Peroxisomal Boundary Membrane

In the photorespiratory pathway eight different metabolites have to cross the peroxisomal membrane. Previous attempts by several research groups to identify specific metabolite translocators in the peroxisomal membrane similar to those of mitochondria and chloroplasts have been unsuccessful. Instead, using the planar lipid bilayer technique, it was found that leaf peroxisomes as well as glyoxysomes contain a pore forming protein (Reumann et al., 1995, 1996, 1997). Compared with the porin from the outer membrane of mitochondria and chloroplasts, the peroxisomal pore has a comparatively small opening, since its single channel conductance (350 pS in 1 M KCl) is much lower than those formed by the porins of mitochondria and chloroplasts (Table 2). As a special feature, the peroxisomal pore contains an anion binding site with a high affinity for short chain dicarboxylates, such as malate, oxaloacetate and 2-oxoglutarate (Reumann et al., 1998). Glycolate and glyceral were also found to permeate the pore (Reumann et al., 1998).

Table 2. Comparison of single channel conductance of the peroxisomal and glyoxysomal pore forming activity with that of other eukaryotic porins in 1 M KCl

Porin from	Single channel conductance [ns]	Reference
Peroxisome (spinach)	0.35	Reumann et al., 1995
Glyoxysome (castor bean)	0.34	Reumann et al., 1997
Mitochondrion (pea)	3.7	Schmid et al., 1993
Chloroplast (spinach)	7.8	Flügge and Benz, 1984

Apparently, the peroxisomal channel is not a wide, water-filled channel like the channels of the mitochondrial and the chloroplast porins but has specific properties comparable to specific and inducible porins, which have been characterized in some gram-negative bacteria. These findings indicate that the porin-like channel which is slightly anion selective in accordance with its physiological function, represents the major permeability pathway of the peroxisomal membrane.

VII. Concluding Remarks

The oxygenation of RuBP by Rubisco is looked upon as a side reaction, which nature was unable to avoid (Andrews and Lorimer, 1978). The reason is probably that early evolution of Rubisco occurred at a time when there was no oxygen in the atmosphere. When, more than a billion years later, due to photosynthesis, oxygen appeared in the atmosphere in higher concentrations, the complexity of Rubisco protein probably made it too difficult to change the catalytic center to eliminate oxygenase activity linked to glycolate-2-P production. Rubisco inevitably initiates inefficiencies of carbon assimilation (Osmond and Grace, 1995). Unlike respiration producing ATP, photorespiration which is a consequence of the oxygenation of RuBP by Rubisco consumes ATP. The recycling of glycolate-2-P into glyceral-3-P via the C₂ cycle and then further to RuBP is not only a very costly reaction, consuming one third or more of the total energy requirement of CO₂ fixation, it also requires a large machinery, consisting of more than 15 enzymes and translocators, distributed over three different organelles, i.e. the chloroplast, peroxisome and mitochondrion (fig. 1). Photorespiratory metabolism is able to prevent the dramatic accumulation of glycolate as well as the formation of the excited triplet state of chlorophyll

and excess reactive O₂ species (superoxide radicals and singlet oxygen) which would be damaging to the chloroplast membranes (photoinhibition) (Osmond and Grace, 1995). In other words photorespiration, in concert with other reactions (Halliwell-Asada cycle), potentially mitigates chronic photoinhibition. Once glycolate-2-P is formed, the photorespiratory cycle works forward to convert all the carbon diverted out of the C₃ cycle back to photosynthesis as rapidly as possible (Lorimer and Andrew, 1981). Indeed several reactions occurring in chloroplasts and peroxisomes strongly favor product formation: that is the forward motion of the photorespiratory cycle. In addition, the rapid utilization of NADH, and the immediate utilization of NH₃ (via glutamate synthase and glutamine synthetase operating in a concerted manner) and CO₂ (via Rubisco) during the course of mitochondrial glycine oxidation continuously shift the equilibrium of both reactions catalyzed by glycine decarboxylase and SHMT towards serine production even though the reaction are readily reversible in vitro. Likewise, serine is rapidly removed from the mitochondria in order to allow the continuous production of serine by SHMT, the equilibrium of which would otherwise be unfavorable (Besson et al., 1993). Obviously the only control step in the photorespiratory cycle is at the level of competition between O₂ and CO₂ for binding to Rubisco.

It appears certain that the introduction of a genetic approach complements the more classical methods used in the study and regulation of photorespiration. Two major problems remaining for the future are: a) despite few impressive advances (Weber et al., 1996), it is fair to say that we still do not have a clear idea as to how any of these transporters directly involved in photorespiratory cycle function at the molecular level to translocate substrates across the chloroplastic and mitochondrial membranes. In addition, the purification and functional reconstitution as well as the completion of detailed kinetic analyses of most of

these transporters should be undertaken. Such information will prove valuable to our understanding of the full spectrum of molecular mechanisms that chloroplasts and mitochondria utilize to catalyze the rapid metabolite flows across the inner membranes during the course of photorespiration.

Finally, an intriguing question is how the co-ordinated control of a multitude of genes in a precise spatial and temporal program, can lead to the development of this exquisite photorespiratory cycle.

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References

- Andrews TJ and Lorimer GH (1978) Photorespiration—still unavoidable? *FEBS Lett.* 90: 1–9
- Asada K (1992) Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. *Physiol Plant* 85: 235–241
- Bainbridge G, Madgwick P, Parmar S, Mitchell R, Paul M, Pitts J and Keys AJ (1996). Engineering Rubisco to change its catalytic properties. *J Exp Bot* 46: 1269–127
- Banjoko A and Trelease RN (1995) Development and application of an *in vivo* plant peroxisome import system. *Plant Physiol* 107: 1201–1208
- Becker TW, Perrot-Rechenmann C, Suzuki A and Hirel B (1993) Subcellular and immunocytochemical localization of the enzymes involved in ammonia assimilation in mesophyll cells and bundle sheath cells of maize leaves. *Planta* 191: 129–136
- Beevers H (1979) Microbodies in plants. *Ann Rev Plant Physiol* 30: 159–193
- Belanger FC and Ogren WL (1987) Phosphoglycolate phosphatase: purification and preparation of antibodies. *Photosynth Res* 14: 3–13
- Bertoni GP and Becker WM (1996) Expression of the cucumber hydroxypyruvate reductase gene is down-regulated by elevated CO_2 . *Plant Physiol* 112: 599–605
- Besson V, Rébeillé F, Neuburger M, Douce R and Cossins EA (1993). Effects of tetrahydrofolate polyglutamates on the kinetic parameters of serine hydroxymethyltransferase from pea leaf mitochondria. *Biochem J* 292: 425–130
- Besson V, Neuburger M, Rébeillé F and Douce R (1995) Evidence for three serine hydroxymethyltransferase in green leaf cells. Purification and characterization of the mitochondrial and chloroplastic isoforms. *Plant Physiol Biochem.* 33: 665–673
- Blackwell RD, Murray AJS, Joy KW and Lea PJ (1987) Inhibition of photosynthesis in barley with decreased levels of chloroplastic glutamine synthetase. *J Exp Bot* 38: 1799–1809
- Blackwell RD, Murray AJS, Lea PJ, Kendall AC, Hall N.P., Turner JC and Wallsgrove (1988) The value of mutant unable to carry out photorespiration. *Photosynth Res* 16: 155–176
- Bourguignon J, Neuburger M and Douce R (1988). Resolution and characterization of the glycine cleavage reaction in pea leaf mitochondria. Properties of the forward reaction catalyzed by glycine decarboxylase and serine hydroxymethyltransferase. *Biochem J* 255: 169–178
- Bourguignon J, Macherel D, Neuburger M and Douce R (1992) Isolation, characterization, and sequence analysis of a cDNA clone encoding L-protein, the dihydrolipoamide dehydrogenase component of the glycine cleavage system from pea-leaf mitochondria. *Eur J Biochem* 204: 865–873
- Bourguignon J, Vauclare P, Mérand V, Forest E, Neuburger M and Douce R (1993). Glycine decarboxylase complex from higher plants: Molecular cloning, tissue distribution and mass spectrometry analyses of the T-protein. *Eur J Biochem* 217: 377–386
- Bourguignon J, Mérand V, Rawsthorne S, Forest E and Douce R (1996). Glycine decarboxylase and pyruvate dehydrogenase complexes share the same dihydrolipoamide dehydrogenase in pea leaf mitochondria: Evidence from mass spectrometry and primary structure analysis. *Biochem J* 313: 229–234
- Bunkelman J and Trelease RN (1996) Ascorbate peroxidase: A prominent membrane protein in oilseed glyoxysomes. *Plant Physiol* 110: 589–598
- Chamnongpol S, Willekens H, Langebartels C, van Montagu M, Inze D, and van Camp W (1996) Transgenic tobacco with a reduced catalase activity develops necrotic lesions and induces pathogenesis-related expression under high light. *Plant J* 10: 491–503
- Cheng SH, Moore BD, Edwards GE and Ku MSB (1988) Photosynthesis in *Flavaria brownii*, a C_4 -like species. Leaf anatomy, characteristics of CO_2 exchange, compartmentation of photosynthetic enzymes, and metabolism of $^{14}\text{CO}_2$. *Plant Physiol* 87: 867–873
- Chollet R and Ogren WL (1975) Regulation of photorespiration in C_3 and C_4 species. *Bot Rev* 41: 137–179
- Cleland WW, Andrews TJ, Gutteridge S, Hartman FC and Lorimer G (1998) Mechanism of Rubisco: The carbamate as general base. *Chem Rev* 98: 549–561
- Cohen-Addad C, Pares S, Sieker L, Neuburger M and Douce R (1995) The lipoamide arm in the glycine decarboxylase complex is not freely swinging. *Nature Struct Biol* 2: 63–68
- Day DA, Neuburger M and Douce R (1985a) Biochemical characterization of chlorophyll-free mitochondria from pea leaves. *Aust J Plant Physiol* 12: 219–228
- Day DA, Neuburger M and Douce R (1985b) Interactions between glycine decarboxylase, the tricarboxylic acid cycle and the respiratory chain in pea leaf mitochondria. *Aust J Plant Physiol* 12: 119–130
- Douce R and Neuburger M (1989) The uniqueness of plant mitochondria. *Annu Rev Plant Physiol Plant Mol Biol* 40: 371–414
- Douce R, Moore AL and Neuburger M (1977) Isolation and oxidative properties of intact mitochondria isolated from spinach leaves. *Plant Physiol* 60: 625–628
- Douce R, Bourguignon J, Macherel D and Neuburger M (1994) the glycine decarboxylase system in higher plant mitochondria: Structure, function and biogenesis *Biochem Soc Trans* 22: 184–188
- Ebbighausen H, Chen J and Heldt HW (1985) Oxaloacetate

- translocator in plant mitochondria. *Biochim Biophys Acta* 810: 184–199
- Edwards GE and Walker DA (1983) **C₃, C₄:** Mechanisms, and cellular and environmental regulation of photosynthesis. Blackwell Scientific, Oxford and London
- Edwards JW and Coruzzi GM (1989) Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. *Plant Cell* 1: 241–248
- Edwards JW, Walker EL and Coruzzi GM (1990) Cell-specific expression in transgenic plants reveals non-overlapping roles for chloroplast and cytosolic glutamine synthetase. *Proc Natl Acad Sci USA* 87: 3459–3463
- Flügge UI and Benz R (1984) Pore-forming activity in the outer membrane of the chloroplast envelope. *FEBS Lett* 169: 85–89
- Flügge UI, Freisl M and Heldt HW (1980) The mechanism of the control of carbon fixation by the pH in the chloroplast stroma. *Planta* 149: 48–51
- Flügge UI, Weber A, Fisher K, Häusler R and Kammerer B (1996) Molecular characterization of plastid transporters. *C R Acad Sci* 319: 849–852
- Fujiwara K, Okamura-Ikeda K and Motokawa Y (1994). Purification and characterization of lipoyl-AMP: **N^ε-lysine** lipoyltransferase from bovine liver mitochondria. *J Biol Chem* 269: 16605–16609
- Gardesmö P and Edwards GE (1985) Leaf mitochondria (**C₃ + C₄ + CAM**). In: Douce R and DADay (eds) Encyclopedia of Plant Physiology, Vol. 18, Higher plant cell respiration, pp 314–346. Springer-Verlag, Berlin
- Gardesmö P, Bergman A, Sahlström S, Edman K-A and Ericson I (1983). A comparison of the membrane composition of mitochondria isolated from spinach leaves and leaf petioles. *Plant Sci. Lett.* 31: 173–180
- Gerhardt B (1981). Enzyme activities of the β -oxidation pathway in leaf peroxisomes. *FEBS Lett.* 126: 71–73
- Givan CV (1980) Aminotransferases in higher plants. In: Miflin BJ (ed) The Biochemistry of Plants, Vol. 5, Amino acids and Derivatives, pp 329–357. Academic Press, New York
- Givan CV, and Kleczkowski LA (1992) The enzymic reduction of glyoxylate and hydroxypyruvate in leaves of higher plants. *Plant Physiol* 100: 552–556
- Givan CV, Joy KW and Kleczkowski LA (1988) A decade of photorespiratory nitrogen cycling. *Trends in Biochem Sci* 13: 433–437
- Greenler JM, Sloan JS, Schwartz BW and Becker WM (1989) Isolation, characterization and sequence analysis of a full length cDNA encoding NADH-dependent hydroxypyruvate reductase from cucumber. *Plant Mol Biol* 13: 139–150
- Gutteridge S and Gatenby AA (1995) Rubisco synthesis, assembly, mechanism and regulation. *Plant Cell* 7: 809–819
- Hall NP, Reggiani R and Loa G (1985). Molecular weights of glycolate oxidase from **C₃** and **C₄** plants determined during early stages of purification. *Phytochemistry* 25: 1645–1648
- Hanning I and Heldt HW (1993). On the function of mitochondrial metabolism during photosynthesis in spinach leaves (*Spinacia oleracea* L.). Partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. *Plant Physiol.* 103: 1147–1154
- Hardy P and Baldy P (1986) Corn phosphoglycolate phosphatase: purification and properties. *Planta* 168: 245–252
- Hatch MD, Dröscher L, Flügge UI and Heldt HW (1984). A specific translocator for oxaloacetate transport in chloroplasts. *FEBS Letts.* 178: 15–19
- Havir EA, Brisson LF and Zelitch I (1996) Distribution of catalase isoforms in *Nicotiana tabacum*. *Phytochemistry* 41: 699–702
- Heineke D, Riens B, Grosse H, Hoferichter P, Peter U, Flügge UI and Heldt HW (1991) Redox transfer across the inner chloroplast envelope membrane. *Plant Physiol* 95: 1131–1137
- Heupel R and Heldt HW (1994) Protein organization in the matrix of leaf peroxisomes: A multi-enzyme complex involved in photorespiratory metabolism. *Eur J Biochem*, 220: 165–172
- Heupel R, Markgraf Th, Robinson DG and Heldt HW (1991) Compartmentation studies on spinach leaf peroxisomes. Evidence for channeling of photorespiratory metabolites in peroxisomes devoid of intact boundary membrane. *Plant Physiol* 96: 971–979
- Hondred D, Wadle DM, Titus DE and Becker WM (1987) Light-stimulated accumulation of the peroxysomal enzymes hydroxypyruvate reductase and serine:glyoxylate aminotransferase and their translatable mRNAs in cotyledons and cucumber seedlings. *Plant Mol Biol* 9: 259–275
- Hornig JT, Behari R, Burke CA and Baker A (1995) Investigation of the energy requirement and targeting signal for the import of glycolate oxidase into glyoxysomes. *Eur J Biochem* 230: 157–163
- Howitz KT and McCarty RE (1985) Substrate specificity of the pea chloroplast glycolate transporter. *Biochemistry* 24: 3645–3650
- Howitz KT and McCarty RE (1985) Kinetic characteristics of the chloroplast envelope glycolate transporter. *Biochemistry* 24: 2645–2652
- Howitz KT and McCarty RE (1991) Solubilization, partial purification and reconstitution of the glycolate/glycerate transporter from chloroplast inner envelope membranes. *Plant Physiol* 96: 1060–1069
- Huang AHC, Trelease RN and Moore TS (1983) Plant peroxisomes. Academic Press, New York
- Husic DW, Husic HD and Tolbert NE (1987). The oxidative photosynthetic carbon cycle or C₂ Cycle. *CRC Critical Reviews in Plant Sciences* 5: 45–100
- Imeson HC, Zheng L and Cossins EA (1990) Fatty polyglutamate derivatives of *Pisum sativum* L. Determination of polyglutamate chain lengths by high performance liquid chromatography following conversion to p-aminobenzoyl polyglutamates. *Plant Cell Physiol.* 31: 223–231
- Ireland RJ and Joy KW (1983) Purification and properties of an asparagine aminotransferase from *Pisum sativum* leaves. *Arch Biochem Biophys.* 223: 291–296
- Kikuchi G and Hiraga K (1982) The mitochondrial glycine cleavage system—unique features of the glycine decarboxylation. *Mol. Cell. Biochem.* 45: 137–149
- Kim DE and Smith SM (1994) Expression of a single gene encoding microbody NAD-malate dehydrogenase during glyoxysome and peroxisome development in cucumber. *Plant Mol Biol* 26: 1833–1841
- Kim Y and Oliver D (1990) Molecular cloning, transcriptional characterization, and sequencing of the cDNA encoding the H-protein of the mitochondrial glycine decarboxylase complex in peas. *J Biol Chem* 265: 848–853
- Kim Y, Shah K and Oliver DJ (1991) Cloning and light-dependent expression of the gene coding for the P-protein of the glycine decarboxylase complex from peas. *Physiologia*

- Plant. 81: 501–506
- Kleczkowski LA and Randall DD (1988) Purification and characterization of a novel NADPH(NADH)-dependent hydroxypyruvate reductase from spinach leaves. Biochem J 250: 145–152
- Kleczkowski LA, Randall DD and Zahler WL (1985) The substrate specificity, kinetics, and mechanism of glycerate-3-kinase from spinach leaves. Arch Biochem Biophys 236: 185–194
- Kleczkowski LA, Edwards GE, Blackwell RD, Lea PJ and Givan CV (1990) Enzymology of the reduction of hydroxypyruvate and glyoxylate in a mutant of barley lacking peroxisomal hydroxypyruvate reductase. Plant Physiol 94: 819–825
- Klein SM and Sagers RD (1966) Glycine metabolism: 1. Properties of the system catalyzing the exchange of bicarbonate with the carboxyl group of glycine in *Peptococcus glycinophilus*. J Biol Chem 241: 197–205
- Kochi H and Kikuchi G (1969) Reactions of glycine synthesis and glycine cleavage catalyzed by extracts of *Arthrobacter globiformis* grown in glycine. Arch Biochem Biophys 132: 359–369
- Kohn LD, Warren W.A and Carroll WR (1970) The structural properties of spinach leaf glyoxylic acid reductase. J Biol Chem 245: 3821–3230
- Krömer S and Heldt HW (1991a) Respiration of pea leaf mitochondria and redox transfer between the mitochondrial and extramitochondrial compartment. Biochim Biophys Acta, 1057: 42–50
- Krömer S and Heldt HW (1991b) On the role of mitochondrial phosphorylation in photosynthesis metabolism as studied by the effect of oligomycin on photosynthesis in protoplasts and leaves of barley (*Hordeum vulgare*). Plant Physiol, 95: 1270–1276
- Kunce C and Trelease R (1986) Heterogeneity of catalase in maturing and germinating cotton seeds. Plant Physiol 81: 1134–1139
- Lazarow PB and Fujiki F (1985) Biogenesis of peroxisomes. Annu Rev Cell Biol. 1: 489–530
- Lea PJ and Forde BG (1994) The use of mutants and transgenic plants to study amino acid metabolism. Plant Cell Environ 17: 541–556
- Lea PJ, Walls-grove RM and Miflin BJ (1978) Photorespiratory cycle. Nature 257: 741–743
- Leegood RC, Lea PJ, Adcock MD and Häusler RE (1995) The regulation and control of photorespiration. J Exp Bot 46: 1397–1414
- Lilley RMC, Ebbighausen H, Heldt HW (1987) The simultaneous determination of carbon dioxide release and oxygen uptake in suspensions of plant leaf mitochondria oxidizing glycine. Plant Physiol 83: 349–353
- Lindqvist Y, Branden CI, Mathews FS and Lederer F (1991) Spinach glycolate oxidase and yeast flavocytochrome-*b*₂ are structurally homologous and evolutionarily related enzymes with distinctly different function and flavin mononucleotide binding. J Biol Chem 266: 3198–3207
- Lorimer GH (1981) The carboxylation and oxygenation of ribulose 1,5-bisphosphate: the primary events in photosynthesis and photorespiration. Annu Rev Plant Physiol 32: 349–383
- Lorimer GH and Andrews TJ (1981) The C2 chemo- and photorespiratory carbon oxidation cycle. In: Hatch MD and Boardman NK (eds) The Biochemistry of plants, Vol 8, Photosynthesis, pp 329–374. Academic Press, New York
- Macherel D, Lebrun M, Gagnon J, Neuburger M and Douce R (1990) Primary structure and expression of H-protein, a component of the glycine cleavage system of pea leaf mitochondria. Biochem J 268: 783–789
- Macherel D, Bourguignon J and Douce R (1992) Cloning of the gene (gdc H) encoding H-protein, a component of the glycine decarboxylase complex of pea (*Pisum sativum*). Biochem J 286: 627–630
- Macherel D, Bourguignon J, Forest E, Faure M, Cohen-Addad C, and Douce R (1996) Expression, lipoylation and structure determination of recombinant pea H-protein in *Escherichia coli*. Eur J Biochem 236: 27–33
- Macko V, Wolpert TJ, Acklin W, Jaun B, Seibl J, Meili J and Arigoni D (1985). Characterization of victorin C, the major host selective toxin from *Cochliobolus victorius*: structure of degradation products. Experientia 41: 1366–1370
- McHale NA, Havir EA and Zelitch I (1988) A mutant of Nicotiana sylvestris deficient in serine:glyoxylate aminotransferase activity. Theor Appl Genet 76: 71–75
- Mérand V, Forest E, Gagnon J, Monnet C, Thibault P, Neuburger M and Douce R (1993). Characterization of the primary structure of H-protein from *Pisum sativum* and location of a lipoic acid residue by combined LC-MS and LC-MS-MS. Biol Mass Spectrometry 22: 447–456
- Murray AJS, Blackwell RD, Joy KW and Lea PJ (1987) Photorespiratory N donors, aminotransferase specificity and photosynthesis in a mutant of barley deficient in serine: glyoxylate aminotransferase activity. Planta 172: 106–113
- Murray AJS, Blackwell RD and Lea PJ (1989) Metabolism of hydroxypyruvate in a mutant of Barley lacking NADH-dependent hydroxypyruvate reductase, an important photorespiratory enzyme activity. Plant Physiol 91: 395–400
- Nakamura Y and Tolbert NE (1983) Serine: glyoxylate, alanine: glyoxylate and glutamate: glyoxylate amino transferase reactions in peroxisomes from spinach leaves. J Biol Chem 258: 7631–7638
- Navarre DA and Wolpert TJ (1995) Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. Plant Cell 7: 463–471
- Neuburger M, Jourdain A and Douce R (1991) Isolation of H-protein loaded with methylamine as a transient species in glycine decarboxylase reactions. Biochem J 278: 765–769
- Neuburger M, Day DA and Douce R (1985) Transport of NAD⁺ in percoll-purified potato tuber mitochondria. Inhibition of NAD⁺ influx and efflux by N-4-azido-l-nitrophenyl-4-aminobutyryl-3'-NAD⁺. Plant Physiol 78: 405–410
- Neuburger M, Bourguignon J and Douce R (1986) Isolation of a large complex from the matrix of pea leaf mitochondria involved in the rapid transformation of glycine into serine. FEBS Lett 207: 18–22
- Neuburger M, Rébeillé F, Jourdain A, Nakamura S and Douce R (1996) Mitochondria are a major site for folate and thimidylate synthesis in plants. J Biol Chem 271: 9466–9472
- Ninnemann O, Jauniaux J-C and Frommer WB (1994) Identification of a high affinity NH₃ transporter from plants. EMBO J. 13: 3464–3471
- Noguchi T and Hayashi S (1980) Peroxisomal localization and properties of tryptophan aminotransferase in plant leaves. J Biol Chem 255: 2267–2269
- Noguchi T and Hayashi S (1981) Plant leaf alanine:2-oxoglutarate amino transferase. Peroxisomal localization and identity with

- glutamate:glyoxylate amino transferase. *Biochem J* 195:235–239
- Ogren WL (1984) Photorespiration: Pathways, regulation and modification. *Annu Rev Plant Physiol* 35: 415–442
- Okinaka O and Iwai K (1970). The biosynthesis of folic acid compounds in plants *J Vitaminol* 16: 196–209
- Oliver DJ (1987) Glycine uptake by pea leaf mitochondria: A proposed model for the mechanism of glycine-serine exchange. In: Moore AL and Beechey RB (eds) *Plant mitochondria. Structural, Functional and Physiological Aspects*, pp 219–22. Plenum Press, New York
- Oliver DJ (1994) The glycine decarboxylase complex from plant mitochondria. *Annu Rev Plant Physiol Plant Mol Biol* 45: 323–338
- Oliver DJ and Raman R (1995) Glycine decarboxylase: Protein chemistry and molecular biology of the major protein in leaf mitochondria. *J Bioenergetics Biomembranes* 27: 407–414
- Oliver DJ., Neuburger M, Bourguignon J and Douce R (1990) Glycine metabolism by plant mitochondria. *Physiologia Plantarum* 80: 487–491
- Ohnishi J and Kanai R (1983) Differentiation of photorespiratory activity between mesophyll and bundle sheath cells of C4 plants. I. Glycine oxidation by mitochondria. *Plant Cell Physiol* 24: 1411–1420
- Osmond CB and Grace SC (1995) Perspectives on photoinhibition and photorespiration in the field: Quintessential inefficiencies of the light and dark reactions of photosynthesis, *J Exp Bot* 46: 1351–1362
- Pares S, Cohen-Addad C, Sieker L, Neuburger M and Douce R (1994). X-ray structure determination at 2.6 Å-resolution of a lipoate containing protein: The H-protein of the glycine decarboxylase from pea leaves. *Proc Natl Acad Sci USA* 91: 4850–4853
- Parry R.J (1983) Biosynthesis of some sulfur-containing natural products. Investigations of the mechanism of carbon-sulfur bond formation. *Tetrahedron* 39: 1215–1238
- Pierce J, Lorimer GH and Reddy GS (1986) The kinetic mechanism of ribulose-1,5-bisphosphate carboxylase: Evidence for an ordered, sequential reaction. *Biochemistry* 25: 1636–1644
- Prabhu V, Chatson KB, Abrams GD and King J (1996) ^{13}C nuclear magnetic resonance detection of interactions of serine hydroxymethyltransferase with C_1 -tetrahydrofolate synthase and glycine decarboxylase activities in *Arabidopsis*. *Plant Physiol* 112: 207–216
- Rawsthorne S (1992) C3-C4 intermediate photosynthesis: Linking physiology to gene expression. *Plant J* 2: 267–274
- Rawsthorne S, Douce R and Oliver D (1995) The glycine decarboxylase complex in higher plant mitochondria: Structure, function and biogenesis. In: Wallsgrove RM (ed) *Amino Acids and Their Derivatives in Higher Plants*, pp 87–109. Academic Press, London
- Rébeillé F and Hatch MD (1986a) Regulation of NADP-malate dehydrogenase in C4 plants: Effect of varying NADPH to NADP ratios and thioredoxin redox state on enzyme activity in reconstituted systems. *Arch Biochem Biophys* 249: 164–170
- Rébeillé F and Hatch MD (1986b) Regulation of NADP-malate dehydrogenase in C4 plants: Relationship among enzyme activity, NADPH to NADP ratios, and thioredoxin redox states in intact amize mesophyll chloroplasts. *Arch Biochem Biophys* 249: 171–179
- Rébeillé F, Neuburger M and Douce R (1994) Interaction between glycine decarboxylase, serine hydroxymethyltransferase and tetrahydrofolate polyglutamates in pea leaf mitochondria. *Biochem J* 302: 223–228
- Rébeillé F, Macherel D, Mouillon J.M, Garin J and Douce R (1997) Folate biosynthesis in higher plants: purification and molecular cloning of a bifunctional 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase localized in mitochondria *EMBO J.* 16: 947–957
- Redinbaugh MG, Sabre M and Scandalios JG (1990) The distribution of catalase activity, isozyme protein and transcript in the tissues of the developing maize seedling. *Plant Physiol* 92: 375–380
- Rehfeld DW and Tolbert NE (1972) Aminotransferases in peroxisomes from spinach leaves. *J Biol Chem* 247: 4803–4811
- Reumann S, Heupel R, and Heldt HW (1994) Compartmentation studies on spinach leaf peroxisomes. II. Evidence for the transfer of reductant from the cytosol to the peroxisomal compartment via malate shuttle. *Planta* 193: 167–173
- Reumann S, Maier E, Benz R, and Heldt HW (1995) The membrane of leaf peroxisomes contains a porin-like channel. *J Biol Chem* 270: 17559–17565
- Reumann S, Maier E, Benz R, and Heldt HW (1996) A specific porin is involved in the malate shuttle of leaf peroxisomes. *Biochemical Society Transactions* 24: 754–757
- Reumann S, Bettermann M, Benz R and Heldt HW (1997) Evidence for the presence of a porin in the membrane of glyoxysomes of castor bean. *Plant Physiol* 115: 891–899
- Reumann S, Maier E, Heldt HW and Benz R (1998) Permeability properties of the porin of spinach leaf peroxisomes. *Eur J Biochem* 251: 359–366
- Rogers WJ, Jordan B, Rawsthorne S and Tobin AK (1991) Changes to the stoichiometry of glycine decarboxylase subunits during wheat (*Triticum aestivum* L.) and pea (*Pisum sativum* L.) leaf development. *Plant Physiol* 96: 952–956
- Rolland N, Dorne AJ, Amoroso G, Süttemeyer DF, Joyard J and Rochaix JD (1997) Disruption of the plastid *ycf10* open reading frame affect uptake of inorganic carbon in the chloroplast of *Chlamydomonas*. *EMBO J* 16: 6713–6726
- Sandalova T and Lindqvist Y (1993) Crystal structure of apoglycolate oxidase. *FEBS Lett* 327: 361–365
- Sarojini G and Oliver D (1985) Inhibition of glycine oxidation by carboxymethoxyamine, methoxylamine, and acethydrazide. *Plant physiol.* 77:786–789
- Scandalios JG (1994) Regulation and properties of plant catalases. In: Foyer CH and Mullineaux PM (eds) *Causes of Photo-oxidative Stress and Amelioration of Defense Systems in Plants*, pp 275–315. CRC Press, Boca Raton
- Scheibe R (1987) NADP-malate dehydrogenase in C₃ plants: Regulation and role of a light activated enzyme. *Physiologia Plantarum* 71: 393–400
- Scheibe R (1990) Light/dark modulation: Regulation of chloroplast metabolism in a new light. *Botanica Acta* 103: 327–334
- Schirch LV (1984) Folates in glycine and serine metabolism. In: Blakley RL and Benkovic SJ (eds) *Folates and Pterins*, Vol 1, pp 399–131. Wiley (Interscience), New York
- Schmidt A, Krömer S, Heldt HW and Benz R. (1992) Identification of two general diffusion channels in the outer membrane of pea mitochondria. *Biochim Biophys Acta*, 1112: 174–180
- Schmitt MR and Edwards, GE (1983) Provision of reductant for

- the hydroxypyruvate to glycerate conversion in leaf peroxisomes. *Plant Physiol.* 72: 728–734
- Sharkey TD (1988) Estimating the rate of photorespiration in leaves. *Physiol Plant* 73: 147–152
- Sinclair DA Hong SP and Dawes IW (1996) Specific induction by glycine of the gene for the P-subunit of glycine decarboxylase from *Saccharomyces cerevisiae*. *Mol Microbiol.* 19: 611–623
- Somerville CR (1982) Genetic modification of photorespiration. *Trends Biochem. Sci.* 7, 171–174
- Somerville, CR and Ogren WL (1979) A phosphoglycolate phosphatase-deficient mutant of *Arabidopsis*. *Nature* 280:833–836
- Somerville CR and Ogren WL (1980) Photorespiration mutants of *Arabidopsis thaliana* deficient in serine:glyoxylate aminotransferase activity. *Proc. Natl. Acad. Sci USA* 77: 2684–2687
- Somerville CR and Ogren WL (1981) Photorespiration-deficient mutants of *Arabidopsis thaliana* lacking mitochondrial serine transhydroxymethylase activity. *Plant Physiol* 67: 666–671
- Somerville CR and Ogren WL (1982) Mutants of the cruciferous plant *Arabidopsis thaliana* lacking glycine decarboxylase activity. *Biochem J* 202: 373–380
- Somerville SC and Somerville CR (1983) Effects of oxygen and carbon dioxide on photorespiratory flux determined from glycine accumulation in a mutant of *Arabidopsis thaliana*. *J Exp Bot* 34: 415–424
- Spronk A.M and Cossins EA (1972) Folate derivative of photosynthetic tissues. *Phytochemistry* 11: 3157–3165
- Srinivasan R and Oliver DJ (1995) Light-dependent and tissue specific expression of the H-protein of the glycine decarboxylase complex. *Plant Physiol* 109: 161–168
- Srinivasan R, Kraus C and Oliver DJ (1992) Developmental expression of the glycine decarboxylase multienzyme complex in greening pea leaves. In: Lambers H and van der Plas LHW (eds) Molecular, Biochemical, and Physiological Aspects of Plant Respiration, pp 323–334. SBP Academic publishing, The Hague
- Stenberg K, Clausen T, Lindqvist Y and Macheroux P (1995) Involvement of Tyr24 and Trp 108 in substrate binding and substrate specificity of glycolate oxidase. *Eur J Biochem* 228: 408–416
- Ta TC and Joy KW (1986) Metabolism of some amino acids in relation to the photorespiratory nitrogen cycle of pea leaves. *Planta*, 169: 117–122
- Titus DE, Honored D. and Becker WM (1983) Purification and characterization of hydroxypyruvate reductase from cucumber cotyledons. *Plant Physiol.* 72: 402–408
- Tobin AK and Rogers WJ (1992) Metabolic interactions of organelles during leaf development. In: Tobin AK (ed) *Plant Organelles: Compartmentation of Metabolism in Photosynthetic Tissue*, pp 293–324, Society for Experimental Biology Seminar Series 50. Cambridge University Press, Cambridge
- Tolbert NE (1980) Microbodies—peroxisomes and glyoxysomes. In Stumpf PK and Conn EE (eds) *The Biochemistry of Plants*, Vol 1, pp 359–388. Academic Press, New York
- Tsugeki R, Hara-Nishimura I, Mori H and Nishimura M (1993) Cloning and sequencing of cDNA for glycolate oxidase from pumpkin cotyledons and northern blot analysis. *Plant Cell Physiol* 34: 51–57
- Turner SR, Ireland RJ and Rawsthorne S (1992a) Cloning and characterization of the P-subunit of glycine decarboxylase from pea. *J Biol Chem* 267: 5355–5360.
- Turner SR, Ireland RJ, Morgan CL and Rawsthorne S (1992b) Identification and localization of multiple forms of serine hydroxymethyltransferase in pea (*Pisum sativum*) and characterization of a cDNA encoding a mitochondrial isoform. *J Biol Chem* 267: 13528–13534
- Vauclare P, Diallo N, Bourguignon J, Macherel D and Douce R (1996) Regulation of the expression of the glycine decarboxylase during pea leaf development. *Plant Physiol* 112: 1523–1530
- Volotika, M. (1991) The carboxy-terminal end of glycolate oxidase directs a foreign protein into tobacco leaf peroxisomes. *Plant J.* 1:361–366
- Volokita M and Sommerville CR (1987). The primary structure of spinach glycolate oxidase deduced from the cDNA sequences of a cDNA clone. *J Biol Chem* 262: 15825–15828
- Wada H, Shintani D and Ohlrogge J (1997) Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production. *Proc Natl Acad Sci USA* 94: 1591–1596
- Walker GH, Sarojini G and Oliver DJ (1982) Identification of a glycine transporter from pea leaf mitochondria. *Biochem Biophys Res Comm* 107: 856–861
- Walker JL and Oliver D (1986) Glycine decarboxylase multienzyme complex. Purification and partial characterization from pea leaf mitochondria. *J Biol Chem* 261: 2214–2221
- Walls-grove RM, Turner JC, Hall NP, Kendall AC and Bright SWJ (1987) Barley mutants lacking chloroplastic glutamine synthetase—biochemical and genetic analysis. *Plant Physiol* 83: 155–158
- Weber A, Menzlaff E, Arbinger B, Guttensohn M, Eckerskorn C and Flügge UI (1995) The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: Molecular cloning of a transporter containing a 12-helix motif and expression of the functional protein in yeast cells. *Biochemistry* 34:2621–2627
- Wendler C, Putzer A and Wild A (1992) Effect of glufosinate (phosphinothricin) and inhibitors of photorespiration on photosynthesis and ribulose-1,5-bisphosphate carboxylase activity. *J Plant Physiol* 139: 666–671
- Wolpert TJ, Navarre D.A., Moore DL and Macko V (1994) Identification of the 100-kD victorin binding protein from Oats. *Plant Cell* 6: 1145–1155
- Woo KC, Flügge UI and Heldt HW (1987) A two-translocator model for the transport of 2-oxoglutarate and glutamate in chloroplasts during ammonia assimilation in the light. *Plant Physiol.* 84: 624–632
- Yu C, Claybrook DL and Huang AHC (1983) Transport of glycine, serine and proline into spinach leaf mitochondria. *Arch Biochem Biophys* 227: 180–187

Chapter 6

Metabolite Transport Across the Chloroplast Envelope of C₃-Plants

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Summary

During photosynthesis, energy from solar radiation is used to convert atmospheric CO₂ into intermediates that are used within, as well as outside, the chloroplast for a series of metabolic pathways. These intermediates have to be exchanged between the chloroplast and the cytosol. The envelope membrane of chloroplasts contains various specific translocators that are involved in these transport processes. The basic properties of several of these translocators have been extensively studied in the past. The elucidation of the molecular structure of some of these translocators during the last years opened the way to study the function of particular translocators in more detail. This chapter focuses on the progress achieved in this field with emphasis on the characterization of different classes of phosphate translocators and on translocators that are specific for dicarboxylates, adenylates and hexoses.

I. Introduction

Communication between chloroplasts and the surrounding cytosol occurs via the plastid envelope membrane. The outer envelope possesses pore forming proteins (porins) that allow the nonspecific diffusion of hydrophilic solutes. The inner envelope membrane contains various metabolite transporters that mediate the exchange of metabolites between both compartments (Flügge and Heldt, 1991). The carbon fixed during the day can be exported from the chloroplasts into the cytosol for the synthesis of sucrose which is subsequently allocated to heterotrophic organs of the plant such as roots, seeds, fruits or tubers. When the rate of sucrose biosynthesis and export falls below that of CO_2 assimilation, the fixed carbon is retained in the chloroplasts and directed into the biosynthesis of assimilatory starch. Remobilization of starch during the following dark period and export of the starch breakdown products ensures a continuous supply of photosynthates to heterotrophic tissues in the dark. Two chloroplast translocators are involved in these processes: (1) The triose phosphate/phosphate translocator (TPT), a member of the phosphate translocator family, that exports the fixed carbon in form of triose phosphates and 3-phosphoglycerate from the chloroplasts in exchange for inorganic phosphate. This translocator is present in photosynthetic tissues only. (2) A glucose translocator exporting the products of amyloytic starch breakdown. This chapter focuses on the characterization of the different classes of phosphate translocators, the glucose translocator, and on chloroplast translocators involved in nitrogen assimilation and amino acid biosynthesis.

II. Plastidic Phosphate Translocators

A. The Triose Phosphate-3-Phosphoglycerate-Phosphate Translocator

1. Structure and Function

The triose phosphate-3-phosphoglycerate-phosphate

translocator (TPT) mediates the export of the fixed carbon (in the form of trioseP and 3-PGA) from the chloroplast into the cytosol, where it is converted into other substances, e.g. sucrose and amino acids (see Fig. 1). The inorganic phosphate released during these biosynthetic processes is reimported into the chloroplast via the TPT for the formation of ATP catalyzed by the thylakoid ATP synthase.

Sucrose and amino acids are the main products for the supply of heterotrophic tissues with photo-assimilates. They are actively loaded into the sieve element/companion cell complex by specific H^+ /symporters. The isolation and elucidation of the molecular structures of transporters for sucrose and various amino acids have been recently achieved using a yeast complementation system (for review see Frommer and Ninnemann, 1995; Ward et al., 1997; Fischer et al., 1998).

Transport mediated by the TPT was first investigated in intact chloroplasts (Fliege et al., 1978) and later, after purification and cloning of the corresponding cDNA, in artificial membranes containing the recombinant expressed protein (see below). In its functional form, the TPT is a dimer composed of two identical subunits (Flügge, 1985; Wagner et al., 1989). As substrates, the TPT accepts either inorganic phosphate or a phosphate molecule attached to the end of a three-carbon chain, such as trioseP or 3-PGA. C3-compounds with the phosphate molecule at C-atom 2 (phosphoenolpyruvate, 2-PGA) are only poorly transported (see below). Under physiological conditions, the substrates are transported via a strict 1:1 exchange. Transport proceeds via a ping-pong type of reaction mechanism, i.e. the first substrate has to be transported across the membrane and then leave the transport site before the second substrate can be bound and transported to the opposite direction (Flügge, 1992). In intact chloroplasts, unidirectional transport of phosphate can be observed but with a V_{\max} that is two to three orders of magnitude lower as compared to the antiport mode (Fliege et al., 1978; Neuhaus and Maass, 1996). Using the reconstituted system in which the concentrations of phosphate in both the internal and the external compartments are accessible to experimental variations, it could be demonstrated that the transport activity of the reconstituted TPT can reach values that exceed those measured for an antiport mode by at least one order of magnitude. It is suggested that transport under these conditions proceeds by a mechanism different from the antiport mode, probably by a (channel-like) uniport mechanism. Evidence for ion channel

Abbreviations: DiT1 – 2-oxoglutarate/malate translocator; DiT2 – glutamate/malate translocator; GS – glutamine synthetase; OEP – outer envelope protein; PEP – phosphoenolpyruvate; 2-PGA – 2-phosphoglycerate; 3-PGA – 3-phosphoglycerate; PPT – phosphoenolpyruvate/phosphate translocator; TPT – triose phosphate/3-phosphoglycerate/phosphate translocator; TrioseP – triose phosphates; VDAC – voltage-dependent anion channel

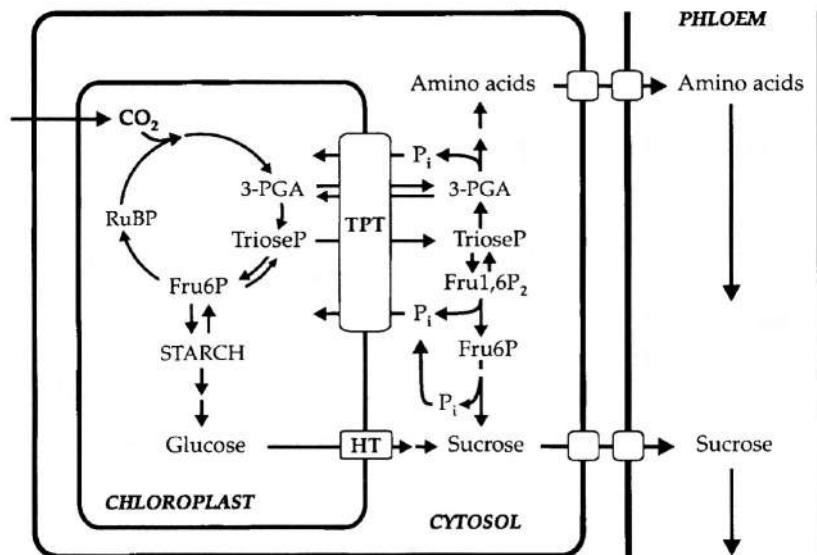


Fig. 1. Transport processes involved in the export of fixed carbon from chloroplasts. 3-PGA, 3-phosphoglycerate; Fru6P, fructose 6-phosphate; Fru1,6P₂, fructose 1,6-bisphosphate; HT, hexose translocator; P_i, inorganic phosphate; RuBP, ribulose 1,5-bisphosphate; TPT, triose phosphate/3-phosphoglycerate translocator; TrioseP, triose phosphates.

properties of the TPT is provided, (i) by a decrease of the activation energy for phosphate transport from 46 kJ/mol (antiport mode) to 18 kJ/mol (uniport mode), a value that is in the range observed for ion channels and, (ii) by measuring the TPT-mediated unidirectional transport by the patch-clamp technique (Schwarz et al., 1994). It can be concluded from these electrophysiological experiments that the PT can behave as a voltage-dependent ion channel, preferentially permeable to anions, as well as an antiporter. Different classes of transporters might share common structural motifs and may have arisen from a common ancestor. A small structural change within the translocation pathway might then allow transport via an ion channel mode or might result in strong coupling of substrate binding with conformational changes as observed for transporters operating in the antiport mode.

The spinach TPT was the first plant membrane transport system for which the primary sequence could be determined (Flügge et al., 1989). Meanwhile, TPT-sequences from various plants are available, e.g., those from *Arabidopsis*, pea, potato, maize, *Flaveria* and tobacco that all have a high similarity to each other (Knight and Gray, 1994; Fischer et al., 1994a, 1997). All TPTs are nuclear-encoded and possess N-terminal transit peptides (about 80 amino acid residues) that direct the adjacent protein to the chloroplasts (Brink et al., 1995; Knight and Gray, 1995). Import of the translocator into chloroplasts is

driven by ATP and depends on the translocation machinery of the envelope membrane. The mature part of these transporters consists of about 330 amino acid residues per monomer, is highly hydrophobic, and contains information (envelope insertion signals) for the integration of the protein within the inner envelope membrane. It is composed of five to seven hydrophobic segments in an α -helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic loops. The TPT thus belongs to the group of translocators with a 6 + 6 helix folding pattern, as is the case for mitochondrial carrierproteins (Maloney, 1990). Based on a tentative model for the arrangement of the TPT in the membrane, it is probable that all twelve α -helices take part in forming a hydrophilic translocation channel through which the substrates are transported across the membrane. According to the proposed three-dimensional structural model of the TPT (Wallmeier et al., 1992), an asymmetry in the structure of the substrate binding sites on either membrane side is suggested. This structural asymmetry was verified experimentally by demonstrating that only the cytosolic side of the translocator is accessible to specific inhibitors, namely pyridoxal 5'-phosphate and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (Flügge, 1992). Bisubstrate initial velocity studies of the reconstituted translocator further showed that the TPT possesses a higher affinity (about five-fold) toward its substrates on the cytosolic side

compared to the stromal side.

The availability of the TPT-cDNA also opened the way to produce the corresponding protein in heterologous expression systems. Since prokaryotic systems failed to express the hydrophobic translocator protein, yeast cells (*Schizosaccharomyces pombe*) were used. It could be shown that the TPT was produced in yeast cells in a functional state representing about 1% of the total protein (Loddenkötter et al., 1993). To facilitate the isolation of the TPT from the cells, a recombinant protein was engineered containing a C-terminal tag of six consecutive histidine residues (His₆-tag). This was initially achieved by overlapping PCR; however, in the meanwhile, various commercially available vectors can be used for introducing different kinds of tags. Despite the low level of expression, the TPT-His₆ protein could be purified to apparent homogeneity by a single chromatography step on metal-affinity columns. It could be demonstrated that the recombinant protein possessed transport characteristics almost identical to those of the authentic chloroplast protein (Loddenkötter et al., 1993).

2. Physiological Role of the TPT

The TPT is an important link between metabolism in the chloroplast and the cytosol. It can be calculated that only about 10% of the total transport activity of the TPT can be used for (productive) net trioseP export in order to provide the carbon skeleton for further biosynthetic processes. Since both the cytosol and the stroma contain trioseP, 3-PGA and inorganic phosphate competing for transport in either direction, it appears feasible that most of the TPT activity is used for catalyzing (non-productive) homologous exchanges (Flügge, 1987).

From subcellular metabolite concentrations in intact spinach leaves, it has been proposed that the TPT can exert a kinetic limitation during sucrose biosynthesis in vivo (Gerhardt et al., 1987). We have assessed the role of the TPT on photosynthetic metabolism by means of antisense repression of its corresponding mRNA. Transgenic potato (*Solanum tuberosum* L.) plants were created that showed a dramatically reduced expression of the TPT at the RNA level (Riesmeier et al., 1993). In all transgenic plants analyzed, the maximum reduction of both the amount and the activity of the TPT was about 25–30%. Under ambient CO₂ and light conditions, there was no significant effect on photosynthetic rates (Table 1), growth, and tuber development. However, even under ambient conditions, the reduction of the TPT activity resulted in a marked perturbation of leaf metabolism. Most remarkably, the content of 3-PGA was greatly increased compared to the corresponding value in wild-type plants and this increase was found to be almost entirely due to an increase of the stromal 3-PGA content with the cytosolic values remaining almost unchanged. Also, the sum of organic phosphates (3-PGA plus sugar phosphates) was about twice as large in the transformants as in the wild-type plants. This should result in a large decrease of the stromal content of inorganic phosphate since the TPT mediates a strict counter-exchange of substrates. Thus, reduction of the TPT activity in vivo resembles the situation of chloroplasts performing photosynthesis under phosphate limitation (Heldt et al., 1977): the increase in 3-PGA (and the 3-PGA/phosphate ratio) results in a large increase of starch synthesis due to the allosteric activation of the ADP-glucose pyrophosphorylase (AGPase) (Preiss and Levi, 1980). Indeed, both at the end of the day and end of the night the starch content

Table 1. Antisense repression of the triose phosphate/phosphate translocator (TPT) in transgenic potato plants. Rates of phosphate transport and photosynthesis, and starch levels in wild-type and transformant leaves. Data from Riesmeier et al. (1993).

	Plants			
	Wild-type	TPT 1	TPT 7	TPT 39
Uptake of [³² P] phosphate into chloroplasts (% of V _{max})	= 100	72	69	83
Photosynthetic rate, ambient conditions (mmol O ₂ per m ² per h)	17.5	21.9	14.3	19.8
Starch (end of the day) (μatom C per mg Chl.)	761	1310	2101	1336
Starch (end of the night) (μatom C per mg Chl.)	330	654	1291	505

in transformants was much greater than in wild-type plants (Table 1). It appears that due to the restriction of the TPT, the daily assimilated carbon is mainly maintained within the plastids in which it is directed into the accumulation of starch (Table 2). Since there was no obvious effect on plant growth, the transformants were obviously able to efficiently compensate for their deficiency in TPT activity. A detailed analysis of the transgenic potato plants indeed showed that these plants mobilized and exported the major part of the daily accumulated carbon during the following night period (Heineke et al., 1994). By contrast, wild-type plants generally export the major part of the fixed carbon during ongoing photosynthesis (Table 2).

The question arises how the mobilization and export of photoassimilates is achieved in the antisense TPT plants during the night. Mobilization of starch and export of the resulting breakdown products can, in principle, either be performed via the TPT (after phosphorolytic starch breakdown resulting in trioseP) or via a hexose translocator (after amyloytic starch breakdown resulting in hexoses, see Fig. 1). Due to the reduced activity of the TPT, the mobilized carbon cannot be exported (at least not at high rates) from the chloroplasts as C₃-compounds that are end-products from phosphorylytic starch breakdown. Obviously, the transformants follow the hydrolytic starch breakdown pathway and preferentially export the mobilized carbon as hexoses via the hexose translocator. This is in line with the observation that transgenic antisense TPT plants from tobacco showed increased rates of amyloytic starch mobilization which process is accompanied by a higher transport capacity for glucose across the envelope membrane (Häusler et al., 1998). It can be concluded that plants can efficiently compensate for their deficiency in TPT activity provided that a carbon sink (i.e., starch)

can be generated during photosynthesis which can be mobilized during the following night period. Interestingly, transgenic potato plants with a reduced ability to synthesize assimilatory starch (e.g., by anti-sense repression of the AGPase) also show no effect on growth and productivity. Export of the daily fixed carbon via the TPT is obviously so efficient in these plants that heterotrophic tissues can be supplied sufficiently with reduced carbon. However, if both starch formation and the activity of the TPT are reduced, the corresponding transformants show a dramatic phenotype (Hattenbach et al., 1997). On the one hand, the transformants are not able to export sufficient amounts of fixed carbon during the day (due to antisense repression of the TPT) and on the other hand, they do not have a carbon store which they could use during the dark period (due to antisense repression of the AGPase).

B. The Phosphoenolpyruvate/Phosphate Translocator

1. Structure and Function

Another type of phosphate translocator has been described recently (Fischer et al., 1997). This transporter, named PPT (phosphoenolpyruvate/phosphate translocator) was initially identified in non-green tissues, but it is also present in photosynthetic tissues. As in the case of the TPT, the PPTs are nuclear-encoded and consist of a transit peptide (about 80 amino acid residues) and a mature part (about 320 amino acid residues). Sequence comparisons between the TPT and the PPTs from different plants and plant tissues showed that the PPTs represent a second class of phosphate translocators (Table 3). Within each group of transporters, the mature proteins are highly homologous to each other. In contrast, the

Table 2. Antisense repression of the triose phosphate/phosphate translocator (TPT) in transgenic potato plants. Effect of antisense repression of the TPT on the accumulation and export of photoassimilates. TPT39, antisense TPT potato plant. Data from Heineke et al. (1994).

	Plants	
	Wild-type	TPT 39
1. Amount of assimilate		
a) assimilated during the day	1085	975
b) accumulated during the day	466 (= 43%)	866 (= 89%)
2. Rate of assimilate export		
a) during the day	52 (= 57%)	9 (= 11%)
b) during the night	39 (= 43%)	72 (= 89%)
		$\mu\text{atom C mg Chl}^{-1}$
		$\mu\text{atom C mg Chl}^{-1}\text{h}^{-1}$

Table 3. Homologies (in %) between the chloroplast triose phosphate/phosphate translocators (TPT) and the phosphoenolpyruvate/phosphate translocators (PPT) from different plants.

	TPTs from			PPTs from			
	Spinach	Cauliflower	Maize	Cauliflower PPT3	Arabidopsis PPT12	Tobacco PPT10	Maize PPT1
Spinach TPT	(100)	88	85	35	34	35	35
Cauliflower TPT		(100)	81	35	34	36	37
Maize TPT			(100)	34	35	36	35
Cauliflower PPT3				(100)	93	77	75
Arabidopsis PPT12					(100)	80	73
Tobacco PPT10						(100)	76
Maize PPT1							(100)

homologies between members of the TPT family and the PPT family, respectively, are only about 35%.

To study the transport characteristics of the cloned PPT proteins, the cDNA from the cauliflower PPT3 clone was fused to a DNA fragment encoding a tag of six consecutive histidine residues (**His₆-tag**) and was then subcloned into a yeast expression vector. As outlined above, this procedure enables the one-step purification of the produced transporter to apparent homogeneity by metal-affinity chromatography for the subsequent analysis of the transport characteristics by reconstitution experiments (Loddenkötter et al., 1993).

Figure 2 shows the substrate specificities of the purified PPT and the TPT, both purified from yeast cells, as determined by measuring [³²P]phosphate transport into proteoliposomes that had been preloaded with the indicated counter-substrates. Both classes of phosphate translocators greatly differ in their transport characteristics. The PPT protein transports inorganic phosphate preferentially in exchange with PEP. This is in sharp contrast to the transport characteristics of the TPT. This transporter accepts either inorganic phosphate or C3-compounds that are phosphorylated at C-atom 3 i.e., triose phosphates and 3-phosphoglycerate (see above). These substrates, however, are only poorly transported by the PPT protein. Both types of transporters strictly rely on the presence of an exchangeable substrate within the vesicles (i.e., they function as antiport systems) and do not transport hexose phosphates like glucose 6-phosphate at all.

2. Physiological Role of the PPT

Do plastids rely on externally produced PEP or can PEP be generated inside the organelles? Work from

several laboratories has shown that the conversion of hexose phosphates and/or triose phosphates via glycolysis cannot proceed further than to 3-phosphoglycerate due to the absence (or low activities) of phosphoglycerate mutase and/or enolase in most plastids (Stitt and ap Rees, 1979; Schulze-Siebert et al., 1984; Journet and Douce, 1985; Van der Straeten et al., 1991; Miernyk and Dennis, 1992; Borchert et al., 1993). These plastids depend therefore on the provision of externally produced PEP. This fact has been overlooked to date, or it has been assumed that the transport of PEP into chloroplasts is facilitated by the TPT. This, however, appears unlikely because PEP is only poorly accepted as a substrate by the TPT (Fig. 2) and has, under physiological conditions, to compete with inorganic phosphate, trioseP, and 3-phosphoglycerate for binding to the TPT. Hence, the presence of a PPT that bypasses the TPT is the most likely alternative for an efficient provision of the chloroplasts with PEP.

Several processes inside the plastids rely on PEP (see Fig. 3). This compound is an immediate substrate for the shikimate pathway which leads to the biosynthesis of aromatic amino acids and to a large number of secondary compounds that are important in plant defense mechanisms and stress responses (for review see Herrmann, 1995). It has also been shown that the plastid-located fatty acid biosynthesis can be driven by exogenous pyruvate (Liedvogel and Bäuerle, 1986). Plastidic acetyl-CoA can subsequently be formed by the action of either acetyl-CoA synthetase or via the plastidic pyruvate dehydrogenase complex. In those plastids missing a complete pathway from 3-phosphoglycerate to pyruvate, PEP could be imported into the organelles via the PPT and pyruvate kinase could subsequently convert PEP into pyruvate.

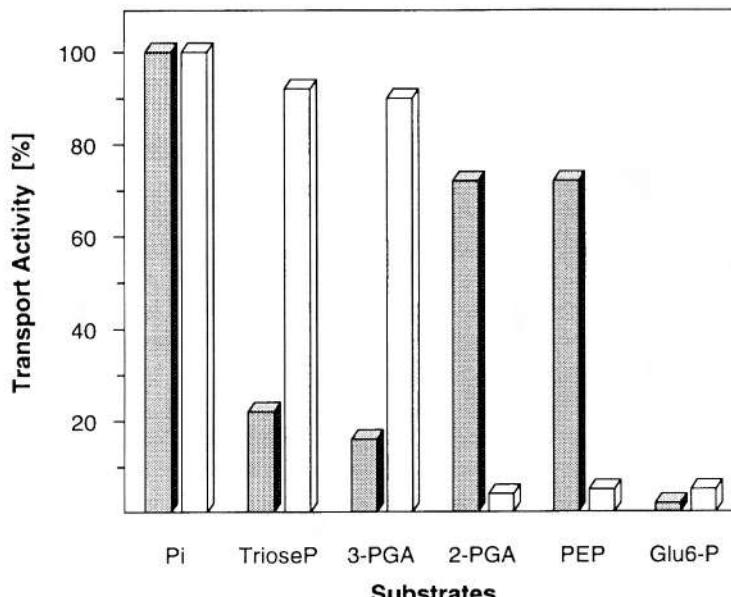


Fig. 2. Substrate specificities of the TPT and the PPT. The cauliflower PPT3-His₆ protein and the spinach TPT-His₆ protein were expressed in *Schizosaccharomyces pombe* cells and purified from these cells by metal affinity chromatography (Loddenkötter et al., 1993). The recombinant proteins were reconstituted into liposomes that had been preloaded with 25 mM substrates as indicated. [³²P]phosphate transport activity was measured as described by Fischer et al. (1997) and is given as a percentage of the activity measured for proteoliposomes preloaded with inorganic phosphate. The 100% exchange activities ($\mu\text{mol mg}^{-1}$ protein $\times \text{min}^{-1}$) were 1.5 (PPT; dark grey, left), and 0.85 (TPT; light grey, right). 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; TrioseP, dihydroxyacetone phosphate; Glu6-P, glucose 6-phosphate.

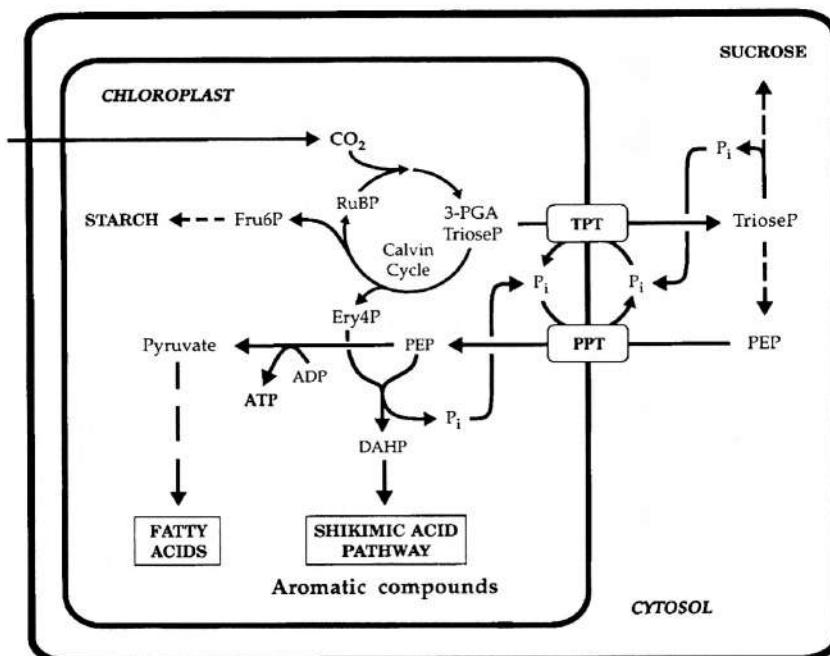


Fig. 3. Proposed function of the PPT protein in photosynthetically active tissues. The combined action of the TPT and the PPT results in the supply of the organelle with PEP generated from photosynthetically fixed carbon. 3-PGA, 3-phosphoglycerate; DAHP, 3-deoxy-D-arabino-heptulonate-7-phosphate; Ery4P, erythrose 4-phosphate; Fru6P, fructose 6-phosphate; RuBP, ribulose 1,5-bisphosphate; TrioseP, triose phosphates.

Figure 3 outlines the proposed physiological function of the PPT in chloroplasts. The fixed carbon is exported from the chloroplast by the TPT and partially converted into PEP in the cytosol. The PPT can provide the chloroplast stroma with PEP for the shikimate pathway. In addition, PEP can be converted into pyruvate as a precursor for fatty acid biosynthesis, concomitantly providing the plastids with ATP. The latter issue is probably important in chloroplasts during night or in non-photosynthetic tissues. Because inorganic phosphate is used as a counter-substrate by both the TPT and the PPT, the combined action of both translocators would result in an exchange of triose phosphate with PEP without net phosphate transport.

With the identification of a phosphate translocator specific for PEP, it is proposed that plastids contain a set of phosphate translocators with different structures but overlapping substrate specificities. Chloroplasts, or at least a subtype of chloroplasts, contain members of both the TPT and the PPT family. The TPT is present only in photosynthetic tissues (Schulz et al., 1993, Flügge, 1995), whereas PPT-specific transcripts can be detected in both leaves and non-green tissues although they are more abundant in non-green tissues (Fischer et al., 1997). The presence of different phosphate translocators with different substrate specificities would allow the uptake of any particular phosphorylated substrates even in the presence of high concentrations of other phosphorylated metabolites, which would otherwise compete for the binding site of a single phosphate translocator.

C. The Hexose Phosphate/Phosphate Translocator

It has been shown that chloroplasts from guard-cells contain a phosphate translocator that also transports glucose 6-phosphate (Overlach et al., 1993). Stomatal opening is associated with the production of malate as a counterion for potassium. Malate can derive from starch mobilization, the products of which are exported from the chloroplasts and used for malate formation. For the biosynthesis of starch, guard-cells have to import reduced carbon. It has been proposed that trioseP and/or 3-PGA, imported via the chloroplast phosphate translocator, can be used for the formation of hexose phosphates (Preiss et al., 1985). However, guard-cells are devoid of fructose 1,6-bisphosphatase activity (Hedrich et al., 1985),

the key enzyme for the conversion of trioseP into hexose phosphates, and therefore, rely on the provision of hexose phosphates for starch biosynthesis. In this respect, guard-cell chloroplasts behave like non-green plastids that also lack fructose 1,6-bisphosphatase activity (Entwistle and ap Rees, 1988) and have to import hexose phosphates for starch biosynthesis. Both organelles obviously contain a phosphate translocator that can import hexose phosphates in exchange with inorganic phosphate.

A hexose phosphate/phosphate transport activity can also be detected in spinach chloroplasts after feeding of detached leaves with glucose (Quick et al., 1995). The chloroplasts of these leaves are photosynthetically active but contain large quantities of starch. As is the case for heterotrophic plastids in sink tissues, the precursor for starch biosynthesis is imported into the chloroplasts in the form of glucose 6-phosphate by a novel hexose phosphate/phosphate translocator. Glucose-feeding has obviously induced a switch in the function of the chloroplasts from carbon-exporting source organelles to carbon-importing sink-organelles. These experiments show that the physiological state of a tissue can greatly alter its biochemical properties and demonstrate the high flexibility of plant metabolism.

We have recently identified a translocator that mediates the transport of glucose 6-phosphate in exchange with either inorganic phosphate or triose phosphates (glucose 6-phosphate/phosphate translocator, GPT) (Kammerer et al., 1998). Glucose 6-phosphate imported into the plastids via the GPT can thus be used either for starch biosynthesis, during which process inorganic phosphate is released, or as a substrate for the oxidative pentose phosphate pathway, yielding triose phosphates. The GPT shares about 38% identical amino acids with members of the TPT and PPT families and thus represents a third group of plastidic phosphate antiporters. The GPT gene is predominantly expressed in heterotrophic tissues but is most probably also responsible for the above described hexose phosphate transport activities in chloroplasts.

III. Transport of Glucose

The amyloytic degradation of starch at night results in the formation of glucose that can be exported from the chloroplasts by a glucose translocator and

converted to sucrose in the cytosol (see Fig. 1). As mentioned above, transgenic anti-sense TPT potato plants export carbon during the night preferentially via this translocator to compensate for their deficiency in TPT activity (Heineke et al., 1994). Evidence that the preferred route for exporting carbon from chloroplasts proceeds via the glucose translocator comes from studies on a high starch *Arabidopsis* mutant (starch-excess mutant TC265, Caspar et al., 1991). This mutant is able to degrade starch but obviously unable to export the products of starch degradation. These are therefore redirected into the synthesis of starch leading to the observed starch-excess phenotype. The chloroplasts possess a functional TPT but obviously lack a functional chloroplast glucose translocator for carbon export (Trethewey and ap Rees, 1994). Little is known about this translocator that had first been described in the late seventies (Schäfer et al., 1977). The availability of starch-excess mutants will presumably open the way to isolate the corresponding gene, e.g., by map-based cloning.

IV. Dicarboxylate Translocators

The photosynthetically fixed carbon that is exported from the chloroplasts via the TPT as trioseP and 3-PGA can also be used for the formation of carbon skeletons (2-oxoglutarate) via processes occurring in the cytosol and in the mitochondria. 2-oxoglutarate can then be imported into the chloroplasts for the fixation of ammonia that derives from nitrate reduction or photorespiration. Fixation of ammonia within the chloroplast is achieved via the stromal glutamine synthetase (GS2)/glutamate synthase cycle. Glutamate synthesized during this cycle is the key compound of nitrogen metabolism in plants. It can be subsequently exported into the cytosol (Fig. 4). Two different dicarboxylate antiport systems are involved in this process. Both translocators mediate an exchange with malate: (i) a 2-oxoglutarate/malate translocator (DiT1) that transports 2-oxoglutarate into the chloroplasts in exchange with malate and that does not transport glutamate or aspartate and (ii) a glutamate/malate translocator (DiT2) exporting glutamate in exchange with cytosolic malate. DiT2 can be considered as a general dicarboxylate

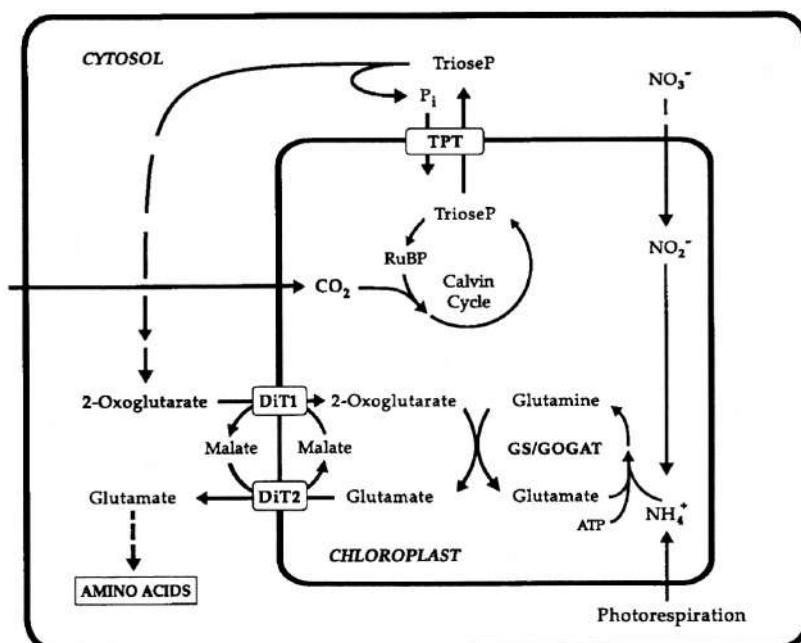


Fig. 4. Two plastidic dicarboxylate translocators are involved in ammonia assimilation. DiT1, 2-oxoglutarate/malate translocator; DiT2, glutamate/malate translocator; GS/GOGAT, glutamine synthetase/glutamate synthase; P_i, inorganic phosphate; RuBP, ribulose 1,5-bisphosphate; TrioseP, triose phosphates.

translocator. Because both translocators use malate as the substrate for counter-exchange, the resulting 2-oxoglutarate/glutamate transport proceeds without net malate transport (Woo et al., 1987; Flügge et al., 1988). Glutamate and other amino acids can then be further loaded into the sieve tubes via specific amino acid transporters.

Mutants of *Arabidopsis* deficient in dicarboxylate transport activities are not viable under photorespiratory conditions (Somerville and Somerville, 1985). The defect is probably caused by the general dicarboxylate translocator (DiT2). However, attempts to clone the corresponding gene have failed so far.

The identification of the 2-oxoglutarate/malate translocator (DiT1) as a 45 kDa component of the inner envelope membrane has recently been described (Menzlaff and Flügge, 1993). Subsequently, a cDNA clone was obtained that codes for the precursor protein with a relative molecular mass of 60 kDa (Weber et al., 1995). Interestingly, there is no detectable homology to any other known protein, not even to the mitochondrial 2-oxoglutarate/malate carrier with transport characteristics similar to DiT1 (Runswick et al., 1990).

The mature part of DiT1 contains 13-14 hydrophobic segments; 12 of them are long enough to span the membrane as transmembrane α -helices. These helices are obviously arranged in such a way that two clusters of six helices each are separated by an intervening hydrophilic loop. This transmembrane topology with an 12-helix motif resembles that of other plasmamembrane transporters from prokaryotes and eukaryotes that presumably all function as monomers. The recently identified plastidic ADP/ATP translocator also belongs to this type of transporter (see below). All other organellar transporters, including those from mitochondria, belong to the second group of transporters having 5-7 transmembrane helices like the chloroplast phosphate translocators. These translocators probably function as homodimers with a '6 + 6' transmembrane helix pattern. Thus, the inner envelope membrane possesses two different types of transporters which may all have, in their functional state, a comparable number of membrane-spanning segments.

To verify the identity of the cloned gene, the full-length cDNA sequence was expressed in the fission yeast *Schizosaccharomyces pombe*. Yeast transformants harboring the DNA coding for DiT1 indeed displayed reconstituted malate transport activities at rates exceeding those of the endogenous transporters

by at least two orders of magnitude. It could be further shown that substrate specificities of the recombinant protein were almost identical to the translocator purified from envelope membranes (Weber et al., 1995). The activity of the recombinant protein is thus in accordance with its proposed function as a supplier of the chloroplasts with 2-oxoglutarate for ammonia assimilation.

It has been shown that chloroplasts contain, in addition to DiT1 and DiT2, a specific translocator for glutamine with glutamate as the preferential countersubstrate. Glutamine is not transported by DiT1, and by DiT2 only at low rates. It has been proposed that the glutamine translocator is involved in the refixation of photorespiratory ammonia by mediating uptake of glutamine formed by the cytosolic isoform of glutamine synthetase (GS1) (Yu and Woo, 1988). However, it has been shown that the presence of GS1 is exclusively restricted to the cytoplasm of phloem companion cells whereas the chloroplast-located GS2 is localized in mesophyll cells in which it is involved in nitrogen assimilation (Edwards et al., 1990; Carvalho et al., 1992). Since the phloem companion cells do not contain photosynthetically active chloroplasts, uptake of GS1-produced glutamine into the plastids is not possible. Although the location of the glutamine/glutamate translocator has not yet been determined, it is suggested that this translocator resides in the mesophyll. In these cells, glutamine, produced by the stromal GS2, could be exported from the chloroplasts in exchange for cytosolic glutamate. This would allow a supply of assimilated nitrogen to the cell.

Chloroplasts are also able to transport oxaloacetate (OAA). Import of OAA into chloroplasts can be closely linked to an export of malate. This malate/OAA shuttle plays an important role in the transfer of reducing equivalents from the chloroplasts into the cytosol (Heineke et al., 1991). In principle, OAA can be transported by DiT1 and DiT2. However, this appears unlikely because the concentrations of OAA in both the cytosol and the stroma are at least one order of magnitude lower than that of malate and other dicarboxylates with which substrates OAA has to compete for binding to the translocator. Measurements of the uptake of OAA into chloroplasts of C₃-as well of C₄-plants revealed the presence of a specific translocator with a high affinity for OAA. This translocator is able to transport OAA even in the presence of a large excess of other dicarboxylates

(Hatch et al., 1984). Imported OAA can also be used for transamination to aspartate which is, in turn, the precursor for the biosynthesis of various amino acids (Bryan, 1990). The molecular structure of the OAA translocator is yet unknown.

C₃-chloroplasts also contain an antiport system for monocarboxylates, i.e. glycerate and glycolate, which are both intermediates of the photorespiratory cycle. This transporter will be dealt with in the chapter on photorespiration.

V. Transport of Nucleotides

Plant cells contain a highly active ADP/ATP translocator (AAT) in the inner membranes of mitochondria that exports the ATP generated by oxidative phosphorylation. Heldt (1969) was the first to show that chloroplasts are also capable of transporting ADP and ATP in a counterexchange mode. The proposed physiological function of this translocator in mature leaves is to supply chloroplasts with mitochondrial ATP during the night whereas nongreen plastids depend on the supply with ATP as the driving force for biosynthetic processes, e.g., starch and fatty acids (Hill and Smith, 1991; Kleppinger-Sparace et al., 1992; Neuhaus et al., 1993). A detailed analysis of the kinetic constants of the AAT showed that ATP and ADP are preferentially transported. AMP and other nucleotides showed only a poor interaction with the translocator (Schünemann et al., 1993). It has been claimed that ADP-glucose can also be transported via the AAT into both chloroplasts and amyloplasts (Pozueta-Romero et al., 1991). However, the affinity of AAT toward ADP-glucose is 2 orders of magnitude lower than toward ATP. Because ADP-glucose has to compete with ATP and ADP for binding to the translocator, the amount of ADP-glucose transported by the AAT cannot be of any physiological significance (Schünemann et al., 1993). Nevertheless, a mechanism for the transport of ADP-glucose is required in the endosperm of some cereals. It has been shown recently that in these tissues the AGPase is mainly present in the cytosol but not in the plastids (Denyer et al., 1996; Thorbjørnsen et al., 1996). The cytosolically formed ADP-glucose is presumably transported into the plastids for starch biosynthesis via an ADP-glucose/ADP antiporter which is distinct from the AAT. Most probably, the Bt1 protein, the cDNA of which has already been cloned, serves this

function (Sullivan et al., 1991; Sullivan and Kaneko, 1995). The corresponding *Bt1* mutation results in an accumulation of ADP-glucose and a reduction of starch biosynthesis in maize, probably reflecting the lack of the ADP-glucose/ADP translocator.

The molecular structure of the plastidic AAT remained elusive until Kampfenkel et al. (1995) happened to isolate a partial cDNA clone from *A. thaliana* that exhibited similarities to the ADP/ATP translocase from the bacterium *Rickettsia prowazekii*. Isolation and characterization of the full-length clone revealed no homology to the known mitochondrial adenylate translocators. The corresponding hydrophobic membrane protein (AATP1) possesses an N-terminal region with typical features of plastidic transit peptides. Interestingly, the AATP1 protein contains 12 potential transmembrane helices and thus belongs to the group of transporters with a 12 helix motif as is the case for DiT1 (see above). The transit peptide of AATP1 was able to target the adjacent protein to isolated chloroplasts (Neuhaus et al., 1997). In purified chloroplast envelope membranes, the AATP1 protein (62 kDa) could be detected immunologically. Expression of the AATP1-cDNA in heterologous systems and subsequent transport measurements demonstrates the identity of AATP1 as an ATP translocator. AATP1-specific transcripts could be detected both in photosynthetic and nongreen tissues. Taken together, the results suggest that AATP1 represents the plastidic adenylate translocator that is present in all plant tissues.

VI. Other Translocators

It is well established that chloroplasts are the major site of the synthesis of many amino acids including those of the aspartate and the pyruvate family (Bryan, 1990). In addition, the plastid-located shikimate pathway leads to the formation of the aromatic amino acids phenylalanine, tyrosine and tryptophan. The obvious reason for the chloroplast-located biosynthesis of amino acids is that reduced nitrogen and reducing equivalents, required for some of the biochemical reactions, are produced within the chloroplasts via nitrite reductase and photosynthetic electron transport, respectively, and carbon skeletons (pyruvate (phosphoenolpyruvate), OAA, 2-oxoglutarate) can be imported from the cytosol via specific translocators. The synthesized amino acids are either used for the plastid-located protein biosynthesis or

are, in the main part, exported into the cytosol for intercellular nitrogen transport. Unfortunately, almost nothing is known about plastidic translocators that are involved in these transport processes.

VII. Channels in Chloroplast Envelope Membranes

So far, translocator proteins of the inner chloroplast envelope as the major osmotic barrier between the chloroplast and the cytosol have been dealt with. Both the inner and the outer envelope membrane also contain channels and pores to mediate the flux of ions across these membranes. The outer envelope membrane appears to be permeable to low molecular weight solutes, mediated by the chloroplast porin (Flügge and Benz, 1984). Porins are voltage-dependent channels (VDACs) that are also present in the outer membrane of bacteria and mitochondria (Benz, 1994). In planar bilayers containing reconstituted chloroplast outer envelope membrane proteins, a large conductance channel ($\Lambda \approx 720 \text{ pS}$, in 100 mM KCl) was found (Flügge and Benz, 1984). Heiber et al. (1995) reported the presence of a slightly cation-selective high conductance channel ($\Lambda \approx 1.14 \text{ nS}$) in the outer envelope membrane. Weak anion and cation-selective large conductance pathways ($\Lambda \approx 512 \text{ pS}$ and $\Lambda \approx 1016 \text{ pS}$ in 100 mM KCl) have also been detected in the chloroplast envelope membrane by direct patch-clamping of chloroplasts from the green alga *Nitelopsis obtusa* (Pottosin, 1992, 1993). It has been suggested that the weakly anion-selective channel with a conductivity of $\Lambda \approx 512 \text{ pS}$ may be related to the mitochondrial VDAC (Pottosin, 1993).

Although the identities of these electrophysiologically characterized channels are not yet known, primary sequences of one class of porins from non-green plastids were recently obtained (Fischer et al., 1994b). These porins showed only a 20–25% identity to the known mitochondrial porins from eukaryotes. However, the plant porins contain 16 antiparallel transmembrane β -strands as is the case for porins from other organisms. The plant porin could be functionally expressed in bacterial cells and the recombinant protein revealed, after reconstitution into planar lipid bilayers, single channel conductances and voltage dependencies almost identical to the authentic protein, 3 and 1.5 nS in 1 M KCl (Benz et al., 1997). These values are similar to those obtained

for plant mitochondrial porins indicating that both non-green plastids and mitochondria possess one type of closely related porin proteins. This conclusion is corroborated by direct alignments of porin sequences from plant mitochondria (Heins et al., 1994).

More recently, the primary sequences of two chloroplast outer envelope membrane proteins were obtained (OEP16, OEP24) that both possess channel-like activities but do not show similarities to bacterial or mitochondrial porins (Pohlmeyer et al., 1997, 1998). Both proteins act as selectivity filters of the outer envelope membrane allowing the passage of amino acids (OEP16) and phosphate, dicarboxylates or adenylates (OEP24), respectively. It is suggested that the outer envelope membrane may contain various channel-like proteins with different selectivities and that the combined action of these channel proteins defines the permeability properties of the chloroplast outer envelope membrane.

Ion channels are also present in the inner envelope membrane. It has already been mentioned that the TPT can function as an anion-selective channel. Ion channel activity of the TPT, however, can only be observed if the concentrations of the monovalent ions are much higher than those of the physiological substrates (Schwarz et al., 1994). Recently, a weakly anion-selective high conductance channel with a single channel conductance of $\Lambda \approx 525 \text{ pS}$ has been discovered in proteoliposomes containing purified inner envelope membranes from spinach chloroplasts (Fuks and Homble, 1995). A similar weak anion-selective high conductance channel ($\Lambda \approx 540 \text{ pS}$) was observed by Heiber et al. (1995) in proteoliposomes containing inner and outer envelope membranes from spinach chloroplasts. To prevent uncontrolled dissipation of ion gradients across the inner envelope membrane, these porin-like channels have to be highly regulated.

The inner envelope membrane also contains a chloride channel (Heiber et al., 1995) that might be responsible for the rather high chloride permeability of this membrane (Demmig and Gimmler, 1983). Similar anion-selective channels have been observed in the chloroplast membranes from green algae (Pottosin, 1992; Thaler et al., 1992).

The presence of a potassium channel in the inner chloroplast envelope has been demonstrated by Berkowitz and coworkers (Mi et al., 1994) and Heiber et al. (1995). This K^+ -channel revealed an inward rectifying I/V-relationship, was blocked by millimolar

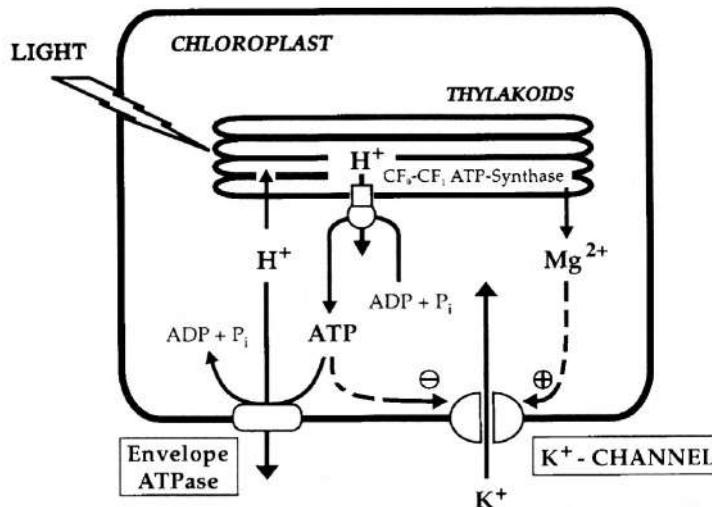


Fig. 5. Proposed function of the K^+ -channel of the chloroplast inner envelope membrane in energy-dependent fluxes of H^+ and K^+ -ions. Modulation of the K^+ -channel activity by photosynthetically generated ATP and by Mg^{2+} -ions takes part in the pH-stasis across the chloroplast envelope during photosynthesis. For details, see text. P_i , inorganic phosphate.

concentrations of ATP and activated by Mg^{2+} (Heiber et al., 1995). The proposed physiological function of the K^+ -channel inphotosynthetic ion fluxes is outlined in Fig. 5. Light-driven H^+ -transport into the thylakoids can be counterbalanced by a release of Mg^{2+} -ions from the thylakoid lumen. Elevated levels of Mg^{2+} and alkalization of the chloroplast stroma are both required for full activity of the photosynthetic carbon cycle. Stromal Mg^{2+} activates the envelope K^+ -channel leading to an increased K^+ -influx that is electrically balanced by H^+ -efflux via the envelope H^+ -ATPase. On the other hand, the K^+ -channel is blocked by increasing concentrations of photosynthetically produced ATP. This effect results in a decreased H^+ -efflux from the chloroplasts and an acidification of the chloroplast stroma. It is proposed that the opposing modulation of the K^+ -channel by Mg^{2+} -ions and ATP play a major role in the pH-stasis across the chloroplast envelope and the regulation of photosynthesis.

VIII. Concluding Remarks

Much progress has been achieved during the last years in studying translocators at biochemical and, more recently, molecular levels. When the corresponding gene is available, its cell- and tissue-specific expression can be studied (e.g., by RNA gel blot analysis, by in situ hybridization, or by expression studies of the corresponding promoter fused to a

reporter gene). Furthermore, transgenic plants with a controlled expression of the transporter protein can be used to elucidate the precise role of these translocators in plant metabolism. On the other hand, our knowledge of the identity of other plastidic translocators is still rudimentary. There are multiple reasons for this. First, the biochemical identification and isolation of non-abundant translocators is difficult. Second, the identification of unknown plastidic transporters appears not to be possible using the heterologous complementation system of yeast mutants. As mentioned above, this system has been used extensively to isolate transporters of the plasma membrane. Third, the primary sequences of plastidic translocators known to date revealed only limited homologies to transporters known in other systems. Thus, genes coding for plastidic translocators are rarely obtained by screenings of DNA libraries with heterologous probes.

In the foreseeable future, the *Arabidopsis* and rice genomic sequencing programs will provide the sequences of complete higher plant genomes. In addition, 30,000 *Arabidopsis* ESTs (Expressed Sequence Tags) tagging about half of the expected *Arabidopsis* genes are already available. It appears evident that future work will then concentrate on the identification of the functions of genes coding for putative envelope translocators.

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References

- Bagge P and Larsson C (1986) Biosynthesis of aromatic amino acids by highly purified spinach chloroplasts—Compartmentation and regulation of the reactions. *Physiol Plantarum* 68: 641–647
- Benz R (1994) Permeation of hydrophilic solutes through mitochondrial outer membranes. *Biochim Biophys Acta* 1197: 167–196
- Borchert S, Harborth J, Schünemann D, Hoferichter P and Heldt HW (1993) Studies of the enzymatic capacities and transport properties of pea root plastids. *Plant Physiol* 101: 303–312
- Brink S, Fischer K, Klösgen R-B and Flügge UI (1995) Sorting of nuclear-encoded chloroplast membrane proteins to the envelope and the thylakoid membrane. *J Biol Chem* 270: 20808–20815
- Bryan JK (1990) Advances in the biochemistry of amino acids biosynthesis. In: Miflin BJ and Lea PJ (eds) *The Biochemistry of Plants*, Vol 16, pp 161–195. Academic Press, New York
- Caspar T, Lin T-S, Kakefuda G, Benbow L, Preiss J and Somerville C (1991) Mutants of *Arabidopsis* with altered regulation of starch metabolism. *Plant Physiol* 95: 1181–1188
- Demmig B and Gimmler H (1993) Properties of the isolated intact chloroplast at cytoplasmic K^+ concentrations. *Plant Physiol* 73: 169–174
- Dennis DT and Miernyk JA (1982) Compartmentation of nonphotosynthetic carbohydrate metabolism. *Ann Rev Plant Physiol* 33: 27–50
- Denyer K, Dunlap F, Thorbjørnsen T, Keeling P and Smith AM (1996) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiol* 112: 779–785
- Edwards JW, Walker EL and Coruzzi GM (1990) Cell-specific expression in transgenic plants reveals non-overlapping roles for chloroplast and cytosolic glutamine synthetase. *Proc Natl Acad Sci USA* 87: 3459–3463
- Entwistle G and ap Rees T (1988) Enzymatic-capacities of amyloplasts from wheat (*Triticum aestivum*) endosperm. *Biochem J* 255: 391–396
- Fischer K, Arbinger B, Kammerer K, Busch C, Brink S, Wallmeier H, Sauer N, Eckerskorn C and Flügge UI (1994a) Cloning and *in vivo* expression of functional triose phosphate/phosphate translocators from C_3 - and C_4 -plants: Evidence for the putative participation of specific amino acids residues in the recognition of phosphoenolpyruvate. *Plant J* 5: 215–226
- Fischer K, Weber A, Brink S, Arbinger B, Schünemann D, Borchert S, Heldt HW, Popp B, Benz R, Eckerskorn C and Flügge UI (1994b) Porins from plants: Molecular cloning and functional characterization of two new members of the porin family. *J Biol Chem* 269: 25754–25760
- Fischer W-N, André B, Rentsch D, Krolkiewicz S, Tegeder M, Breitkreuz K and Frommer WB (1998) Plant amino acid transport. *Trends Plant Sci* 3: 188–195
- Fliege R, Flügge UI, Werdan K and Heldt HW (1978) Specific transport of inorganic phosphate, 3-phosphoglycerate and triosephosphates across the inner membrane of the envelope in spinach chloroplasts. *Biochim Biophys Acta* 502: 232–247
- Flügge UI (1987) Physiological function and physical characteristics of the chloroplast phosphate translocator. In: Biggins J (ed) *Progress in Photosynthesis Research*, Vol III, pp. 739–746. Martinus Nijhoff Publishers, Dordrecht
- Flügge UI (1992) Reaction mechanism and asymmetric orientation of the reconstituted chloroplast phosphate translocator. *Biochim Biophys Acta* 1110: 112–118
- Flügge UI (1995) Phosphate translocation in the regulation of photosynthesis. *J Exp Bot* 46: 1317–1323
- Flügge UI and Benz R (1984) Pore forming activity in the outer membrane of the chloroplast envelope. *FEBS Lett* 169: 85–89
- Flügge UI and Heldt HW (1991) Metabolite translocators of the chloroplast envelope. *Annu Rev Plant Physiol Plant Mol Biol* 42: 129–144
- Flügge UI and Weber A (1994) A rapid method for measuring organelle-specific substrate transport in homogenates from plant tissues. *Planta* 194: 181–185
- Flügge UI, Woo KC and Heldt HW (1988) Characteristics of 2-oxoglutarate and glutamate transport in spinach chloroplasts. Studies with a double-silicone-layer centrifugation technique and in liposomes. *Planta* 174: 534–541
- Flügge UI, Fischer K, Gross A, Sebald W, Lottspeich F and Eckerskorn C (1989) The triose phosphate-3-phosphoglycerate-phosphate translocator from spinach chloroplasts: Nucleotide sequence of a full-length cDNA clone and import of the *in vitro* synthesized precursor protein into chloroplasts. *EMBO J* 8: 39–46
- Frommer WB and Ninnemann O (1995) Heterologous expression of genes in bacterial, fungal, animal, and plant cells. *Annu Rev Plant Physiol Plant Mol Biol* 46: 419–444
- Fuks B and Homble F (1995) A voltage-dependent porin-like channel in the inner envelope membrane of plant chloroplasts. *J Biol Chem* 270: 9947–9952
- Gerhardt R, Stitt M and Heldt HW (1987) Subcellular metabolite levels in spinach leaves. Regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. *Plant Physiol* 83: 399–407
- Häusler RE, Schlieben NH, Schulz B and Flügge UI (1998) Compensation of decreased triose phosphate/phosphate transport activity by accelerated starch turnover and glucose transport in transgenic tobacco. *Planta* 204: 366–376
- Hatch MD, Dröscher L, Flügge U-I and Heldt HW (1984) A specific translocator for oxaloacetate transport in chloroplasts. *FEBS Lett* 178: 15–19
- Hattenbach B, Müller-Röber B, Nast G and Heineke D (1997) Antisense repression of both ADP-glucose pyrophosphorylase and triose phosphate translocator modifies carbohydrate partitioning in potato leaves. *Plant Physiol* 115: 471–475
- Hedrich R, Raschke K and Stitt M (1985) A role for fructose-2,6-bisphosphate in regulating carbohydrate metabolism in guard cells. *Plant Physiol* 79: 977–982
- Heiber T, Steinkamp T, Schwarz M, Flügge UI, Weber A and Wagner R (1995) Ion channels in the chloroplast envelope

- membrane. *Biochemistry* 34: 15906–15917
- Heineke D, Riens B, Hoferichter P, Peter U, Flügge UI and Heldt HW (1991) Redox transfer across the inner chloroplast envelope membrane. *Plant Physiol* 95: 1131–1137
- Heineke D, Kruse A, Flügge UI, Frommer WB, Riesmeier JW, Willmitzer L and Heldt HW (1994) Effect of antisense repression of the chloroplast triose-phosphate translocator on photosynthetic metabolism in transgenic potato plants. *Planta* 193: 174–180
- Heldt HW, Chon CJ, Maronde D, Herold A, Stancovic ZS, Walker DA, Kraminer A, Kirk MR and Heber U (1977) Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol* 59: 1146–1155
- Heldt HW, Flügge UI and Borchert S (1991) Diversity of specificity and function of phosphate translocators in various plastids. *Plant Physiol* 95: 341–343
- Herrmann KM (1995) The shikimate pathway: Early steps in the biosynthesis of aromatic compounds. *Plant Cell* 7: 907–919
- Hill LM and Smith AM (1991) Evidence that glucose 6-phosphate is imported as the substrate for starch biosynthesis by the plastids of developing pea embryos. *Planta* 185: 91–96
- Journet EP and Douce R (1985) Enzymic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. *Plant Physiol* 79: 458–467
- Kammerer B, Fischer K, Hilpert B, Schubert S, Guttensohn M, Weber A and Flügge UI (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: The glucose 6-phosphate/phosphate antiporter. *Plant Cell* 10: 105–117
- Kampfenkel K, Möhlmann T, Batz O, Van Montagu M, Inzé D and Neuhaus HE (1995) Molecular characterization of an *Arabidopsis thaliana* cDNA encoding a novel putative adenylate translocator of higher plants. *FEBS Lett* 374: 351–355
- Kleppinger-Sparace KF, Stahl RJ and Sparace SA (1992) Energy requirements for fatty acid biosynthesis and glycerolipid biosynthesis from acetate by isolated pea root plastids. *Plant Physiol* 98: 723–727
- Knight JS and Gray JC (1994) Expression of genes encoding the tobacco chloroplast phosphate translocator is not light regulated and is repressed by sucrose. *Mol Gen Genet* 242: 586–594
- Knight JS and Gray JC (1995) The N-terminal hydrophobic region of the mature phosphate translocator protein is sufficient for targeting to the chloroplast inner envelope membrane. *Plant Cell* 7: 1421–1432
- Liedvogel B and Bäuerle R (1986) Fatty-acid synthesis in chloroplasts from mustard (*Sinapis alba* L.) cotyledons: Formation of acetyl coenzyme A by intraplastid glycolytic enzymes and a pyruvate dehydrogenase complex. *Planta* 169: 481–489
- Loddenkötter B, Kammerer B, Fischer K and Flügge UI (1993) Expression of the functional mature chloroplast triose phosphate translocator in yeast internal membranes and purification of the histidine-tagged protein by a single metal-affinity chromatography step. *Proc Natl Acad Sci USA* 90: 2155–2159
- Maloney PC (1990) A consensus structure for membrane transport. *Res Microbiol* 141: 374–383
- Mannella CA (1992) The ‘ins’ and ‘outs’ of mitochondrial membrane channels. *Trends Biochem Sci* 17: 315–320
- Menzlaff E and Flügge UI (1993) Purification and functional reconstitution of the 2-oxoglutarate/malate translocator from spinach chloroplasts. *Biochim Biophys Acta* 1147: 13–18
- Mi F, Peters JS and Berkowitz GA (1994) Characterization of a chloroplast inner envelope K^+ channel. *Plant Physiol* 105: 955–964
- Miernyk JA and Dennis DT (1992) A developmental analysis of the enolase isoenzymes from *Ricinus communis*. *Plant Physiol* 99: 748–750
- Neuhaus HE and Maass U (1996) Unidirectional transport of orthophosphate across the envelope of isolated cauliflower-bud amyloplasts. *Planta* 198: 542–548
- Neuhaus HE, Thom E, Batz O and Scheibe R (1993) Purification of highly intact plastids from various heterotrophic plant tissues. Analysis of enzyme equipment and precursor dependency for starch biosynthesis. *Biochem J* 296: 495–501
- Neuhaus HE, Thom E, Möhlmann T, Steup M and Kampfenkel K (1997) Characterization of a novel ATP/ADP translocator located in the plastid envelope of *Arabidopsis thaliana* L. *Plant J* 11: 73–82
- Overlach S, Diekmann W and Raschke K (1993) Phosphate translocator of isolated guard-cell chloroplasts from *Pisum sativum* L. transports glucose-6-phosphate. *Plant Physiol* 101: 1201–1207
- Pohlmeier K, Soll J, Steinkamp T, Hinnah S and Wagner R (1997) Isolation and characterization of an amino acid-selective channel protein present in the chloroplastic outer envelope membrane. *Proc Natl Acad Sci USA* 94: 9504–9509
- Pohlmeier K, Soll J, Grimm R, Hill K and Wagner R (1998) A high-conductance solute channel in the chloroplastic outer envelope from pea. *Plant Cell* 10: 1207–1216
- Popp B, Gebauer S, Fischer K, Flügge UI and Benz R (1997) Study of structure and function of recombinant pea root plastid porin by biophysical methods. *Biochemistry* 36: 2844–2852
- Pottosin II (1992) Single channel recording in the chloroplast envelope. *FEBS Lett* 308: 97–90
- Pottosin II (1993) One of the chloroplast envelope ion channels is probably related to the mitochondrial VDAC. *FEBS Lett* 330: 211–214
- Pozueta-Romero J, Frehner M, Viale AM and Akazawa T (1991) Direct transport of ADPglucose by an adenylate translocator is linked to starch biosynthesis in amyloplasts. *Proc Natl Acad Sci USA* 88: 5769–5773
- Preiss J and Levi C (1980) Starch biosynthesis and degradation. In: Stumpf PK and Conn EE (eds) *The Biochemistry of Plants*, Vol 3, pp 371–424. Academic Press, New York
- Preiss J, Robinson N, Spilatro S and McNamara K (1985) Starch synthesis and its regulation. In: Heath RL and Preiss J (eds) *Regulation of Carbon Partitioning in Photosynthetic Tissue*, pp 1–126. Symposium in Plant Physiology, American Society of Plant Physiologists, Rockville, MD
- Quick WP, Scheibe R and Neuhaus HE (1995) Induction of a hexose-phosphate translocator activity in spinach chloroplasts. *Plant Physiol* 109: 113–121
- Runswick MJ, Powell SJ, Bisaccia F, Iacobazzi V and Palmieri F (1990) Sequence of the bovine 2-oxoglutarate/malate carrier protein: Structural relationship to other mitochondrial transport proteins. *Biochemistry* 29: 11033–11040
- Schäfer G, Heber U and Heldt HW (1977) Glucose transport into spinach chloroplasts. *Plant Physiol* 60: 286–289
- Schulz B, Frommer WB, Flügge UI, Hummel S, Fischer K and Willmitzer L (1993) Expression of the triose phosphate

- translocator gene from potato is light dependent and restricted to green tissues. *Mol Gen Genet* 238: 357–361
- Schulze-Siebert D, Heineke D, Scharf H and Schulz G (1984) Pyruvate-derived amino acids in spinach chloroplasts: Synthesis and regulation during photosynthetic carbon metabolism. *Plant Physiol* 76: 465–471
- Schünemann D, Borchert, S, Flügge, UI and Heldt HW (1993) A DP/ATP translocator from pea root plastids: comparison with translocators from spinach chloroplasts and pea leaf mitochondria. *Plant Physiol* 103: 131–137
- Schwarz M, Gross A, Steinkamp T, Flügge UI and Wagner R (1994) Ion channel properties of the reconstituted chloroplast triose phosphate/phosphate translocator. *J Biol Chem* 269: 29481–29489
- Somerville SC and Somerville CR (1985) A mutant of *Arabidopsis* deficient in chloroplast dicarboxylate transport is missing an envelope protein. *Plant Sci Lett* 37: 217–220
- Stitt M and ap Rees T (1979) Capacities of pea chloroplasts to catalyse the oxidative pentose phosphate pathway and glycolysis. *Phytochemistry* 18: 1905–1911
- Sullivan T and Kaneko Y (1995) The maize brittle 1 gene encodes amyloplast membrane polypeptides. *Planta* 196: 477–484
- Sullivan TD, Strelow LI, Illingworth CA, Phillips CA and Nelson OE (1991) Analysis of the maize *brittle-1* alleles and a defective *Suppressor-mutator*-induced mutable allele. *Plant Cell* 3: 1337–1348
- Thorbjørnsen T, Villand P, Denyer K, Olsen O-A and Smith AM (1996) Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J* 10: 243–250
- Trethewey RN and ap Rees T (1994) A mutant of *Arabidopsis thaliana* lacking the ability to transport glucose across the chloroplast envelope. *Biochem J* 301: 449–454
- Wallmeier H, Weber A, Gross A and Flügge UI (1992) Insights into the structure of the chloroplast phosphate translocator protein. In: Cooke DT and Clarkson DT (eds) *Transport and Receptor Proteins of Plant Membranes*, pp 77–89. Plenum Press, New York
- Ward JM, Kühn C, Tegeder M and Frommer WB (1997) Sucrose transport in higher plants. *Intern Rev Cytol* 178: 41–71
- Weber A, Menzlaff E, Arbinger B, Gutensohn M, Eckerskorn C and Flügge UI (1995) The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: Molecular cloning of a transporter protein containing a 12-helix motif and expression of the functional protein in yeast cells. *Biochemistry* 34: 2621–2627
- Woo KC, Flügge UI and Heldt HW (1987) A two-translocator model for the transport of 2-oxoglutarate and glutamate in chloroplasts during ammonia assimilation in the light. *Plant Physiol* 84: 624–632
- Yu J and Woo KC (1988) Glutamine transport and the role of the glutamine translocator in chloroplasts. *Plant Physiol* 88: 1048–1054

Chapter 7

Photosynthesis, Carbohydrate Metabolism and Respiration in Leaves of Higher Plants

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Summary

The relationships between photosynthesis, carbohydrate metabolism and respiration in leaves of C₃ plants are reviewed. We first provide an overview of how mitochondrial respiration relies on, and responds to, the supply of photosynthetic products in the light. The pathways by which the various substrates (glycine, oxaloacetate, malate and/or pyruvate) enter the mitochondria and are oxidized, are discussed. We also provide an overview of the pathways of mitochondrial electron transport, with particular attention being paid to the non-phosphorylating alternative oxidase (AOX). We then discuss what is known about leaf respiration rates in light versus darkness (both O₂ consumption and CO₂ release). The extent to which mitochondrial O₂ consumption continues in the light is highly variable, being inhibited, not affected or even stimulated in various reports. On the other hand, non-photorespiratory mitochondrial CO₂ release (R) is invariably inhibited by light (5–80% inhibition). R is sensitive to the lowest irradiance values, and is inhibited rapidly. Three methods via which R in the light is measured are outlined and mechanisms via which light might inhibit R are discussed. The effect that light to dark transitions have on respiration are also discussed: we distinguish the initial, photorespiratory post-illumination burst (PIB) from the post-illumination rise in respiration (LEDR, light-enhanced-dark-respiration) which occurs following the PIB. The chapter also considers the demand for mitochondrially-derived ATP for photosynthesis and carbohydrate metabolism and the potential role of respiration during over-reduction of the chloroplast is highlighted. Mitochondrial respiration appears to be critical for the provision of ATP necessary for energy demanding processes in the light. Moreover, there is growing evidence that respiration helps a plant cope with excess photosynthetic redox equivalents, which otherwise can result in photo-oxidative stress.

Finally, the interactions between nitrogen metabolism, respiration and photosynthesis are also reviewed. Respiration provides the redox equivalents and carbon skeletons necessary for nitrogen assimilation in the light. The inhibitory effect that intermediary nitrogen oxides (NO_x; produced during light to dark transitions) have on mitochondrial electron transport via the cytochrome pathway is discussed.

I. Introduction

Photosynthesis uses light energy and water to convert atmospheric CO₂ into carbon-rich compounds such as carbohydrates. Respiration oxidizes these compounds, releasing CO₂, generating useable energy and forming carbon intermediates needed for biosynthesis. On a whole plant basis, up to 70% of the CO₂ fixed during photosynthesis can be released back into the atmosphere by mitochondrial respiration (Van Der Werf et al., 1994), depending on growing

conditions. A thorough understanding of both photosynthesis and respiration, and the interplay between them, is necessary, therefore, if we are to fully understand biomass production in plants.

This chapter describes the relationships between photosynthesis, carbohydrate metabolism and respiration in leaves of C₃ plants. Although leaf mitochondria fulfill important specific functions in C₄ photosynthesis this is outside the scope of this chapter. For this aspect the reader is referred to the excellent review on this topic by Hatch and Carnal

Abbreviations: AOX – alternative oxidase; c_i – internal CO₂ concentration; CI – complex I; CII – complex II (succinate dehydrogenase); CIII – complex III (cytochrome b/c₁ complex); CIV (cytochrome oxidase); CV – complex V (ATP synthase); Cyt – cytochrome; DHAP – dihydroacetone 3-phosphate; F16BP – fructose 1,6 bisphosphate; GDC – glycine decarboxylase; GDH – glutamate dehydrogenase; GS – glutamine synthase; GOGAT – glutamate synthase; G6P – glucose 6-phosphate; IS – intermembrane space; IM – innermembrane; LEDR – light-enhanced dark respiration; M – matrix; MDH – malate dehydrogenase; ME – malic enzyme; NADH – nicotinamide-adenine dinucleotide, reduced form; NADPH – nicotinamide-adenine dinucleotide phosphate, reduced form; NDex – external NAD(P)H dehydrogenase; NDin – internal, rotenone-insensitive NADH dehydrogenase; NR – nitrate reductase; NiR – nitrite reductase; OAA – oxaloacetate; OPP – oxidative pentose phosphate; Pi – inorganic phosphate; PDC – pyruvate dehydrogenase complex; PEP – phosphoenolpyruvate; PIB – photorespiratory post-illumination burst; 3-PGA – 3-phosphoglycerate; P-glycolate – phosphoglycolate; PK, pyruvate kinase; Q_t – ubiquinol; RQ – respiratory quotient, ratio of CO₂ release to O₂ uptake; R – non-photorespiratory mitochondrial CO₂ release; R_d – R in the light; R_d – R in the dark; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP – ribulose-1,5-bisphosphate; SPS – sucrose phosphate synthase; SDH – succinate dehydrogenase; TCA cycle, tricarboxylic acid cycle; UTP – uridine triphosphate; UDP – uridine diphosphate; Γ* – gamma star, c_i value where v_e equals 0.5v_o; v_c – rate of carboxylation by Rubisco; v_o – rate of oxygenation by Rubisco

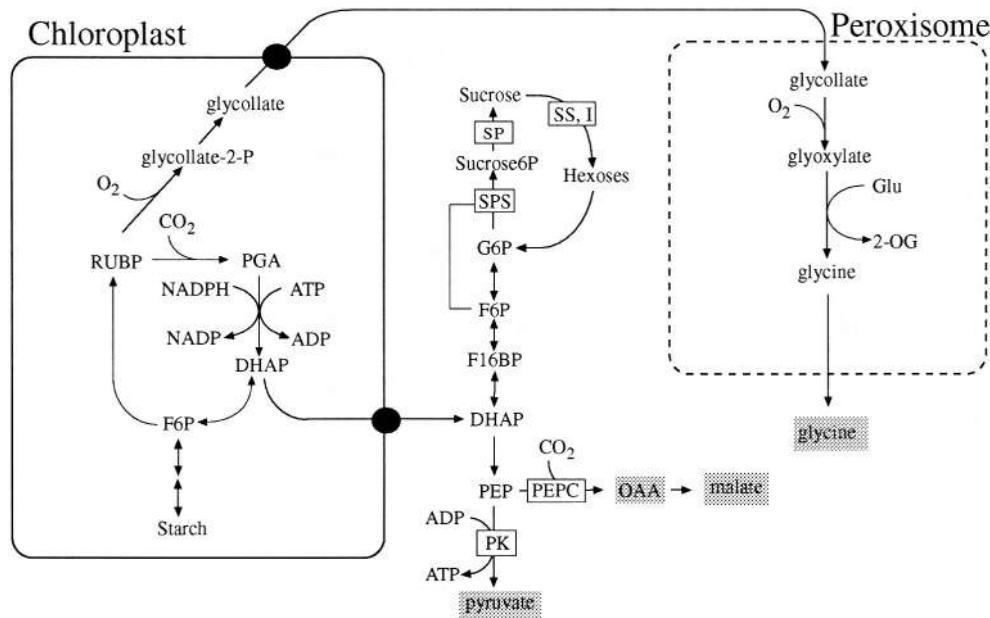


Fig. 1. Provision of mitochondrial substrates in the light. Shaded compounds represent the most likely substrates provided to mitochondria from the chloroplasts in leaf cells. Solid circles represent carriers on the chloroplast envelope.

(1992). We start with an overview of how mitochondrial respiration relies on, and responds to, the supply of photosynthetic products. We then consider what is known about leaf respiration rates in light versus darkness and the mechanisms by which light can affect mitochondrial CO_2 release. The demand for mitochondrially-derived ATP for photosynthesis and carbohydrate metabolism is considered and the potential role of respiration during over-reduction of the chloroplast (photo-oxidative stress) is highlighted. The interactions between nitrogen metabolism, respiration and photosynthesis are also reviewed.

II. Supply and Utilization of Mitochondrial Substrates in Leaves

In the light, photorespiration provides glycine as a mitochondrial substrate while carbon fixation in the chloroplast yields triose-phosphate in the cytosol, which, after conversion to phosphoenolpyruvate (PEP), can subsequently be converted either to pyruvate via pyruvate kinase (PK.) or oxaloacetate (OAA) via PEP carboxylase (Fig. 1). The OAA may be reduced to malate in the cytosol if sufficient NADH is available. These substrates can all be oxidized in mitochondria. Pärnik and Keerberg (1995)

defined such substrates as primary products of photosynthesis/photorespiration. The major primary photosynthetic product used as a substrate by mitochondria in the light appears to be glycine, while products from PEP represent ca. 3–15% of primary substrates that are oxidized (Ellsworth and Reich, 1996; Hurry et al., 1996). The time taken for these substrates to be exported from the chloroplast to the respiratory apparatus is likely to be in the order of seconds to minutes. For example, the period between Rubisco oxygenation in the chloroplast and the initiation of photorespiratory mitochondrial CO_2 release in tobacco is approximately 15–20 s (Atkin et al., 1998a).

Starch and sucrose generated by photosynthetic carbon fixation minutes, hours or days previously can also serve as respiratory substrates in leaves. Such photosynthetic end-products (Ellsworth and Reich, 1996) can represent 40–50% of total substrate oxidation in the light (Ellsworth and Reich, 1996; Hurry et al., 1996). In the presence of sufficient Pi, starch degradation in the light can occur at rates comparable to the rate of starch synthesis (Stitt and Heldt, 1981). Darkness eliminates the production of primary carbon products and respiration then relies completely on degradation of starch and sucrose reserves.

A. Export of Carbon From Chloroplasts

Carbon is exported from the chloroplasts mainly as triose-phosphates on the phosphate/triose-phosphate exchange translocator (Flügge and Heldt, 1991). In the cytosol, triose-phosphates can be further oxidized through glycolysis or combined to form fructose 1,6 bisphosphate (F1,6BP). Subsequent dephosphorylation produces hexose phosphates which serve as substrates for gluconeogenesis or for oxidation and decarboxylation via the pentose phosphate pathway. The synthesis of sucrose is regulated by the availability of UTP for UDP-glucose synthesis and also by the phosphorylation state of sucrose phosphate-synthase (SPS) which can be modulated allosterically by changes in G6P and Pi concentrations (Krömer, 1995). The sucrose formed can be transported to other parts of the plant, or stored locally and subsequently degraded by invertase or sucrose synthase for entry into oxidative pathways. These reactions are summarized in Fig. 1. Of the hexose phosphates oxidized, approximately 70–95% are degraded via phosphofructokinase and aldolase in glycolytic reactions; the remainder are oxidized to triose-phosphates via the OPP pathway (ap Rees, 1980).

B. Products of Glycolysis

Triose-phosphates are oxidized through glycolysis, yielding ATP and NADH, to form PEP. PEP carboxylase and PK compete for cytosolic PEP and both reactions are essentially irreversible. The activity of both PEP carboxylase and PK can be finely controlled, allowing the plant cell to regulate the availability of OAA, malate and pyruvate for oxidation by mitochondria. PK is inhibited by high concentrations of ATP (Hatzfeld and Stitt, 1991; Podstá and Plaxton, 1994) but is activated by dihydroacetone-3-phosphate (DHAP) in algal cells (Lin et al. 1989). In contrast, PEP carboxylase activity is not regulated by the cell's energy status but is inhibited by malate, aspartate and glutamate (Lepiniec et al., 1994; Krömer et al., 1996). The degree of inhibition can be regulated by phosphorylation of PEP carboxylase by a protein kinase (Van Quy et al., 1991), with the phosphorylated form being less sensitive to inhibition by metabolites (Krömer et al., 1996). PEP carboxylase activity is also increased by G6P, DHAP, 3-phosphoglycerate (3-PGA) and inorganic phosphate (P_i) (Krömer et al., 1996). At physiological concentrations of malate

and G6P, the activation by G6P overrides malate inhibition (Krömer et al., 1996). The regulatory properties of PEP carboxylase may be summarized as a feed-forward activation by primary products of photosynthetic carbon fixation and a feed-back inhibition from malate and amino acids.

The ability of the PEP carboxylase route to supply OAA and/or malate to the mitochondria is shown by transgenic PK-deficient tobacco plants which exhibit a phenotype not significantly different from wildtype plants (Gottlob-McHugh et al., 1992). The question of whether PEP carboxylase activity results in OAA or malate being imported into the mitochondria, depends on whether OAA is converted to malate by cytosolic MDH (which may happen when the cytosolic NADH/NAD ratio is high).

C. Export of Redox Equivalents from Chloroplasts

Photosynthetic redox equivalents can be exported from the chloroplast to the cytosol in the form of triose-phosphates via the DHAP:3-PGA shuttle in the inner envelope of the chloroplast (Heber, 1975; Heineke et al., 1991). If the export of DHAP is coupled to the synthesis of 3-PGA in the cytosol, then one molecule of both ATP and NADH, or NADPH alone, will be liberated into the cytosol for each molecule of DHAP exported, depending on the cytosolic enzymes that are involved (Krömer, 1995). 3-PGA can be imported subsequently into the chloroplast thus completing the cycle. This shuttle mechanism appears to be controlled by the relative rates of chloroplastic 3-PGA reduction and cytosolic DHAP oxidation (Heineke et al., 1991).

Reductants can also be exported from the chloroplasts via malate/OAA exchange (Fig. 2). Chloroplasts contain a **NADP⁺-malate dehydrogenase (NADP⁺-MDH)** which is light inducible (Scheibe, 1987). Under conditions where the **NADPH/NADP⁺** ratio in the chloroplast is high (Scheibe, 1987; Heineke et al., 1991), OAA will be converted to malate. Malate can subsequently be exported via the malate-OAA translocator present in the inner envelope membrane of the chloroplast (Hatch et al., 1984). It has been suggested that this shuttle allows excess photosynthetic redox equivalents to be exported from the chloroplast in the form of malate (Heineke et al., 1991). The operation of the shuttle appears to be driven by a redox gradient between the chloroplast and the cytosol, which will be substantial

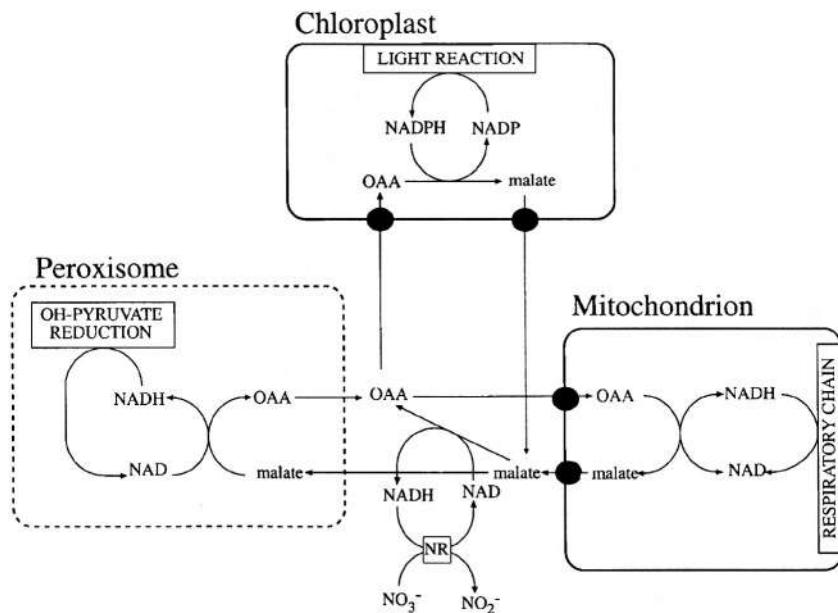


Fig. 2. Exchange of reducing power between leaf cell compartments. NR: nitrate reductase. Solid circles represent transporters; note that malate and OAA transport may be catalysed as an exchange on the OAA translocator, or on separate carriers as shown.

in the light. For example, the ratio of NADPH:NADP⁺ in the stroma of spinach chloroplasts is approximately 0.5, whereas the NADH/NAD⁺ ratio in the cytosol is far lower (0.001; Heineke et al., 1991). Since the chloroplast NADP⁺-MDH is only active at high stromal NADPH/NADP⁺, and is inactivated in the dark, the shuttle only operates in the light when there is excess reductant available. In this way NADP⁺-MDH in the chloroplast acts as a 'redox valve' (Kröamer and Scheibe 1996). In addition to being controlled by redox gradients between the chloroplast and the cytosol, malate export also is regulated by the translocating step across the envelope membrane (Heineke et al., 1991). The malate thus released into the cytosol could either:

- 1) be oxidized in peroxisomes to support hydroxypyruvate reductase during operation of the photorespiratory cycle;
- 2) be oxidized in mitochondria, allowing ATP to be synthesized (which may be important for rapid sucrose synthesis—see below); or
- 3) be oxidized in the cytosol to support nitrate reduction.

In all cases, the OAA formed in the cytosol must

return to the chloroplast to complete the shuttle. Figure 2 illustrates these reactions.

D. Oxidation of Organic Acids by Mitochondria

1. Pathways of Oxidation

Pyruvate, malate and OAA can be imported into the mitochondrial matrix via carriers present on the inner mitochondrial membrane (Day and Wiskich, 1984; Fig. 3). Malate uptake could occur via the dicarboxylate carrier which catalyses malate/Pi exchange. On the other hand, plant mitochondria also contain a specific transporter which catalyses OAA uptake in exchange for either malate or citrate (Heldt and Flügge 1987; Hanning et al., 1999), and this transporter may be involved in transfer of reductant and carbon skeletons out of the mitochondrion (Woo and Osmond, 1976; Day and Wiskich, 1981; Zoglowek et al., 1988).

Malate is present in high concentrations in leaves (although much of it is in the vacuole) (Gerhardt and Heldt, 1984; Heineke et al., 1991) and can serve as an important respiratory substrate (Lance and Rustin, 1984). Malate levels increase during photosynthesis (Gerhardt et al., 1987; Heineke et al., 1991; Xue et al., 1996), as a consequence of both the export of redox equivalents from the chloroplast via the malate/

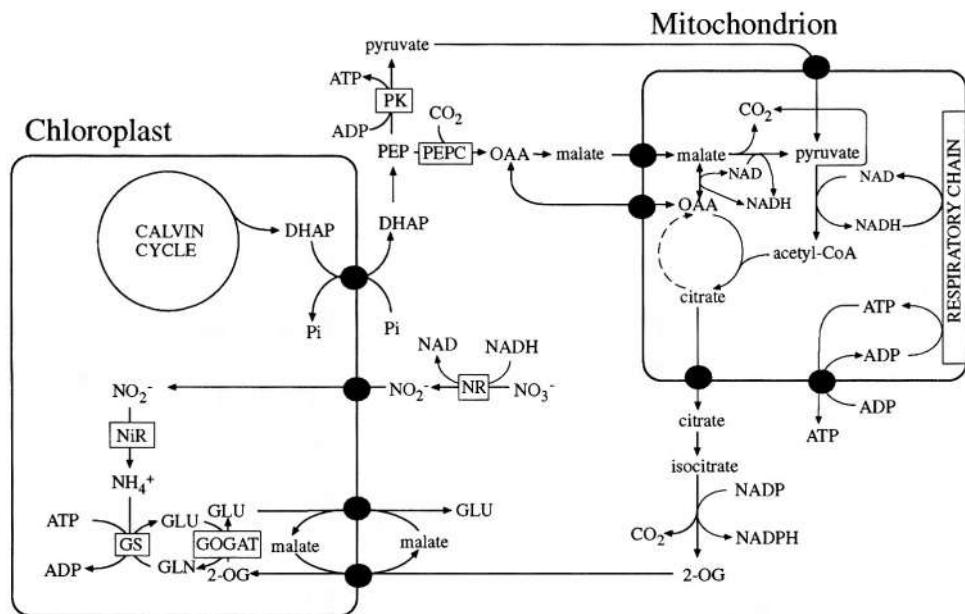


Fig. 3. Metabolic interactions between mitochondria and chloroplasts in leaf cells. Solid circles represent transporters; note that citrate and malate transport across the mitochondrial inner membrane could occur via the tricarboxylate and dicarboxylate carriers, respectively, or via the OAA carrier in exchange for OAA. It is also possible that 2-oxoglutarate leaves the mitochondrion directly, in place of citrate. see text for details.

OAA shuttle (Heineke et al., 1991) and PEP carboxylase/MDH activity (Hill and Bryce, 1992). Martinoia and Rentsch (1994) concluded that because cellular concentrations of malate decrease in the dark, vacuolar malate is likely to be used by the TCA cycle as a substrate. However, the capacity for malate import into isolated mitochondria appears to be considerably lower than the capacity for OAA import (Zoglowek et al., 1988; Krömer, 1995) and OAA is potentially an alternative substrate for mitochondria. The greater activity of the OAA transporter may be off-set in vivo by the very low concentration of this metabolite in the cytosol.

If malate is imported, it can be converted both to OAA by MDH and to pyruvate via NAD-ME. The same enzymes can make pyruvate from OAA if the matrix NADH concentration is high. The advantage of producing both substrates within the mitochondrion is that carbon intermediates can be exported from the TCA cycle; for example, citrate or 2-oxoglutarate can be exported to support GOGAT activity in the chloroplast (Fig. 3). Pyruvate can also be imported into the mitochondrion, to feed into a complete or partial TCA cycle (Fig. 3).

2. Mitochondrial Electron Transport

Partial or complete operation of the TCA cycle yields

NADH which is oxidized by the respiratory chain to produce ATP (Fig. 4). The amount of ATP formed will depend on the activity of the non-phosphorylating by-passes of the respiratory chain.

Two NADH-oxidizing enzymes are available from the matrix of mitochondria. A rotenone-insensitive NADH dehydrogenase exists on the matrix side of the inner mitochondrial membrane in plants, which does not pump protons and bypasses the energy-conserving complex I (Fig. 4). The activity of this bypass appears to be largely governed by the concentration of NADH in the matrix, since it has a significantly lower affinity for NADH than does complex I (Møller and Lin, 1986). When substrate supply to the mitochondrion is high, the bypass will contribute to NADH oxidation and decrease ATP production rates. These conditions (high matrix NAD(P)H, high internal pyruvate concentration and reduced ubiquinone) will also lead to activation of the non-phosphorylating alternative oxidase (AOX) of the plant respiratory chain, further decreasing ATP production. These two bypasses of the proton translocating respiratory complexes could allow leaf cells to oxidize mitochondrial NADH when cytosolic adenylate levels limit the cytochrome path (or it is overwhelmed) and may act as safety valves, preventing over reduction of the mitochondrial NAD and ubiquinone pools and avoiding generation of

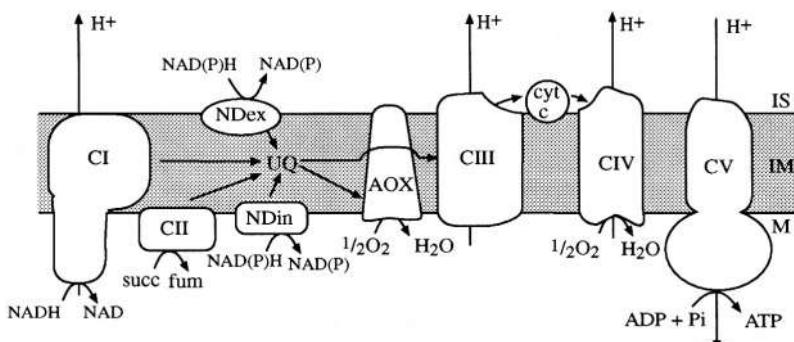


Fig. 4. Schematic representation of the plant respiratory electron transport chain. CI: complex I; CII: complex II (succinate dehydrogenase); CIII: complex III (cytochrome b/c_1 complex); CIV: complex IV (cytochrome oxidase); CV: complex V (ATP synthase); NDex: external NAD(P)H dehydrogenase(s); NDin: internal, rotenone-insensitive NADH dehydrogenase; IS: intermembrane space; IM: inner membrane; M: matrix; succ: succinate; fum: fumarate; AOX: alternative oxidase; Cyt: cytochrome.

reactive oxygen species (Purvis and Shewfelt, 1993, Wagner and Krab, 1995, Millar and Day 1997).

AOX activity appears to be regulated by a sophisticated feed-forward activation mechanism. AOX exists as a dimer which under oxidizing conditions is covalently linked by disulfide bonds (Umbach and Siedow, 1993; Umbach et al., 1994). The AOX protein must be reduced in order to be activated (Umbach and Siedow, 1993), probably via matrix NADPH in a thioredoxin-mediated reaction (Vanlerberghe et al., 1995; Day and Wiskich, 1995). This reduction process may involve the interconversion of S-S to -SH groups in a particular cysteine residue on the protein, although this has not been conclusively demonstrated. The reduced form of AOX can be stimulated by μM concentrations of pyruvate and some other 2-oxo acids, such as glyoxylate (Millar et al., 1993; Day et al., 1995; Millar et al., 1996). The substrate for AOX is ubiquinol (Q_r). Stimulators such as pyruvate appear to alter the interaction between Q and AOX, so that the oxidase is active at lower levels of reduced Q_r. The cytochrome and alternative oxidases have been shown to compete for electrons from Q when AOX is activated by pyruvate (Hoefnagel et al., 1995; Ribas Carb et al., 1995).

Not all species express AOX constitutively in their leaves (in such plants AOX synthesis is usually triggered by environmental stress: McIntosh, 1994). In those that do, the oxidase is likely to be active under photosynthetic conditions when the cell redox pools are highly reduced, particularly in combination with plentiful supply of substrates and metabolites such as pyruvate and glyoxylate. The latter may be an important feed-forward activator during photorespiration. Recent studies of respiration in a period

following illumination indicate that AOX is activated in the light (Igamberdiev et al., 1997a). There is also some evidence that AOX synthesis and contribution to respiratory oxygen uptake increases in photosynthetic organs during greening. For example, exposure of etiolated Belgian endives to light causes induction of cyanide-insensitive respiration within 24 h (Atkin et al., 1993). In soybeans, measurements using the non-invasive ^{18}O discrimination technique (Guy et al., 1992) have shown that AOX contribution to dark respiration increases during greening of cotyledons (Ribas-Carb et al., 1997; Robinson et al., 1995) and the amount of AOX protein also increases (Finnegan et al., 1997).

E. Glycine Oxidation During Photorespiration and Interactions with the TCAC

The photorespiratory glycolate cycle provides a major mitochondrial substrate in photosynthetic tissues. Previous chapters have outlined how under ambient atmospheric conditions P-glycolate is produced via the oxygenase reaction of Rubisco in the chloroplast: this oxygenase reaction accounts for 20–35% of the net photosynthetic activity (Lorimer and Andrews, 1981; Dry et al., 1987). P-glycolate is converted to glycolate and exported to the peroxisome, where the glycolate is converted to glycine; this in turn is exported to mitochondria to be oxidized as a respiratory substrate.

The degree to which photorespiration contributes to mitochondrial electron transport (and thus ATP synthesis) will depend on the degree to which NADH produced by glycine decarboxylation is exported to the cytosol (via the OAA-malate shuttle mechanism) or oxidized within the mitochondria. Maintenance of

the photorespiratory cycle requires that equal amounts of NADH be reoxidized in the peroxisome (during the conversion of OH-pyruvate to glycerate) as is released during mitochondrial glycine decarboxylation (Leegood et al., 1995). In vivo, it is likely that at least some of the NADH produced by glycine oxidation is oxidized in the peroxisome, since the malate/OAA shuttle capacity in isolated mitochondria exceeds the capacity of GDC (Ebbighausen et al., 1987). However, if the peroxisome requirements for NADH can be met partly by glycolysis or the chloroplast (see above), then oxidation of glycine can contribute to mitochondrial ATP synthesis. In experiments with isolated mitochondria under simulated *in vivo* conditions, Krömer et al., (1992) estimated that 25%–50% of NADH from glycine oxidation was shuttled out of the mitochondria. In line with this, the cytosolic ATP/ADP ratio in barley protoplasts was higher under photorespiratory than in non-photorespiratory conditions (Gardeström and Wigge, 1988).

In vivo, it is probable that photorespiration and the TCA cycle operate side-by-side. However, it is imperative that glycine oxidation in the mitochondria proceeds as rapidly as possible to prevent accumulation of toxic intermediates in the photorespiratory cycle. In isolated leaf mitochondria, glycine oxidation takes precedence over the oxidation of other mitochondrial substrates (Dry et al., 1983; Day et al., 1985; Igamberdiev et al., 1997b). This preferential oxidation of glycine is partly achieved by a dominance of complex I over both complex II (SDH) and the external NADH dehydrogenase of the respiratory chain, ensuring that matrix NADH is rapidly oxidized (Day et al., 1985). GDC also effectively competes against the TCA cycle dehydrogenases for NAD⁺ in the matrix (Day et al., 1985). TCA cycle activity may, therefore, be limited under photorespiratory conditions by NAD⁺ availability and inhibition of succinate oxidation. Measurements on barley protoplasts have shown that the matrix NADH/NAD pool is more reduced under photorespiratory conditions than in non-photorespiratory conditions (Wigge et al., 1993).

Concurrent oxidation of malate and glycine poses other problems for leaf mitochondria if GDC is linked to a malate/OAA shuttle to the peroxisome; under these conditions malate must be both taken up and exported from the mitochondria. This dilemma could be solved by separate but simultaneous operation of the dicarboxylate and OAA carriers.

However, under these conditions mitochondrial MDH must function simultaneously in different directions (malate > OAA in the TCA cycle and OAA > malate in the shuttle). A study of pea leaf mitochondria by Wiskich et al. (1990) points to a possible solution. These authors suggested that the mitochondrial matrix consists of metabolic domains in which groups of MDH enzymes are associated in discrete protein complexes termed ‘metabolons.’ Substrate channeling in these metabolons would allow MDH to operate simultaneously in opposite directions, within the same mitochondrion. Alternatively, OAA could be the main substrate taken up by the mitochondria. An OAA_{in}/malate_{out} exchange could shuttle excess redox equivalents out of the mitochondria and a fraction of OAA could condense with acetylCoA (formed from pyruvate either taken up from the cytoplasm or produced from malate by ME). In such a scheme MDH would only operate in the OAA > malate direction and the uptake of OAA would be linked to either malate or citrate export.

The concept of metabolons offers a possible explanation for the continued operation of the TCA cycle in the face of large amounts of photorespiratory NADH produced in the light in the mitochondrial matrix. High NADH levels can restrict activity of the various TCA cycle dehydrogenases (Dry and Wiskich, 1985) but kinetic separation of the GDC-MDH complex from the other TCA cycle enzymes might reduce this inhibitory potential.

III. Mitochondrial Function in the Light and Dark

Light increases the cellular demand for TCA cycle carbon skeletons (e.g., for ammonia assimilation), NADH (e.g., for the reduction of NO₃⁻ in the cytosol and OH-pyruvate in the peroxisomes) and ATP (e.g., for NH₄⁺ assimilation and sucrose synthesis), in leaf cells. Moreover, decarboxylation of glycine to serine releases substantial amounts of CO₂ and potentially contributes to O₂ consumption. All of this suggests that respiration rates might be greater in the light than in the dark; yet measurements of respiratory CO₂ release often suggest the opposite. In this section we assess the response of mitochondrial CO₂ release and O₂ uptake in leaves in the light.

Measurements of respiratory gas exchange in the dark are relatively straight forward, but the same cannot be said for measurements of respiration in the

light. In the light, photorespiratory and non-photorespiratory reactions result in mitochondrial O_2 consumption, while O_2 is produced via photosynthesis. Moreover, O_2 is also consumed in the chloroplast as a result of Rubisco oxygenation and the Mehler reaction. While definitive measurements of Mehler reaction rates are lacking, available data suggests that it does not contribute significantly to O_2 consumption in the light in leaves of some plants (Peltier and Thibault, 1985) and in algae (Xue et al., 1996). Determinations of TCA cycle CO_2 release in the light are complicated by the presence of other CO_2 releasing and assimilating reactions. For example, in the light CO_2 is fixed by Rubisco and PEP carboxylase, whereas CO_2 is released by glycine decarboxylation, the TCA cycle and the OPP pathway. Measurements of respiratory activity, therefore, need to account for each of the above processes.

A. Mitochondrial O_2 Consumption in the Light

Estimates of mitochondrial O_2 consumption in the light often yield conflicting results and have indicated it to be unaffected, inhibited or even stimulated (Turpin and Weger, 1989). For example, $^{16}O_2/^{18}O_2$ analysis of oxygen exchange in *Dunaliella tertiolecta* indicated that mitochondrial O_2 consumption is inhibited by light (Bate et al., 1988; Canvin et al., 1980). In contrast, similar analysis in carnation cells has indicated that mitochondrial O_2 consumption is similar in the light and dark (Peltier and Thibault, 1985; Avelange et al., 1991), and several studies have reported that light increases mitochondrial O_2 consumption in green algae (Xue et al., 1996).

This conflict in mitochondrial response to light probably reflects the variability in provision of carbon from photosynthesis, the degree to which photorespiratory NADH is consumed in the mitochondria, and the degree to which excess photosynthetic redox equivalents are exported from the chloroplast. These factors in turn are likely to vary with the experimental conditions employed (especially light intensity and temperature). In green alga, the rate of mitochondrial O_2 consumption in the light is greater at high irradiance values than at low irradiance (Xue et al., 1996). Xue et al. (1996) concluded that export of excess photosynthetic redox equivalents provides NADH to the mitochondria in an irradiance-dependent manner. Taken together, the available studies suggest that mitochondrial O_2 consumption can indeed be maintained or even stimulated in the

light, although substantial genetic and environmental variation can occur.

B. Mitochondrial CO_2 Release in the Light

1. ^{14}C -Labeling Measurements

Under photorespiratory conditions (e.g., 21% O_2 , 350 ppm CO_2), total mitochondrial CO_2 release is greater in the light than darkness due to the additional CO_2 release associated with glycine decarboxylation. However, there is growing evidence that non-photorespiratory mitochondrial CO_2 release (R) is inhibited in the light.

Using a ^{14}C -labeling, pulse-chase technique, Pärnik and Keerberg (1995) demonstrated that R is lower in the light than in darkness in several species. This method rests on the assumption that respiration is not affected by decreasing the concentration of oxygen from 21% to 2%. It is able to distinguish between four components of CO_2 release in leaves, depending on the nature of the carbon compounds produced (primary products such as triose-P, malate and glycine released rapidly from the chloroplast vs. secondary end products such as sucrose and starch: see Section II) and the decarboxylation pathway employed to release the CO_2 (i.e. photorespiratory vs. (non-photo)respiratory).

Pärnik and Keerberg (1995) reported that respiratory decarboxylation of end products in the leaves in the dark was 0.64, 1.27 and 0.6 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for wheat, tobacco and barley, respectively. In the light, respiratory decarboxylation of these products was reduced by 60–70% (to 0.27, 0.45 and 0.23 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for wheat, tobacco and barley, respectively (Pärnik and Keerberg, 1995). This decrease was partly offset by the respiratory decarboxylation of photosynthetic primary products, which amounted to 0.28, 0.23 and 0.04 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in the light, respectively for the three species above. Consequently, the total rate of respiratory decarboxylation in the light was 0.55, 0.68 and 0.27 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for wheat, tobacco and barley. Light, therefore inhibited total oxidative decarboxylation by 14, 46 and 55% in wheat, tobacco and barley, respectively (Pärnik and Keerberg, 1995).

The inhibition of total respiratory decarboxylation in these species apparently resulted from a combination of lower rates of non-photorespiratory decarboxylation of end products in the light and relatively low rates of primary product use (or

provision). Separate experiments using ^{14}C labeled carbon have confirmed that R is inhibited by light in wheat (McCashin et al., 1988). However, light was found to stimulate total respiratory decarboxylation in winter rye by 31% (Hurry et al., 1996), showing that light does not necessarily inhibit R in all species.

2. Laisk Method Measurements

The hypothesis that light inhibits R is supported by studies using the Laisk (1977) method for measuring R . For example, light inhibits R in spinach, barley, tobacco, *Eucalyptus* sp., *Poa* spp., as well as in evergreen and deciduous shrubs (Brooks and Farquhar, 1985; Kirschbaum and Farquhar, 1987; Atkin et al., 1998a,b; Villar et al., 1994; 1995). The Laisk method analyzes the rate of net CO_2 exchange (A) at low internal CO_2 concentrations (c_i) and varying irradiances. A is related to R in the light (R_d) according to:

$$A = v_c - 0.5v_o - R_d$$

where v_c and v_o are the rates of carboxylation and oxygenation of Rubisco, respectively. Photorespiration results in one molecule of CO_2 being released for each two molecules of O_2 that are consumed by Rubisco. Figure 5 shows how decreases in c_i lead to decreased CO_2 fixation (v_c) and increased CO_2 release ($0.5v_o$). Decreasing c_i eventually results in the CO_2 fixed by Rubisco being matched by the CO_2 that is released by glycine decarboxylation: i.e., v_c and $0.5v_o$ are equivalent. This point is termed Γ_* . R_d is the rate of CO_2 release at Γ_* . Non-photorespiratory mitochondrial CO_2 release can be measured, therefore, by exposing illuminated leaves to Γ_* and measuring the net assimilation rate (A_{net}).

To determine both Γ_* and R_d , regressions of A_{net} versus c_i at different irradiances must be constructed, as shown in Fig. 6 (Atkin et al., 1997). These regressions intersect at a c_i equal to Γ_* , and R_d is the rate of CO_2 exchange at that c_i . The irradiance values used to construct such regressions should be at least 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, as R_d decreases sharply at lower irradiance values (Brooks and Farquhar, 1985; Atkin et al., 1998a).

The Laisk method assumes that R_d remains constant across a wide range of c_i values. It also assumes that R_d is not substrate limited during prolonged exposure to low CO_2 concentrations. Figure 5 demonstrates that CO_2 uptake by Rubisco is severely limited at Γ_* .

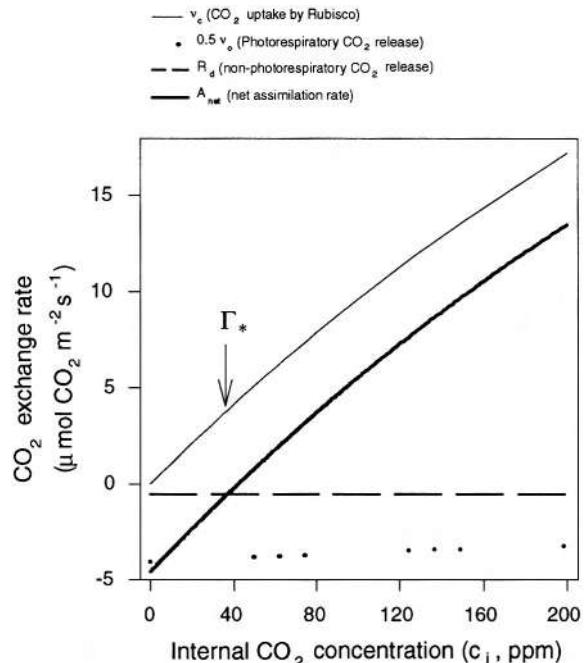


Fig. 5. Modeled CO_2 exchange ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) versus CO_2 internal concentration (c_i) for tobacco leaves. The thin solid line represents the rate of CO_2 uptake by Rubisco (i.e. v_c), the dotted thin line represents the rate of photorespiratory CO_2 release (i.e. $0.5v_o$) and the thin dashed line represents the rate of non-photorespiratory CO_2 release (R_d). The thick solid line represents the net assimilation rate (i.e. $v_c - 0.5v_o - R_d$). Data for this figure was kindly provided by Dr J. Evans, RSBS, ANU.

Hence, supply of primary photosynthetic substrates to the mitochondria could potentially become limiting with time. However, experiments using a fast-response gas exchange system to rapidly expose tobacco leaves to Γ_* , following a period of photosynthesis at 350 ppm CO_2 , have recently demonstrated that R_d is not substrate limited at Γ_* (Atkin et al., 1998a).

3. Kok Method Measurements

Another method for determining the rate of R in the light is the Kok (1948) method. This approach requires that net CO_2 exchange be measured at a set CO_2 partial pressure and at several irradiance levels, as shown in Fig. 7. Decreases in irradiance (starting at 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) initially result in linear decreases in net assimilation (Fig 7). However, there is a break in this linear response as net assimilation approaches zero, with the slope increasing markedly at lower irradiance levels. The measured rate of gas exchange at zero irradiance is R_n (shown as the

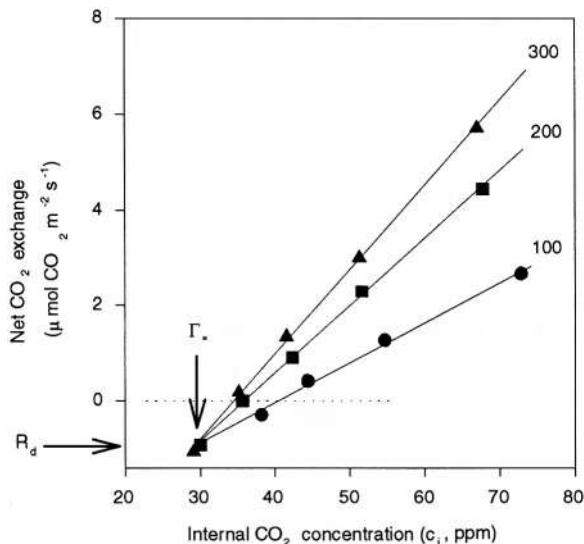


Fig. 6. Net CO_2 exchange rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) versus CO_2 internal concentration (c_i) for *Poa compressa* leaves. Numbers indicate the incident PPFD under which each set of measurements was made. Lines represent the linear regressions at each light intensity. The rate of non-photorespiratory mitochondrial CO_2 release in the light (R_d) and Γ_* are indicated. Based on Atkin et al. (1997).

closed square in Fig 7). Estimates of R in the light (R_d) are obtained by extrapolating the linear section down to zero irradiance (i.e. the intercept with the axis of net assimilation, shown as an open square). The break from linearity is most likely the result of R no longer being inhibited by light at low irradiance levels: as irradiance decreases, the degree of light inhibition of R also decreases. The irradiance at which the plot breaks from linearity provides an estimate of the irradiance at which R is fully inhibited by light. Several studies have used the Kok method to demonstrate that R_d is lower than R_n (Freyer and Wiesberg, 1975; Kirschbaum and Farquhar, 1987; Sharp et al., 1984; Villar et al., 1994).

One problem with the Kok method is that the decrease in irradiance during the measurements can result in a gradual increase in c_i (which suppresses photorespiration and increases carboxylation) and a concomitant relative increase in the rate of net assimilation in the linear region (Villar et al., 1994). As a result, the slope of the linear region plotted through observed data is less than it would be if c_i has stayed constant (Kirschbaum and Farquhar 1987; Villar et al., 1994). In Fig. 8, the solid line represents the regression through the observed data in the linear region, with the dotted line showing the continuation

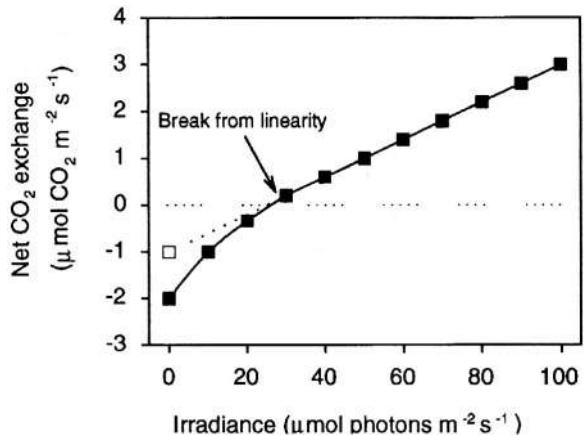


Fig. 7. Net CO_2 exchange rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) versus irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) to demonstrate the Kok effect. Typically observed data points are shown (solid circles), as is the point where the observed data break from linearity. The dotted line indicates the expected rates of net CO_2 exchange if the data points had not broken from linearity. The open square represents the expected rate of non-photorespiratory CO_2 release (R_d) in the light, whereas the solid square shows the rate of respiration in darkness (R_n). The data points are hypothetical examples, based on data reported by Kirschbaum and Farquhar (1987) and Villar et al. (1994).

of the linear region. The three dashed lines present the hypothetical net assimilation rates that would have occurred had c_i been kept constant at the values indicated. The three dashed lines emanate from the true R_d value (as shown) which is more negative than the apparent R_d value estimated from the solid line (Kirschbaum and Farquhar, 1987). The Kok method can, therefore, result in underestimates of the true R_d value. Theoretically, the Kok effect (break from linearity) could be seen even when R_d and R_n are identical (due to the slope at higher irradiances being affected by the changing c_i). The true R_d value can be obtained by correcting the apparent R_d value to a constant c_i using the program reported by Kirschbaum and Farquhar (1987).

Villar et al. (1994) compared the Kok and Laisk methods. They found the Kok method consistently resulted in lower estimates of R_d , even following correction for changing c_i . Moreover, estimates of R_d obtained using the Laisk method were more consistent than estimates using the Kok method and it is sometimes very difficult to detect the change in slope that occur during the Kok measurements. Kok measurements of R_d can take over five hours to complete, whereas measurements of R_d obtained with the Laisk method are complete within two

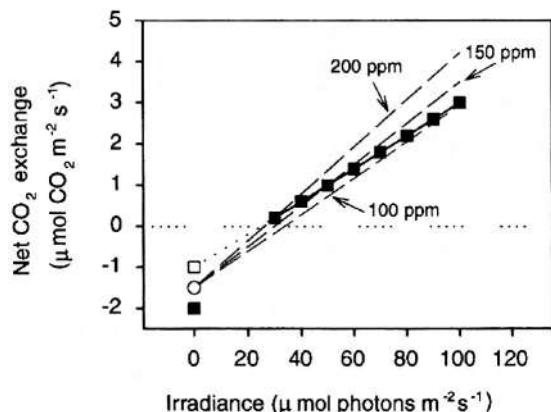


Fig. 8. The effect of different internal CO_2 concentrations on the estimation of R_d , using the Kok method. Net CO_2 exchange ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$) is plotted versus irradiance. Two sets of curves are shown. The solid line represents the regression through observed data in the linear region when c_i is not kept constant, whereas the three dashed lines represent the hypothetical net CO_2 exchange rates that would have occurred had c_i been kept constant at the values indicated. The three dashed lines emanate from the actual R_d value (open square) which is more negative than the apparent R_d value (open circle) estimated from the solid line. The rate of respiration in darkness (R_n) is shown by the closed square. Based on data reported by Kirschbaum and Farquhar (1987).

hours (Villar et al., 1994). It is therefore preferable to use the Laisk method to estimate R_d , but both methods have indicated that mitochondrial CO_2 output is decreased in the light in leaves.

C. Effect of Light to Dark Transitions on Respiration

Illuminated leaves often exhibit large transient increases in O_2 consumption and CO_2 release when first exposed to darkness (Fig. 9). This rapid increase is termed the photorespiratory post-illumination burst (PIB). It is followed by a second slower rise in R , which is termed light-enhanced-dark-respiration (LEDR). Some post-illumination studies have incorrectly assigned PIB to increases in R that are clearly LEDR in nature. The two peaks in respiration occur at separate times, differ in magnitude and are caused by quite separate physiological events.

The PIB peak represents CO_2 release by photorespiratory glycine decarboxylation. While RuBP carboxylation decreases immediately after darkening, glycine decarboxylation does not begin to decrease for several seconds (presumably due to the time taken for photorespiratory products to reach the mitochondria). Usually, the PIB peak is seen about

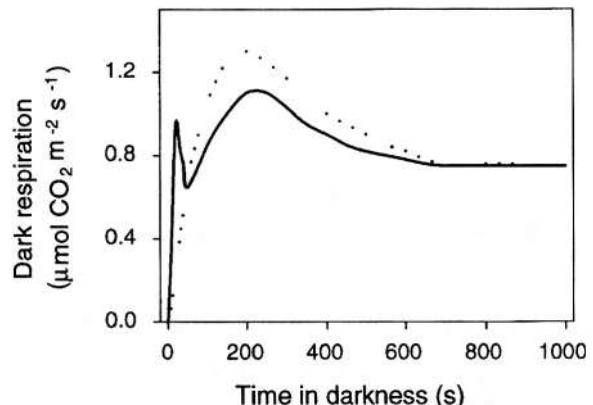


Fig. 9. Effect of darkness on net CO_2 exchange ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$) of a tobacco leaf exposed to 350 ppm CO_2 and 21% O_2 (solid line) or 2% O_2 (dashed line). In both cases the light was switched off at time zero following 10 min of illumination with 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Based on Atkin et al. (1998a)

20 s after the light is switched off (Bulley and Tregunna, 1971; Doehlert et al., 1979; Atkin et al., 1998a,b). The idea that PIB is photorespiratory in origin is supported by the fact that this peak does not occur in leaves exposed to low O_2 (e.g., 2%; Fig 9) or high CO_2 concentrations that inhibit the oxygenation reaction of Rubisco (Bulley and Tregunna, 1971; Doehlert et al., 1979; Atkin et al., 1998a). The size of the PIB peak is increased under conditions of high glycolate production (low CO_2 , high light: Doehlert et al., 1979; Atkin et al., 1998a).

LEDR, on the other hand, reflects an increased supply of non-photorespiratory substrates (e.g. pyruvate and/or malate) which accumulate during photosynthesis (Raghavendra et al., 1994). Although LEDR is often detected as increased O_2 consumption (Azcon-Bieto et al., 1983; Reddy et al., 1991; Gardeström et al., 1992; Hill and Bryce, 1992; Xue et al., 1996; Igamberdiev et al., 1997a), transient increases in CO_2 evolution have also been reported (Xue et al., 1996; Atkin et al., 1998a,b). The magnitude of the LEDR peak is increased when photorespiration is decreased under low O_2 (Atkin et al., 1998a; Igamberdiev et al., 1997a). The magnitude of LEDR also increases with increasing irradiance during the preceding light period (Atkin et al. 1998a,b). LEDR therefore appears to reflect the level of photosynthetic metabolites available to the mitochondria following a period of illumination (Hill and Bryce, 1992; Xue et al., 1996; Igamberdiev et al., 1997a; Atkin et al., 1998a,b; Padmasree and Raghavendra, 1998).

D. Mechanisms Responsible for LEDR

The mechanism leading to sustained, enhanced respiration after a period of illumination has not been determined, but the results of Hill and Bryce (1992) suggest that LEDR may result from an increase in malate oxidation after illumination. Malate pools in leaf cells are high at the commencement of the dark period (Hill and Bryce, 1992; Xue et al., 1996), and the activity of NAD-ME and PDC increase in the dark. Consequently, malate concentrations decrease during lengthy dark periods and respiration rates fall concurrently (Hill and Bryce, 1992; Xue et al., 1996). The corollary of this is that inactivation of ME and PDC underlies the decrease in R in the light (see below).

The mechanism responsible for the light inhibition of NAD-ME is unresolved. The enzyme remains inactive during isolation of mitochondria from illuminated leaves and subsequent gel filtration of mitochondrial extracts. There is no evidence that NAD-ME can be phosphorylated, but other covalent modifications, or altered binding of cofactors, could be involved (Cook et al., 1995). Leaf mitochondrial PDC, on the other hand, can be reversibly inactivated in the light (Randall et al., 1990), due to phosphorylation of the enzyme (Budde and Randall, 1987; Schuller and Randall, 1989). The inhibition of PDC activity mainly occurs under photorespiratory conditions (Budde and Randall, 1990; Gemel and Randall, 1992). For example, exposure of pea leaves to 1500 ppm CO_2 (to inhibit photorespiration) results in a 60% to 80% decrease in the light-dependent inactivation of PDC (Gemel and Randall, 1992). The photorespiration-dependent inhibition of PDC may be due to NH_3 (produced during glycine decarboxylation) stimulation of the protein kinase which phosphorylates PDC (Budde and Randall, 1987; Krömer, 1995). Increased ATP synthesis due to increased electron transport during glycine oxidation could also contribute to PDC inactivation (Moore et al., 1993). In this context, Lernmark and Gardeström (unpublished) found that the degree of photorespiration-dependent inactivation of PDC is less under conditions which decrease mitochondrial ATP synthesis. The light dependent inactivation of PDC has been reported in pea, zucchini, soybean, barley, tobacco and maize (Gemel and Randall, 1992). However, other studies of barley protoplasts showed no inactivation of PDC under photorespiratory conditions (Krömer et al., 1994). The difference

between species might simply reflect varying degrees of contamination with plastid PDC isoenzymes which are not inactivated by phosphorylation (Lernmark and Gardeström, 1994).

E. Relationship Between Light Inhibition of Mitochondrial CO_2 Release (R) and LEDR

The mechanism by which R is inhibited in the light remains to be elucidated. However, it is interesting that both LEDR and light inhibition of R are equally sensitive to increasing irradiances in the light period (Atkin et al., 1998a). R is inhibited at irradiance values as low as 3 μmol photons $\text{m}^{-2} \text{s}^{-1}$ (Brooks and Farquhar, 1985; Atkin et al., 1998a,b), and this is also the lowest irradiance which results in significant LEDR (Atkin et al., 1998a). Moreover, both parameters are insensitive to light quality (Xue et al., 1996; Atkin et al., 1998a) and are tightly correlated (Atkin et al., 1998a,b). It is possible, therefore, that both phenomena have a common cause, and the Hill and Bryce (1992) model for LEDR may also provide a mechanism for inhibition of R in the light. According to this hypothesis, inhibition of R in the light results from limited flux through ME and PDC. Inactivation of PDC alone by light would limit the conversion of pyruvate to acetyl CoA (and thus TCA cycle CO_2 release), regardless of whether the pyruvate resulted from the action of ME or pyruvate kinase (PK). Coordinated inactivation of both ME and PDC would further strengthen the inhibitory effect light has on pyruvate flux into the TCA cycle, especially in tissues where malate is an important carbon source for the mitochondria (see below). Inactivation of ME/PDC might also explain why light inhibits R so rapidly (within 50 seconds; Atkin et al., 1998a,b): the timing of inactivation of ME (Hill and Bryce, 1992) closely mirrors the 50 s taken for light to fully inhibit R (Atkin et al., 1998a,b). Measurements of the irradiance dependence of ME and PDC activation state are needed to test this hypothesis.

F. Role of Nitrogen Assimilation in Light Inhibition of R

Enhanced export of TCA cycle carbon intermediates to the cytosol during light-dependent nitrogen assimilation may also contribute to inhibition of R in the light. TCA cycle CO_2 release would be substantially reduced under conditions where 2-oxoglutarate and/or citrate are removed to support

amino-acid synthesis. In the absence of increased flux of carbon into the TCA cycle, removal of citrate or 2-oxoglutarate would eliminate one site of CO_2 release. Support for this suggestion comes from the fact that the CO_2 compensation point of barley leaves increases when plants are transferred from NO_3^- to NH_4^+ nutrient (Fair et al., 1972). NH_4^+ is not transported from roots to shoots but rather is assimilated in the roots. As a result, transfer to NH_4^+ growth media would largely eliminate leaf N-assimilation and thereby decrease the demand for TCA cycle intermediates in the leaves. This in turn could result in an increase in TCA cycle CO_2 release and the CO_2 compensation point. Further work is required to test this hypothesis.

G. Mitochondrial Electron Transport and ATP Production in the Light

The fact that photosynthesis itself results in ATP synthesis has led several authors in the past to suggest that mitochondrial electron transport might be more limited by high ATP/ADP ratios in the light than in the dark (Graham, 1980; Villar et al., 1995). However, this does not appear to be the case. At saturating CO_2 concentrations, cytosolic ATP/ADP ratios are lower in the light than in darkness (Hampp et al., 1982; Stitt et al., 1982; Krömer, 1995). While cytosolic ATP/ADP ratios are higher in the light than in darkness when CO_2 limits photosynthesis (Gardeström, 1987; Krömer, 1995), they are not sufficiently high to result in adenylate restriction of respiration (Krömer, 1995). Krömer (1995) concluded that, despite some contribution by photophosphorylation, most of the cytosolic ATP pool is maintained by mitochondrial oxidative phosphorylation. Indeed, respiratory chain activity in the light appears to be critical for provision of ATP necessary for energy demanding processes in the light.

There is also mounting evidence that respiratory ATP production in the light is required to maintain maximum photosynthetic activity (Krömer and Heldt, 1991; Krömer et al., 1993; Krömer, 1995). The link between mitochondrial ATP synthesis and photosynthesis probably occurs via the energy demands of sucrose synthesis: ATP is required for the synthesis of UTP, which in turn is required for UDP-glucose synthesis (Fig. 10). Using the mitochondrial electron transport inhibitor oligomycin, Krömer et al. (1993) showed that inhibition of mitochondrial ATP synthesis caused a decline in cytosolic ATP/ADP ratios and a

substantial decrease in SPS activity.

Under high irradiance, the inhibition of sucrose synthesis by low ATP supply results in a feedback inhibition of photosynthetic carbon fixation and photosynthetic electron transport (Krömer et al., 1993). Other studies have also demonstrated that limitations in sucrose synthesis can have a feedback inhibitory effect on photosynthesis (Woodrow and Berry, 1988). The degree of inhibition of regulatory enzymes is, however, dependent on the metabolic status of a leaf. For example, at intermediate light intensities, the oligomycin-mediated decrease in mitochondrial and cytosolic ATP/ADP ratios results in a reduced SPS activity, but does not result in a reduced photosynthetic activity (Krömer et al., 1993). At non-saturating irradiance, photon input limits photosynthesis rather than the activities of the regulatory enzymes (Krömer et al., 1993). As a result, limitations in mitochondrial ATP synthesis do not result in feedback inhibition of photosynthetic activity.

In addition to being required for sucrose synthesis, mitochondrial ATP is also likely to be required for sucrose export from source leaf cells. Sucrose export costs account, on average, for 29% of total dark respiration in several starch storing plant species (Bouma et al., 1995).

H. Role of Mitochondria During Photoinhibition

Respiration may also help a plant cope with excess photosynthetic redox equivalents (i.e. NADPH). A high NADPH/NADP ratio in the chloroplast can lead through other electron transfer components to the generation of reactive oxygen intermediates (ROI) (Purvis and Shewfelt, 1993; Purvis 1997). A number of studies have indicated that the degree of reduction of the NADP pool in the chloroplast can be decreased by export from the chloroplast via the OAA-malate and/or DHAP-PGA shuttles, followed by oxidation in the mitochondria (Saradadevi and Raghavendra, 1992; Shyam et al., 1993; Hurry et al., 1995; Padmasree and Raghavendra, 1998). It has recently been shown that inhibition of mitochondrial electron transport results in a delay in photosynthetic induction accompanied by increased reduction of chloroplast electron carriers (Igamberdiev et al., 1998). When overreduction of the cell leads to photoinhibition, mitochondrial electron transport can be important also. For example, the increase in photoinhibition in the cyanobacteria *Anacystis nidulans* (Shyam et al.,

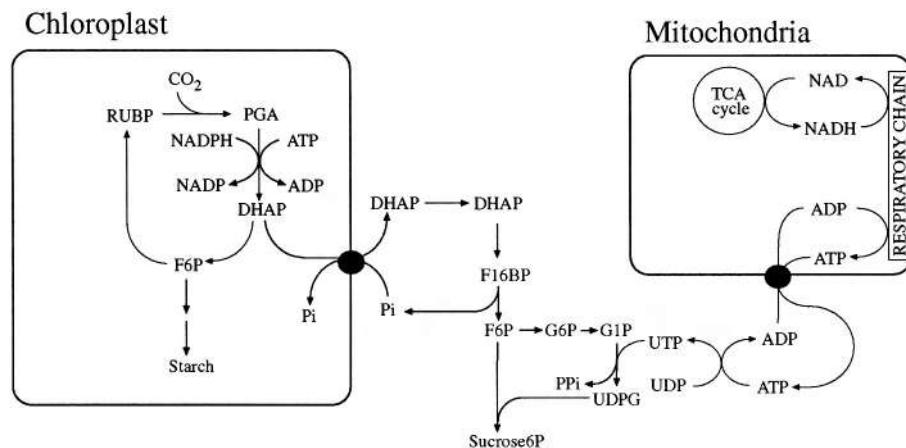


Fig. 10. Mitochondria supply ATP for sucrose synthesis in leaf cells in the light. See text for details.

1993) and pea (Saradadevi and Raghavendra, 1992) following inhibition of mitochondrial respiration, appears to be due in part to the cessation of mitochondrial oxidation of photosynthetic NADPH.

The importance of oxidizing excess photosynthetic redox equivalents in the mitochondria may increase at cold temperatures. Low temperatures decrease the demand for NADPH in the chloroplast and increase the potential for photoinhibition (Ball et al., 1991). Increased oxidation of NADPH by respiration would help reduce the potential for severe photoinhibitory damage. There is evidence that this occurs. Firstly, prolonged exposure to low temperatures often results in an increase in respiratory capacity in leaves (Körner and Larcher, 1988). Secondly, it appears that mitochondria of cold-hardened leaves oxidize greater amounts of photosynthetic redox equivalents in the light than non-hardened leaves (Hurry et al., 1995). The latter conclusion was based on the observation that oligomycin, in winter rye, resulted in a greater decrease in maximum photosynthesis in cold-hardened leaves than in non-hardened leaves. This decrease in photosynthesis was not mediated via a decrease in mitochondrial ATP synthesis. Rather, oligomycin inhibited photosynthesis of the cold-hardened leaves by limiting regeneration of RuBP. However, the mechanism behind this effect was not clear.

The pathway of mitochondrial electron transport via which photosynthetic redox equivalents are oxidized will have a large impact on the amount of ATP synthesized in a cell. For example, ATP synthesis via the cytochrome pathway might be beneficial if there is a demand for additional energy in tissues

exposed to photoinhibitory conditions (e.g., for UTP generation and/or protein repair). Photoinhibition of Photosystem II results when the rate of damage to D₁ protein exceeds the rate of repair and/or protection (Saradadevi and Raghavendra, 1992; Shyam et al., 1993). Shyam et al. (1993) concluded that mitochondrial ATP is necessary for the repair of the D₁ protein, since inhibition of mitochondrial ATP synthesis by azide and/or the uncoupler FCCP caused an increase in the time needed for recovery from photoinhibition in the cyanobacteria *Anacystis nidulans*. However, in many species the demand for ATP decreases at low temperatures. Under these conditions it might be more advantageous to oxidize the excess photosynthetic redox equivalents via the non-phosphorylating alternative oxidase (AOX) (Purvis and Shewfelt, 1993). While this hypothesis has not been proven, there is some evidence to support it. The activity of AOX appears to be increased following greening of etiolated leaves (Atkin et al., 1993; Robinson et al., 1995; Ribas-Carbo et al., 1997) and this is probably due to increased protein synthesis (Finnegan et al., 1997). Extended exposure to low temperatures which cause photoinhibition, also increases AOX protein levels (Vanlerberghe and McIntosh, 1992), as does exposure to reactive oxygen intermediates (Wagner, 1995), which can be produced during photooxidation in chloroplasts. Moreover, Prasad et al. (1994) found that chilling leads to an increase in AOX-mediated respiration in maize. Nonetheless, further work is needed before the role of AOX in ameliorating the effects of photoinhibition is confirmed.

IV. Nitrogen Metabolism, Photosynthesis and Respiration

NO_3^- assimilation and amino acid synthesis in leaves requires a large input of ATP, redox equivalents and carbon intermediates. Before NO_3^- can be assimilated into organic nitrogen in leaves, it must be reduced to NO_2^- in the cytosol and then to NH_4^+ in the chloroplast (Beevers and Hageman, 1983; Campbell, 1988).

A. Demand for Redox Equivalents by NO_3^- and NO_2^- Reduction

The reduction of NO_3^- to NO_2^- is catalyzed by cytosolic nitrate reductase (NR) and requires NAD(P)H. In leaves, these redox equivalents can be derived from three sources: photosynthesis (export of excess NADPH via the DHAP:3-PGA or OAA-malate shuttles; Mann et al., 1978), glycolysis (oxidation of glyceraldehyde 3-phosphate to 1,3bisphosphoglycerate) and/or mitochondrial respiration (via the OAA-malate shuttle; Shaner and Boyer, 1976).

Exposure of a plant to NO_3^- results in a rise in the respiratory quotient (RQ) of some photosynthetic tissues, when measured in darkness (Willis and Yemm, 1955; Bloom et al., 1989). This has led several authors to suggest that mitochondrial redox equivalents are exported to the cytosol and used for NO_3^- reduction (Sawhney et al., 1978; Bloom et al., 1989; Weger and Turpin, 1989). In these tissues, glycolytic NADH supply is insufficient to meet the reductant demands of NO_3^- reduction. It is perhaps not surprising, therefore, that NR activity can be increased in some tissues by the addition of NADH producing metabolites or NADH itself to the NR activity assay media (Klepper et al., 1971; Chanda et al., 1987). However, other studies have reported that reductant supply does not limit NR activity (Tingley et al., 1974; Nicholas et al., 1976; Deane-Drummond et al., 1979; Atkin 1993) or becomes limiting only after a period of extended darkness (Hipkin et al., 1984). Moreover, exposure to NO_3^- does not always result in increased RQ values (Klepper et al., 1971; Deane-Drummond et al., 1979; Hansen, 1980). The degree to which NO_3^- reduction relies on mitochondrial NADH, therefore, varies between species, tissues, and environmental conditions.

An important aspect of the above studies is that RQ was measured in darkness, as was the rate of NO_3^- reduction. In darkness, the NR protein present in leaves is inactivated (Riens and Heldt, 1992; Glaab

and Kaiser, 1996), and as such is unlikely to exert a demand for mitochondrial NADH. However, full inactivation can take 10 min or longer to occur: as a result, measurements of gas exchange performed shortly after exposure to darkness will probably be influenced by the redox equivalent demands of NO_3^- reduction.

To our knowledge, no study has investigated the degree to which in situ NO_3^- reduction relies on photosynthetic versus respiratory redox equivalents. Presumably, NO_3^- reduction could rely more on photosynthetic redox equivalents under high light conditions when the NADP pool in the chloroplast is highly reduced. The reliance on photosynthetic and/or mitochondrial redox equivalents, as opposed to glycolytic NADH, will also probably depend on the specific rate of NO_3^- reduction: the demand for non-glycolytic NADH could well be greater when the rate of NO_2^- reduction is high.

NO_2^- produced by NR activity is transported from the cytosol to the chloroplast where it is reduced to NH_4^+ by nitrite reductase (NiR) (Lee, 1980). Since this reduction is dependent on ferredoxin, it is light dependent. The rate at which NO_2^- can be reduced to NH_4^+ is therefore likely to decrease with decreasing irradiance. Similarly, the reduction of NO_2^- to NH_4^+ ceases almost immediately upon exposure to darkness (Riens and Heldt, 1992). NO_2^- reduction to NH_4^+ therefore represents a sink for excess photosynthetic redox equivalents. This suggests that the need to export NADPH from the chloroplasts to the respiratory apparatus could be lower in illuminated leaves that are assimilating NO_3^- or NH_4^+ .

B. Demand for ATP and Carbon Skeletons by NH_4^+ Assimilation

In photosynthetic tissues, light-dependent NH_4^+ assimilation proceeds in the chloroplast via the coupled activity of ATP-dependent glutamine synthetase (GS) and a Fd_{red} -dependent glutamate synthase (GOGAT), in which glutamate acts as the first recipient of NH_4^+ (Miflin and Lea, 1976). NH_4^+ is normally only assimilated in leaves following the reduction of NO_3^- , as NH_4^+ taken up by roots (or produced during root NO_3^- reduction) is not transported through the xylem due to its toxicity. Rather, NH_4^+ is assimilated into amino acids before it is transported.

In addition to exerting a demand for ATP and redox equivalents in the chloroplast, NH_4^+ assimilation

also requires a continual supply of TCA-cycle derived 2-oxoglutarate (either directly or via citrate). The high rate of amino acid synthesis in the light (Winter et al., 1992) suggests that demand for TCA cycle carbon skeletons is very high. Although the conversion of glutamine to 2 × glutamate requires the input of 2-oxoglutarate, the actual organic acid that is exported from the mitochondria is subject to debate. It has been widely assumed that mitochondria export 2-oxoglutarate, with the concomitant reduction in the release of one molecule of CO_2 , NADH and FADH_2 . However, more recent evidence suggests that plant mitochondria may export citrate (Hanning and Heldt, 1993), which is converted to 2-oxoglutarate by cytosolic isocitrate dehydrogenase (Chen and Gadale, 1990; Krömer, 1995). ^{13}C nuclear magnetic resonance studies using intact leaves have demonstrated that citrate is the major mitochondrial product in the light and that large amounts of citrate accumulate in the vacuole (Gout et al., 1993). If citrate is exported, the TCA cycle will be even more truncated, as two sites of CO_2 and NADH release would be eliminated, in addition to the loss of FAD reduction by succinate dehydrogenase. However, CO_2 would be produced in the cytosol before 2-oxoglutarate could be used for nitrogen assimilation.

C. Requirement for PDC Activity in the Light to Support NH_4^+ Assimilation

The export of large amounts of citrate to the cytosol in the light requires that there must be continuous carbon input into the TCA cycle. However, previous sections of this chapter highlighted the possibility that light can result in an inactivation of the enzymes controlling substrate input into the TCA cycle (e.g. ME and PDC). How, then, can such high rates of citrate synthesis/export occur in the light? The answer to this question probably lies with the stimulatory effect pyruvate has on PDC activity (Schuller and Randall, 1990). Thus, while exposure to light results in a potential phosphorylation-mediated inhibition of PDC activity by photorespiration, these inhibitory effects can be reduced if sufficient pyruvate is present in the mitochondria. For this to occur, additional carbon must be put into the TCA cycle as OAA, since removal of citrate requires input of a C_4 acid. In such a scheme, $\text{OAA}_{\text{in}}/\text{citrate}_{\text{out}}$ exchange would occur via the OAA carrier on the mitochondrial inner membrane (Heldt and Flügge, 1987; Hanning et al., 1999). It is possible, therefore, that in the light pyruvate and

OAA are the sources of carbon for the mitochondria, while immediately following illumination malate may be the major substrate. It is also worth noting that pyruvate influx to the mitochondria will activate AOX (Millar et al., 1993), while citrate accumulation may stimulate AOX synthesis (Vanlerberghe and McIntosh, 1996).

D. Nitric Oxide Production and the Implications for Respiration

Reduction of NO_3^- to NO_2^- and then to NH_4^+ raises the specter of synthesis of intermediary nitrogen oxides that can have major consequences for plant metabolism. Measurements of gaseous emissions from soybean, pea, sugarcane, wheat, corn, sunflower and spinach have demonstrated that NO can be synthesized in plant leaf cells (Klepper, 1979, 1987, 1990; Dean and Harper, 1986; Leshem, 1996; Wildt et al. 1997). NO can be produced enzymatically from NO_2^- in leaves of soybean and other leguminous species by the constitutive NAD(P)H nitrate reductase (NR) enzyme (nr; Dean and Harper, 1988), by non-enzymatic routes (Churchill and Klepper, 1979; Klepper, 1980, 1990), or via putative mammalian-like NO synthases (Sen and Cheema, 1995; Leshem, 1996). Wildt et al. (1997) have shown that NO formation under physiological conditions is positively correlated with the rate of CO_2 fixation in the light, and that substantial NO formation in the dark can occur following a change in the nitrogen regime of plants.

NO formation can provide a signal that the reduction of NO_2^- to NH_4^+ has been partially or fully interrupted. NO acts as a potent inhibitor of cytochrome oxidase with half-maximal inhibition occurring at approximately 25 nM NO, but has a negligible effect on AOX at concentrations of up to 1 μM (Millar and Day, 1996). This differential inhibition may allow AOX, but not cytochrome oxidase, to operate during rapid nitrogen assimilation when the nitrite concentration is high. The significance of this *in vivo* needs to be addressed.

E. Interaction Between Nitrogen Assimilation and Carbohydrate Metabolism

The above sections have highlighted the large demand nitrogen metabolism has for ATP, redox equivalents and carbon skeletons. To meet these requirements, a plant cell must carefully regulate the flux through the

various metabolic pathways of the chloroplast, cytosol and mitochondria.

The coordination of these pathways has been investigated in detail by Turpin and co-workers using chemostat cultures of green algae. In their experiments, addition of NO_3^- and/or NH_4^+ to nitrogen-limited algae results in a rapid, large increase in the demand for carbon skeletons (Elferi and Turpin, 1986; Weger et al., 1988; Elferi et al., 1988) and redox equivalents (Weger and Turpin, 1989). Associated with these demands is a diversion of carbon away from RuBP re-generation (via the export of DHAP) to cytosolic and TCA cycle carbon metabolism (Elferi et al., 1988), resulting in a decrease in net photosynthesis (Elferi and Turpin, 1986) and a concomitant increase in flux through PEP carboxylase (Turpin and Vanlerberghe, 1991), TCA cycle (Elferi and Turpin, 1986) and the respiratory electron transport chain (Weger et al., 1988; Weger et al., 1990). Not surprisingly, respiratory CO_2 release and O_2 uptake increase following induction of nitrogen assimilation (Weger et al., 1988).

The induction of nitrogen assimilation in green algae relies on carbon input from photosynthetic CO_2 fixation via DHAP export from the chloroplast, breakdown of starch/sucrose, and CO_2 fixation by PEP carboxylase (Elferi and Turpin, 1986; Turpin and Vanlerberghe, 1991). The importance of CO_2 fixation by PEP carboxylase is illustrated by the fact that approximately 0.3 moles of carbon are fixed by PEP carboxylase per mole of nitrogen assimilated in the green alga *Selenastrum minutum* (Turpin and Vanlerberghe, 1991). The tight regulation of PEP carboxylase activity by carbon substrates (e.g., activity is increased by DHAP, G6P etc) and some nitrogen products (e.g., inhibition by aspartate, glutamate etc), demonstrates that PEP carboxylase activity can be controlled to meet the nitrogen assimilation requirements of plant cells.

PK also increases in activity following induction of NH_4^+ assimilation (Turpin et al., 1990). The onset of NH_4^+ assimilation in *S. minutum* results in a transient removal of adenylate restriction of PK, thus allowing increased flux through glycolysis (Turpin et al., 1990). Moreover, despite adenylate control of PK subsequently being re-established, PK remains active due to the increased concentration of DHAP (a PK activator) and decreased concentration of glutamine (a PK inhibitor) (Turpin et al., 1990).

The increase in TCA cycle activity that occurs when N-limited green algae are exposed to NH_4^+ is

coupled to increased NADH oxidation by the cytochrome pathway of mitochondrial electron transport (Weger et al., 1988; Weger et al., 1990), despite a large capacity for AOX in these cells (Weger et al., 1990; Weger et al., 1988). The ATP synthesized by the cytochrome pathway is likely to assist in the assimilation of NH_4^+ to amino acids.

Work with nitrogen limited wheat leaves also suggests that carbon is re-directed away from sucrose synthesis to meet the carbon requirements of induced nitrogen assimilation: exposure of N-limited wheat leaves to NO_3^- results in a decrease in SPS activity and an increase in PEP carboxylase activity (Van Quy et al., 1991). More work is needed to better understand the situation in higher plants.

References

- ap Rees T (1980) Assessment of the contributions of metabolic pathways to plant respiration. In: Stumpf PK and Conn EE (eds) *The Biochemistry of Plants*, Vol 2, pp 1–30. Academic Press, New York
- Atkin OK (1993) Nitrogen Source and Arctic Plants: Interactions Between Growth, Nitrogen Metabolism and Respiratory Processes. PhD Thesis. University of Toronto, Canada
- Atkin OK, Cummins WR and Collier DE (1993) Light induction of alternative pathway capacity in leaf slices of Belgian endive. *Plant Cell Environ* 16: 231–235
- Atkin OK, Westbeck MHM, Cambridge ML, Lambers H and Pons TL (1997) Leaf respiration in light and darkness: A comparison of slow- and fast-growing *Poa* species. *Plant Physiol* 113: 961–965
- Atkin OK, Seibke K and Evans JR (1998a) Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Aust J Plant Physiol* 25: 437–443
- Atkin OK, Evans JR, Ball MC, Seibke K, Pons TL and Lambers H (1998b) Light inhibition of leaf respiration: The role of irradiance and temperature. In: Møller IM, Gardeström P, Glimelius K and Glaser E (eds) *Plant Mitochondria: From Gene to Function*, pp 25–32. Backhuys Publishers, Leiden
- Avelange M-H, Thiery JM, Sarrey F, Cans P and Rebeille F (1991) Mass-spectrometric determination of O_2 and CO_2 gas exchange in illuminated higher plant cells. Evidence for light-inhibition of substrate decarboxylations. *Planta* 183: 150–157
- Azcon-Bieto J, Lambers H and Day DA (1983) Effect of photosynthesis and carbohydrate status on respiratory rates and the involvement of the alternative pathway in leaf respiration. *Plant Physiol* 72: 598–603
- Ball MC, Hodges VF and Lauglin GP (1991) Cold-induced photoinhibition limits regeneration of snow gum at tree-line. *Functional Ecol* 5: 663–668
- Bate GC, Söltmeyer DF and Fock HP (1988) $^{16}\text{O}_2/^{18}\text{O}_2$ analysis of oxygen exchange in *Dunaliella tertiolecta*. Evidence for the inhibition of mitochondrial respiration in the light. *Photosynth Res* 16: 219–231

- Beevers L and Hageman RH (1983) Uptake and reduction of nitrate: Bacteria and higher plants. In: Lauchli A and Bielecki RL (eds) Encyclopedia of Plant Physiology, New Series, Volume 15A. Inorganic Plant Nutrition, pp 351–375. Springer-Verlag, New York
- Bloom AJ, Caldwell RM, Finazzo J, Warner RL and Weissbart J (1989) Oxygen and carbon dioxide fluxes from barley shoots depend on nitrate assimilation. *Plant Physiol* 91: 352–356
- Bouma TJ, De Visser R, Van Leeuwen PH, De Kock MJ and Lambers H (1995) The respiratory energy requirements involved in nocturnal carbohydrate export from starch-storing mature source leaves and their contribution to leaf dark respiration. *J Exp Bot* 46: 1185–1194.
- Brooks A and Farquhar GD (1985) Effect of temperature on the $\text{CO}_2\text{-O}_2$ specificity of ribulose-1,5-biphosphate carboxylase/oxygenase and the rate of respiration in the light. Estimates from gas exchange measurements on spinach. *Planta* 165: 397–406
- Budde RJA and Randall DD (1987) Regulation of pea mitochondrial pyruvate dehydrogenase complex activity: Inhibition of ATP-dependent inactivation. *Arch Biochem Biophys* 258: 600–606
- Budde RJA and Randall DD (1990) Pea leaf mitochondrial pyruvate dehydrogenase complex is inactivated *in vivo* in a light-dependent manner. *Proc Nat Acad Sci USA* 87: 673–676
- Bulley NR and Tregunna EB (1971) Photorespiration and the postillumination CO_2 burst. *Can J Bot* 49: 1277–1284
- Campbell WH (1988) Nitrate reductase and its role in nitrate assimilation in plants. *Physiol Plant* 74: 214–219
- Canvin DT, Berry JA, Badger MR, Fock H and Osmond CB (1980) O_2 exchange in leaves in the light. *Plant Physiol* 66: 302–307
- Chanda SV, Joshi AK, Krishnan PN and Singh JD (1987) *In vivo* nitrate reductase activity in leaves of Pearl Millet *Pennisetum americanum* (L.) Leeke. *Aust J Plant Physiol* 14: 125–134
- Chen R-C and Gadwal P (1990) Do the mitochondria provide the 2-oxoglutarate needed for glutamate synthesis in higher plant chloroplasts? *Plant Physiol Biochem* 28: 141–145
- Churchill KA and Klepper LA (1979) Effects of ametryn on nitrate reductase activity and nitrite content of wheat. *Pest Biochem Physiol* 12: 156–162
- Cook RM, Lindsay JG, Wilkins MB and Nimmo HG (1995) Decarboxylation of malate in the crassulacean acid metabolism plant *Bryophyllum (Kalanchoe) fedtschenkoi*—role of NAD-malic enzyme. *Plant Physiol* 109: 1301–1307
- Day DA and Wiskich JT (1981) Glycine metabolism and oxaloacetate transport by pea leaf mitochondria. *Plant Physiol* 68: 425–429
- Day DA and Wiskich JT (1984) Transport processes in isolated plant mitochondria. *Physiol Vég* 22: 241–261
- Day DA and Wiskich JT (1995) Regulation of alternative oxidase activity in higher plants. *J Bioenerg Biomemb* 27: 379–386
- Day DA, Neuburger M and Douce R (1985) Interactions between glycine decarboxylase, the tricarboxylic acid cycle and the respiratory chain in pea leaf mitochondria. *Aust J Plant Physiol* 12: 119–130
- Day DA, Whelan J, Millar AH, Siedow JN and Wiskich, JT (1995) Regulation of the alternative oxidase in plants and fungi. *Aust J Plant Physiol* 22: 497–509
- Dean JV and Harper JE (1986) Nitric oxide and nitrous oxide production by soybean and winged bean during the *in vivo* nitrate reductase assay. *Plant Physiol* 82: 718–723
- Dean JV and Harper JE (1988) The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P)H-nitrate reductase enzyme from soybean. *Plant Physiol* 88: 389–395
- Deane-Drummond CE, Clarkson DT and Johnson CB (1979) Effect of shoot removal and malate on the activity of nitrate reductase assayed *in vivo* in barley roots (*Hordeum vulgare* cv. Midas). *Plant Physiol* 64: 660–662
- Doehlert DC, Ku MSB and Edwards GE (1979) Dependence of the post-illumination burst of CO_2 on temperature, light, CO_2 , and O_2 concentration in wheat (*Triticum aestivum*). *Physiol Plant* 46: 299–306
- Dry IB and Wiskich JT (1985) Characterisation of glycine and malate oxidation by pea leaf mitochondria: Evidence of differential access to NAD and respiratory chains. *Aust J of Plant Physiol* 12: 329–339
- Dry IB, Day DA and Wiskich JT (1983) Preferential oxidation of glycine by the respiratory chain of pea leaf mitochondria. *FEBS Lett* 158: 154–158
- Dry IB, Bryce JH and Wiskich JT (1987) Regulation of mitochondrial respiration. In: Davies DD (ed) *The Biochemistry of Plants*, pp 213–252. Academic Press, San Diego
- Ebbighausen H, Hatch MD, Lilley RMCC, Krömer S, Stitt M and Heldt HW (1987) On the function of malate-oxaloacetate shuttles in a plant cell. In: Moore AL and Beechey RB (eds) *Plant Mitochondria, Structural, Functional and Physiological Aspects*, pp 171–180. Plenum Press, New York
- Elferi IR and Turpin DH (1986) Nitrate and ammonium induced photosynthetic suppression in N-limited *Selenastrum minutum*. *Plant Physiol* 81: 273–279
- Elferi IR, Holmes JJ, Weger HG, Mayo WP and Turpin DH (1988) RuPB limitation of photosynthetic carbon fixation during NH_3 assimilation. Interactions between photosynthesis, respiration, and ammonium assimilation in N-limited green algae. *Plant Physiol* 87: 395–401
- Ellsworth DS and Reich PB (1996) Photosynthesis and leaf nitrogen in five Amazonian tree species during early secondary succession. *Ecology* 77: 581–594
- Finnegan PM, Whelan J, Millar AH, Zhang Q, Smith MK, Wiskich JT and Day DA (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. *Plant Physiol* 114: 455–466
- Fair P, Tew J and Cresswell CF (1972) The effect of age and leaf position on carbon dioxide compensation point Γ and potential photosynthetic capacity, photorespiration and nitrate assimilation in *Hordeum vulgare* L. *J South African Bot* 38: 81–95
- Flügge UI and Heldt HW (1991) Metabolite translocators of the chloroplast envelope. *Ann Rev Plant Physiol Plant Mol Biol* 42: 129–144
- Gardeström P (1987) Adenylate ratios in the cytosol, chloroplasts and mitochondria of barley leaf protoplasts during photosynthesis at different carbon dioxide concentrations. *FEBS Lett* 212: 114–118
- Gardeström P and Wigge B (1988) Influence of photorespiration on ATP/ADP ratios in the chloroplasts, mitochondria and cytosol, studied by rapid fractionation of barley (*Hordeum vulgare*) protoplasts. *Plant Physiol* 88: 69–76
- Gardeström P, Zhou G and Malmberg G (1992) Respiration in barley protoplasts before and after illumination. In: Lambers H and van der Plas LHW (eds) *Molecular, Biochemical and*

- Physiological Aspects of Plant Respiration, pp 261–266. SPB Academic Publishing, The Hague
- Gemel J and Randall DD (1992) Light regulation of leaf mitochondrial pyruvate dehydrogenase complex. Role of photorespiratory carbon metabolism. *Plant Physiol* 100: 908–914
- Gerhardt R and Heldt HW (1984) Measurements of subcellular metabolite levels by fractionation of freeze-stopped material in nonaqueous media. *Plant Physiol* 75: 542–547
- Gerhardt R, Stitt M and Heldt HW (1987) Subcellular metabolite levels in spinach leaves. Regulation by sucrose synthesis during diurnal alterations in photosynthetic partitioning. *Plant Physiol* 83: 399–407
- Glaab J and Kaiser WM (1996) The protein kinase, protein phosphatase and inhibitor protein of nitrate reductase are ubiquitous in higher plants and independent of nitrate reductase expression and turnover. *Planta* 199: 57–63
- Gottlob-McHugh SG, Sangwan RS, Vanlerberghe GC, Ko K, Turpin DH, Plaxton WC, Miki BL and Dennis DT (1992) Normal growth of transgenic tobacco plants in the absence of cytosolic pyruvate kinase. *Plant Physiol* 100: 820–825
- Gout E, Bligny R, Pascal N and Douce R (1993) ^{13}C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells. *J Biol Chem* 268: 3986–3992
- Graham D (1980) Effects of light and ‘dark’ respiration. In: Davies DD (ed) *The Biochemistry of Plants. A Comprehensive Treatise*, pp 525–579. Academic Press, New York
- Guy R, Berry J, Fogel M, Turpin D and Weger H (1992) Fractionation of the stable isotopes of oxygen during respiration in plants—the basis for a new technique. In: Lambers H and van der Plas LHW (eds) *Molecular, Biochemical and Physiological Aspects of Plant Respiration*, pp 442–453. SPB Academic Publishing, The Hague
- Hampp R, Goller M and Zeigler H (1982) Adenylate levels, energy charge, and phosphorylation potential during dark-light and light-dark transition in chloroplasts, mitochondria, and cytosol of mesophyll protoplasts from *Avena sativa* L. *Plant Physiol* 69: 448–55
- Hanning I and Heldt HW (1993) On the function of mitochondrial metabolism during photosynthesis in spinach (*Spinacia oleracea* L.) leaves. Partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. *Plant Physiol* 103: 1147–1154
- Hanning I, Baumgarten K, Schott K and Heldt HW (1999) Oxaloacetate transport into plant mitochondria. *Plant Physiol* 119: 1025–1031
- Hansen GK (1980) Diurnal variation of root respiration rates and nitrate uptake as influenced by nitrogen supply. *Physiol Plant* 48: 421–427
- Hatch MD and Carnal NW (1992) The role of mitochondria in C4 photosynthesis. In: Lambers H and van der Plas LHW (eds) *Molecular, Biochemical and Physiological Aspects of Plant Respiration*, pp 261–266. SPB Academic Publishing, The Hague
- Hatch MD, Dröscher L and Heldt HW (1984) A specific translocator for oxaloacetate in chloroplasts. *FEBS Lett* 178: 15–19
- Hatzfeld W-D and Stitt M (1991) Regulation of glycolysis in heterotrophic cell suspension cultures of *Chenopodium rubrum* in response to proton fluxes at the plasmalemma. *Plant Physiol* 81: 103–110
- Heber U (1975) Energy transfer within leaf cells. In: Avron M (ed) *Proceedings of the 3rd International Congress on Photosynthesis*, Vol 2, pp 1335–1348. Elsevier Scientific Publishing, Amsterdam
- Heineke D, Riens B, Grosse H, Hoferichter P, Peter U, Flügge UI and Heldt HW (1991) Redox transfer across the inner chloroplast membrane. *Plant Physiol* 95: 1131–1137
- Heldt HW and Flügge UI (1987). Subcellular transport of metabolites in a plant cell. In: Stumpf PK and Conn EE (eds) *The Biochemistry of Plants*, Vol 11 pp. 49–85. Academic Press, New York
- Hill SA and Bryce JH (1992) Malate metabolism and light-enhanced dark respiration in barley mesophyll protoplasts. In: Lambers H and van der Plas LHW (eds) *Molecular, Biochemical and Physiological Aspects of Plant Respiration*, pp 221–230. SPB Academic Publishing, The Hague
- Hipkin CR, Gharbi AA and Robertson KP (1984) Studies on nitrate reductase in British angiosperms. II. Variations in nitrate reductase activity in natural populations. *New Phytol* 97: 641–651
- Hoefnagel MHN, Millar AH, Wiskich JT and Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. *Arch Biochem Biophys* 318: 394–400
- Hurry VM, Tobiæson M, Krömer S, Gardeström P and Öquist G (1995) Mitochondria contribute to increased photosynthetic capacity of leaves of winter rye (*Secale cereale* L) following cold-hardening. *Plant Cell Environ* 18: 69–76
- Hurry VM, Keerberg O, Parnik T, Öquist G and Gardeström P (1996) Effect of cold hardening on the components of respiratory decarboxylation in the light and in the dark in leaves of winter rye. *Plant Physiol* 111: 713–719
- Igamberdiev AU, Zhou GQ, Malmberg G and Gardeström P (1997a) Respiration of barley protoplasts before and after illumination. *Physiol Plant* 99: 15–22
- Igamberdiev AU, Bykova NV and Gardeström P (1997b) Involvement of cyanide-resistant and rotenone-insensitive pathways of mitochondrial electron transport during oxidation of glycine in higher plants. *FEBS Lett* 412: 265–269
- Igamberdiev AU, Hurry VM, Krömer S and Gardeström P (1998) The role of mitochondrial electron transport during photosynthetic induction: A study with barley (*Hordeum vulgare*) protoplasts incubated with rotenone and oligomycin. *Physiol Plant* 104:431–439
- Kirschbaum MUF and Farquhar GD (1987) Investigation of the CO_2 dependence of quantum yield and respiration in *Eucalyptus pauciflora*. *Plant Physiol* 83: 1032–1036
- Klepper LA, Flesher D and Hageman RH (1971) Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiol* 48: 580–590
- Klepper LA (1979) Nitric oxide (NO) and nitrogen dioxide (NO_2) emissions from herbicide-treated soybean plants. *Atmos Environ* 13:537–542
- Klepper LA (1980) Nitrate disappearance from excised green leaves in darkness (abstract No 626). *Plant Physiol* 65: S-113
- Klepper LA (1987) Nitric oxide emissions from soybean leaves during *in vivo* nitrate reductase assays. *Plant Physiol* 85: 96–99
- Klepper LA (1990) Comparison between NO_x evolution mechanisms of wild-type and nr_1 mutant soybean leaves. *Plant*

- Physiol 93: 26–32
- Körner C and Larcher W (1988) Plant Life in Cold Environments. In: Long SF and Woodward FI (eds) Plants and Temperature. Symposium of the Society of Experimental Biology, Vol 42, pp 25–57. The Company of Biologists Limited, Cambridge
- Krömer S (1995) Respiration during photosynthesis. Ann Rev Plant Physiol Plant Mol Biol 46: 45–70
- Krömer S and Heldt HW (1991) On the role of mitochondrial oxidative phosphorylation in photosynthesis metabolism as studied by the effect of oligomycin on photosynthesis in protoplasts and leaves of barley (*Hordeum vulgare*). Plant Physiol 95: 1270–1276
- Krömer S and Scheibe R (1996) Function of the chloroplastic malate valve for respiration during photosynthesis. Biochem Soc Trans 24: 761–766
- Krömer S, Hanning I and Heldt HW (1992) On the sources of redox equivalents for mitochondrial oxidative phosphorylation in the light. In: Lambers H and van der Plas LHW (eds) Molecular, Biochemical and Physiological Aspects of Plant Respiration, pp 167–175. SPB Academic Publishing, The Hague
- Krömer S, Malmberg G and Gardestrom P (1993) Mitochondrial contribution to photosynthetic metabolism—a study with barley (*Hordeum vulgare* L) leaf protoplasts at different light intensities and CO_2 concentrations. Plant Physiol 102: 947–955
- Krömer S, Lernmark U and Gardestrom P (1994) In vivo pyruvate dehydrogenase activity studied by rapid fractionation of barley protoplasts. J Plant Physiol 144: 485–490
- Krömer S, Gardestrom P and Samuelsson G (1996) Regulation of the supply of oxaloacetate for mitochondrial metabolism via phosphoenolpyruvate carboxylase in barley leaf protoplasts. II Effects of metabolites on PEPC activity at different activation states of the protein. Biochim Biophys Acta 1289: 351–361
- Laisk AK (1977) Kinetics of Photosynthesis and Photorespiration in C_3 -Plants. Nauka, Moscow
- Lance C and Rustin P (1984) The central role of malate in plant metabolism. Physiol Vég 22: 625–641
- Lee RB (1980) Sources of reductant for nitrate assimilation in non-photosynthetic tissue: A review. Plant Cell Environ 3:65–90
- Leegood RC, Lea PJ, Adcock MD and Häusler RE (1995) The regulation and control of photorespiration. J Exp Bot 46: 1397–1414
- Lepiniec L, Vidal J, Cholet R, Gadal P and Crétin C (1994) Phosphoenolpyruvate carboxylase: Structure, regulation and evolution. Plant Sci Lett 99: 111–124
- Lernmark U and Gardestrom P (1994) Distribution of pyruvate dehydrogenase complex activity between chloroplasts and mitochondria from leaves of different species. Plant Physiol 106: 1633–1638
- Leshem YY (1996) Nitric oxide in biological systems. Plant Growth Regulators 18: 155–159
- Lin M, Turpin DH and Plaxton WC (1989) Pyruvate kinase isozymes from the green alga, *Selenastrum minutum*. II. Kinetic and regulatory properties. Arch Biochem Biophys 269: 228–238
- Lorimer GH and Andrews TJ (1981) The C_2 chemo- and photorespiratory carbon oxidation cycle. In: Stumpf PK and Conn EE (eds) The Biochemistry of Plants, Vol 8, pp 329–374. Academic Press, New York
- Mann AF, Hucklesby DP and Hewitt EJ (1978) Sources of reducing power for nitrate reduction in spinach leaves. Planta 140: 261–263
- Martinoia E and Rentsch D (1994) Malate compartmentation—responses to a complex metabolism. Ann Rev Plant Physiol Plant Mol Biol 45: 447–167
- McCashin BG, Cossins EA and Canvin DT (1988) Dark respiration during photosynthesis in wheat leaf slices. Plant Physiol 87: 155–161
- McIntosh L (1994) Molecular Biology of the alternative oxidase. Plant Physiol 105: 781–786
- Miflin BJ and Lea PJ (1976) The pathway of nitrogen assimilation in plants. Phytochem 15: 873–885
- Millar AH and Day DA (1996). Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. FEBS Lett 398: 155–158
- Millar AH and Day DA (1997) An alternative solution to a radical problem. Trends Plant Sci 2: 289–290
- Millar AH, Wiskich JT, Whelan J and Day DA (1993) Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett 329: 259–262
- Millar AH, Hoefnagel MHN, Day DA and Wiskich JT (1996). Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol 111: 613–618
- Møller IM and Lin W (1986) Membrane bound NAD(P)H dehydrogenases in higher plant cells. Ann Rev Plant Physiol 37: 309–334
- Moore AL, Gemel J and Randall DD (1993) The regulation of pyruvate dehydrogenase activity in pea leaf mitochondria—the effect of respiration and oxidative phosphorylation. Plant Physiol 103: 1431–1435
- Nicholas JC, Harper JE and Hageman RH (1976) Nitrate reductase activity in soybeans (*Glycine max* [L.] Merr.). II. Energy limitations. Plant Physiol 58: 736–739
- Padmasree K, Raghavendra AS (1998) Interaction with respiration and nitrogen metabolism. In: Raghavendra AS (ed) Photosynthesis. A Comprehensive Treatise, pp 197–211. Cambridge University Press, Cambridge
- Pärnik T and Keerberg O (1995) Decarboxylation of primary and end products of photosynthesis at different oxygen concentrations. J Exp Bot 46: 1439–1447
- Peltier G and Thibault P (1985) O_2 uptake in the light in Chlamydomonas. Evidence for persistent mitochondrial respiration. Plant Physiol 79: 225–230
- Podstá F and Plaxton WC (1994) Regulation of cytosolic carbon metabolism in germinating carbon metabolism in germinating *Ricinus communis* endosperm. Planta, 194: 381–387
- Prasad TK, Anderson MD and Stewart CR (1994) Acclimation, hydrogen peroxide, and abscisic acid protect mitochondria against irreversible chilling injury in maize seedlings. Plant Physiol 105: 619–627
- Purvis AC (1997) Role of the alternative oxidase in limiting superoxide production by plant mitochondria. Physiol Plant 100: 165–170
- Purvis AC and Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues [review]. Physiol Plant 88: 712–718
- Raghavendra AS, Padmasree K and Saradadevi K (1994) Interdependence of photosynthesis and respiration in plant cells—interactions between chloroplasts and mitochondria. Plant Sci 97: 1–14

- Randall DD, Miernyk JA, David NR Budde, RJA Schuller KA, Fang TK and Gemel J (1990) Phosphorylation of the leaf mitochondrial pyruvate dehydrogenase complex and inactivation of the complex in the light. *Curr Top Plant Biochem Physiol* 9: 313–328
- Reddy MM, Vani T and Raghavendra AS (1991) Light-enhanced dark respiration in mesophyll protoplasts from leaves of pea. *Plant Physiol* 96: 1368–1371
- Ribas-Carbo M, Berry JA, Yakir D, Giles L, Robinson SA, Lennon AM and Siedow JN (1995) Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. *Plant Physiol* 109: 829–837
- Ribas-Carbo M, Lennon AM, Robinson SA, Giles L, Berry JA and Siedow JN (1997) The regulation of electron partitioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. *Plant Physiol* 113: 903–911
- Riens B and Heldt HW (1992) Decrease of nitrate reductase activity in spinach leaves during a light-dark transition. *Plant Physiol* 98: 573–577
- Robinson SA, Ribas-Carbo M, Yakir D, Giles L, Reuveni Y and Berry JA (1995) Beyond SHAM and cyanide—opportunities for studying the alternative oxidase in plant respiration using oxygen isotope discrimination. *Aust J Plant Physiol* 22: 487–496
- Saradadevi K and Raghavendra AS (1992) Dark respiration protects photosynthesis against photoinhibition in mesophyll protoplasts of pea (*Pisum sativum*). *Plant Physiol* 99: 1232–1237
- Sawhney SK, Naik MS and Nicholas DJD (1978) Regulation of nitrate reduction by light, ATP and mitochondrial respiration in wheat leaves. *Nature* 272: 647–648
- Scheibe R (1987) NADP-malate dehydrogenase in C₃-plants: Regulation and role of a light-activated enzyme. *Physiol Plant* 71: 393–400
- Schuller KA and Randall DD (1989) Regulation of pea mitochondrial pyruvate dehydrogenase complex: Does photorespiration ammonium influence mitochondrial carbon metabolism? *Plant Physiol* 89: 1207–1212
- Schuller KA and Randall DD (1990) Mechanism of pyruvate inhibition of plant pyruvate dehydrogenase kinase and synergism with ADP. *Arch Biochem Biophys* 278: 211–216
- Sen S and Cheema IR (1995) Nitric oxide synthase and calmodulin immunoreactivity in plant embryonic tissue. *Biochem Arch* 11: 221–227
- Shaner DL and Boyer JS (1976) Nitrate reductase activity in maize (*Zea mays* L.) leaves. I. Regulation by nitrate flux. *Plant Physiol* 58: 499–504
- Sharp RE, Matthews MA and Boyer JS (1984). Kok effect and the quantum yield of photosynthesis: light partially inhibits dark respiration. *Plant Physiol* 75: 95–101
- Shyam R, Raghavendra AS and Sane PV (1993) Role of dark respiration in photoinhibition of photosynthesis and its reactivation in the *Cyanobacterium anacystis-nidulans*. *Physiol Plant* 88: 446–452
- Stitt M and Heldt HW (1981) Simultaneous synthesis and degradation of starch in spinach chloroplasts in the light. *Biochim Biophys Acta* 638: 1–11
- Stitt M, Lilley RM and Heldt HW (1982) Adenine nucleotide levels in the cytosol, chloroplast and mitochondria of wheat leaf protoplasts. *Plant Physiol* 70: 971–977
- Tingley DT, Fites RC and Baharsjah J (1974) Factors influencing nitrate reduction in soybean foliage. *New Phytol* 73: 21–29
- Turpin DH and Vanlerberghe GC (1991) The inorganic carbon requirements for nitrogen assimilation. *Can J Bot* 69: 1139–1145
- Turpin DH and Weger HG (1989) Interactions between photosynthesis, respiration and nitrogen assimilation In: Dennis DT and Turpin DH (eds) *Plant Physiology, Biochemistry and Molecular Biology*, pp 422–433. Longman Scientific and Technical, Essex, UK
- Turpin DH, Botha FC, Smith RG, Feil R, Horsey AK and Vanlerberghe GC (1990) Regulation of carbon partitioning to respiration during dark ammonium assimilation by the green alga *Selenastrum minutum*. *Plant Physiol* 93: 166–175
- Umbach AL and Siedow JN (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiol* 103: 845–854
- Umbach AL, Wiskich JT and Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and inter-molecular disulfide bond redox status in soybean seedling mitochondria. *FEBS Lett* 348: 181–184
- Van Der Werf A, Poorter H and Lambers H (1994) Respiration as dependent on a species' inherent growth rate and on the nitrogen supply to the plant. In: Roy J and Gamier E (eds) *A Whole Plant Perspective on Carbon-Nitrogen Interactions*, pp 83–103. SPB Academic Publishing bv, The Hague
- Van Quy L, Foyer CH and Champigny M-L (1991) Effect of light and NO₃[−] on wheat leaf phosphoenolpyruvate carboxylase activity. Evidence for covalent modulation of the C₃ enzyme. *Plant Physiol* 97: 1476–1482
- Vanlerberghe GC and McIntosh L (1992) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. *Plant Physiol* 100: 115–119
- Wigge B, Krömer S and Gardeström P (1993) Estimation of the redox level of subcellular pyridine nucleotide pools in barley protoplasts. *Physiol Plant* 88: 10–18
- Vanlerberghe GC and McIntosh L (1996) Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. *Plant Physiol* 111: 589–595
- Vanlerberghe GC, Day DA, Wiskich JT, Vanlerberghe AE and McIntosh L (1995) Alternative oxidase activity in tobacco leaf mitochondria. Dependence on tricarboxylic acid cycle-mediated redox regulation and pyruvate activation. *Plant Physiol* 109: 353–361
- Villar R, Held AA and Merino J (1994) Comparison of methods to estimate dark respiration in the light in leaves of two woody species. *Plant Physiol* 105: 167–172
- Villar R, Held AA and Merino J (1995) Dark leaf respiration in light and darkness of an evergreen and a deciduous plant species. *Plant Physiol* 107: 421–427
- Wagner AM (1995) A role for active oxygen species as second messengers in the induction of alternative oxidase gene expression in *Petunia hybrida* cells. *FEBS Lett* 368: 339–442
- Wagner AM and Krab K (1995) The alternative respiration pathway in plants: Role and regulation. *Physiol Plant* 95: 318–325
- Weger HG and Turpin DH (1989) Mitochondrial respiration can support NO₃[−] and NO₂[−] reduction during photosynthesis. Interactions between photosynthesis, respiration and N assimilation in the N-limited green alga *Selenastrum minutum*.

- Plant Physiol 89: 409–115
- Weger HG, Birch DG, Elferi IR and Turpin DH (1988) Ammonium assimilation requires mitochondrial respiration in the light A study with the green alga *Selenastrum minutum*. Plant Physiol 86: 688–692
- Weger HG, Chadderton AM Lin M, Guy RD and Turpin DH (1990) Cytochrome and alternative pathway respiration during transient ammonium assimilation by N-limited *Chlamydomonas reinhardtii*. Plant Physiol 94: 1131–1136
- Willis AJ and Yemm EW (1955) The respiration of barley plants. VIII. Nitrogen assimilation and the respiration of the root system. New Phytol 54: 163–181
- Wildt J, Kley D, Rockel A, Rockel P and Segschneider HJ (1997) Emission of NO from several higher plant species. J Geophys Res 102: 5919–5927
- Winter H, Lohaus G and Heldt HW (1992) Phloem transport of amino acids in relation to their cytosolic levels in barley leaves. Plant Physiol 99: 996–1004
- Wiskich JT, Bryce JH, Day DA and Dry IB (1990) Evidence for metabolic domains within the matrix compartment of pea leaf mitochondria. Plant Physiol 93: 611–616
- Woo KC and Osmond CB (1976) Glycine decarboxylation in mitochondria isolated from spinach leaves. Aust J Plant Physiol 3: 771–785
- Woodrow I.E. and Berry JA (1988) Enzymatic regulation of photosynthetic CO_2 fixation in C_3 plants. Ann Rev Plant Physiol Plant Mol Biol 39: 533–594
- Xue XP, Gauthier DA, Turpin DH and Weger HG (1996) Interactions between photosynthesis and respiration in the green alga *Chlamydomonas reinhardtii*—characterization of light-enhanced dark respiration. Plant Physiol 112: 1005–101
- Zoglowek C, Krömer S and Heldt HW (1988) Oxaloacetate and malate transport by plant mitochondria. Plant Physiol 87: 109–115

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Chapter 8

Regulation of Carbon Fluxes in the Cytosol: Coordination of Sucrose Synthesis, Nitrate Reduction and Organic Acid and Amino Acid Biosynthesis

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Summary

Sugars and amino acids fuel plant growth, development and biomass production. They are required in different amounts and stoichiometries according to developmental stage and environmental challenges and constraints. Extensive interdependence of C and N assimilation operates at several levels, necessitating a complex array of reciprocal controls. Export of amino acids occurs in response to active loading and transport of sucrose in the phloem. Sucrose is the currency of energy exchange between organs and provides ATP for transport processes. Coordination of C and N flow into amino acids and carbohydrates is achieved by regulation of crucial enzyme activities. Nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPC) and sucrose phosphate synthase (SPS) are key enzymes in N assimilation, anaplerotic C flow and sucrose synthesis, respectively. Their activities are coordinated by several mechanisms, the most important of which is protein phosphorylation. Intermediates and products of these pathways regulate gene expression, thereby transmitting information on metabolism to orchestrate C/N flow. A number of putative signal metabolites have been characterized. N metabolites are required for synthesis and activation of enzymes involved in C metabolism (especially for PEPC and SPS). C metabolites are necessary for the synthesis and activation of enzymes catalyzing the assimilation of N (for example, sugars promote NR gene expression). The ‘signals’ involved include nitrate per se, as well as intermediates and products of C and N assimilation such as amino acids, organic acids and sugars.

I. Introduction

Photosynthesis produces assimilatory power in the forms of reduced ferredoxin, NADPH and ATP. These are used to drive photosynthetic carbon assimilation in the chloroplasts which yields sugar phosphates as net product, which can be used for starch formation in the chloroplasts or exported to the cytosol for the synthesis of sucrose (Fig. 1). Sucrose and starch synthesis are tightly coupled to the rate of CO₂ assimilation via the exchange of triose phosphate and inorganic phosphate across the chloroplast envelope membrane (see Chapter 6, Flügge; Foyer and Galtier, 1996).

Carbohydrates are central to the carbon and energy

budgets of plants. Sucrose is particularly important since it is a major currency of energy exchange between exporting and importing tissues in higher plants. Starch forms a temporary carbohydrate store in the chloroplasts while sucrose is largely excluded from these organelles being present at the highest concentrations in the cytosol of leaf mesophyll cells. While many plant species predominantly synthesize sucrose and starch, others produce derivatives of sucrose (raffinose saccharides), polymers derived from sucrose (fructans), or acyclic alcohols such as sorbitol or mannitol (see Chapters 12 (Loescher and Everard) and 13 (Cairns et al.); Flora and Madore, 1993; Loescher and Everard, 1996).

In any appreciation of the acquisition of resources for plant growth and development, it is necessary to encompass a wider view, taking into account not only the assimilation of carbon but also the simultaneous assimilation of other essential elements. The most important elements to consider are nitrogen (N) and sulfur (S), as their metabolism is also dependent, either directly or indirectly, on the energy and carbon skeletons provided by photosynthesis. The interdependence between C- and N-metabolism is one major focus of this chapter.

The primary products of photosynthesis in plants and algae are carbohydrates and amino acids. Up to about one third of photosynthetically fixed carbon can be found in amino acids (see Noctor and Foyer, 1998, for review). As well as Gln and Glu which are the products of primary N assimilation, the major amino acids found in plants are: Gly and Ser, which

Abbreviations: ADPGlc PPase – ADPglucosepyrophosphorylase; C – carbon; CAM – Crassulacean acid metabolism; CaMV – cauliflower mosaic virus; CHX – cyclohexamide; cIDH – cytosolic isocitrate dehydrogenase; CoA – coenzyme A; DHAP – dihydroxyacetone phosphate; FC – fusicoccin; Fd – ferredoxin; FNR – ferredoxin NADP reductase; G6PDH – glucose 6-phosphate dehydrogenase; GDH – glutamate dehydrogenase; Glc 6-P – glucose 6-phosphate; GOGAT – glutamate synthase; GS – glutamine synthetase; N – nitrogen; NiR – nitrate reductase; NR – nitrate reductase; 2-OG – 2-oxoglutarate; OAA – oxaloacetate; PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase; PGA – 3 phosphoglycerate; Pi – inorganic phosphate; PK – protein kinase; PM-NR – plasmamembrane bound nitrate reductase; PP – protein phosphatase; rbcS – ribulose 1,5-bisphosphate carboxylase oxygenase small subunit; RPPP – reductive pentose phosphate pathway; Ru5P – ribulose 5 phosphate; RubP – ribulose 1,5 bisphosphate; SPS – sucrose phosphate synthase; Susy – sucrose synthase; TCA – tricarboxylic acid; TP – triose phosphate

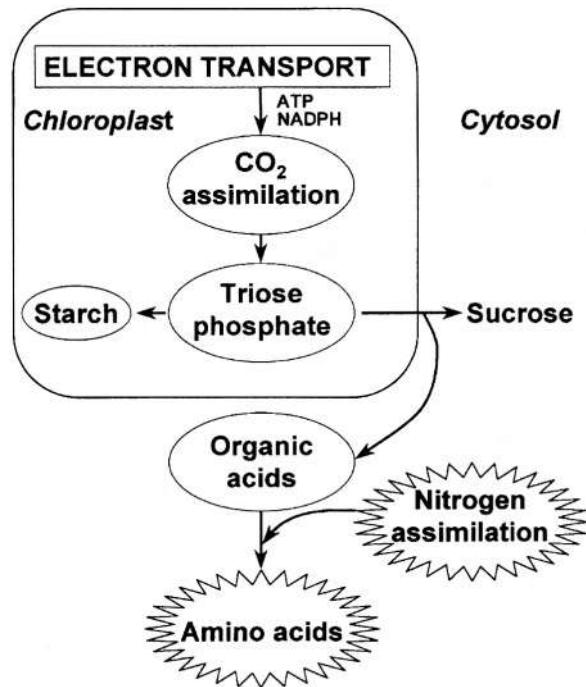


Fig. 1. Representation of the central role of triose phosphate in foliar carbon metabolism.

are largely derived from photorespiration; Ala, which is produced from pyruvate; and Asp and Asn that both arise from oxaloacetate.

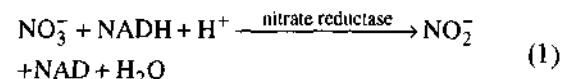
Plant growth and productivity is determined not only by photosynthetic capacity, but also by the way in which energy and carbon skeletons are partitioned, allocated and utilized for the assimilation of inorganic elements, particularly nitrogen. Nitrogen can be used in various forms (nitrate, nitrite, ammonium organic nitrogen) by plants. Recently, much attention has been given to N assimilation via absorption of atmospheric NO_2 and other man-made nitrous pollutants (Rennenberg et al., 1998) but nitrate (taken up by the roots) is considered to be the major N source for higher plants in well aerated soils. Nitrogen uptake mainly occurs across the epidermal and cortical cells of roots. It is subsequently either transported to the vacuole, effluxed out of the cell or transported to other cells. The presence of efficient, membrane-bound transport proteins allows plants and algae to assimilate nitrogen either as nitrate or ammonia (Daniel-Vedele et al., 1998). In many crop species primary N assimilation occurs predominantly in the leaves and is modulated in response to changes in photosynthesis (Kaiser and Forster, 1989; Pace et al., 1990; Ferrario-Méry et al., 1998). In other plants,

particularly wild, ruderal species, nitrate is assimilated in roots using carbon skeletons and energy derived from sucrose exported from the leaves to the roots.

A. The Provision of Energy and Carbon Skeletons

N assimilation involves two processes: (1) the oxidative formation of oxoacids from triose phosphate, and (2) the reductive incorporation of ammonia (derived from nitrate) into oxoacids to form amino acids (equations 1–4). Nitrate assimilation leads firstly to the formation of ammonium (Eqs. (1 and 2)).

Cytosol:

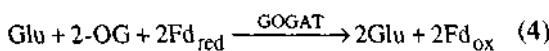
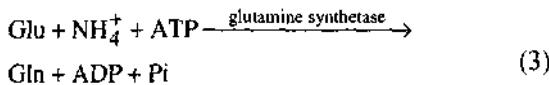


Stroma:



The NR isoforms involved in primary N assimilation are cytosolic proteins that use NADH or NADPH to reduce nitrate to nitrite Eq. (1). In addition, succinate-oxidizing nitrate reductase (PM-NR) isoforms are localized on the plasmamembrane of roots (Stöhr and Ullrich, 1997) and in algae (Stöhr et al., 1993). It is possible that the membrane-bound form of the enzyme may also function to produce nitric oxide (NO^\cdot) from nitrite. NO^\cdot is a powerful regulator of cytochrome *c* oxidase in plant mitochondria (Chapter 7 (Atkin et al.); Millar and Day, 1996). There is mounting evidence that NO^\cdot is a by-product of primary N assimilation in plants, produced when nitrite accumulates (Millar and Day, 1997). The PM-NR isoforms may, therefore, have a role in signal transduction in situations where nitrate reduction exceeds the rate of ammonium formation.

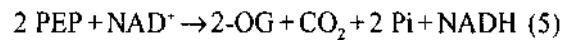
In higher plants, ammonia is assimilated via the chloroplastic enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). The reaction catalyzed by GS Eq. (3) is considered to be the major route facilitating the incorporation of inorganic nitrogen into organic molecules, in conjunction with glutamate synthase Eq. (4), which recycles glutamate and incorporates carbon skeletons into the cycle for the transfer of amino groups (Lea and Miflin, 1974; Coschigano et al., 1998).



The acceptor for the amino groups in the reaction catalyzed by GOGAT is 2-oxoglutarate (2-OG), but a wide range of other organic acids act as the precursors for the synthesis of other amino acids. The shortest metabolic route for generation of 2-OG and other organic acids from triose phosphate is through glycolytic metabolism of triose phosphate and partial anaplerotic tricarboxylic acid (TCA) cycle activity (Fig. 2). It is now widely accepted that respiratory carbon flow must occur in the light to produce metabolic precursors (Noctor and Foyer, 1998). Oxidative phosphorylation is also considered to be at least partially operative in the light and necessary for sucrose synthesis (Krömer et al., 1988; Krömer and Heldt, 1991; Krömer, 1995). Carbon metabolism associated with 2-OG formation not only provides carbon skeletons for the assimilation of ammonium but also organic acids for acid-base regulation and is a source of ATP (Chapter 7, Atkin et al.). The respiratory carbon flow required for photosynthetic N assimilation has the potential to form significant amounts of ATP and may be able to contribute ATP to meet the high ATP/reductant requirements of photosynthetic C assimilation (see Chapter 7, Atkin et al.; Noctor and Foyer, 1998).

While the major route of 2-OG production involves partial operation of the TCA cycle in the mitochondrion, 2-OG synthesis may also occur via a cytosolic isocitrate dehydrogenase (Fig. 2). In the TCA cycle, acetate units are oxidized to CO_2 and cannot, therefore, lead to net synthesis of cycle metabolites. Removal of any of the intermediates for biosynthetic reactions will require replenishment of carbon skeletons subsequent to the steps involving decarboxylation (i.e. as C_4 acids). These could be supplied as succinate, fumarate, malate or oxaloacetate. In mature leaves, the most important input of C_4 acids for 2-OG synthesis is thought to be as oxaloacetate, generated by the enzyme phosphoenolpyruvate (PEP) carboxylase (PEPC). Instead of all of the PEP being converted to pyruvate for oxidative decarboxylation prior to entry into the cycle, approximately half of the PEP is carboxylated to oxaloacetate via PEPC, thus allowing assimilatory

2-OG production through reactions catalyzed by citrate synthase, aconitase and isocitrate dehydrogenase (Fig. 2). The net reaction can be represented simply as:



PEPC carboxylase therefore has a central function in the C and N economy of the leaf. Different PEPC isoforms perform essential roles in photosynthesis in C_4 and CAM plants (Duff and Chollet, 1995) and in anaplerotic metabolism in all plants (O'Leary, 1982). The PEPC isoforms differ in their physiological and biochemical properties but all of the isoforms are regulated by transcriptional and post-translational (protein phosphorylation) controls (Tagu et al., 1991; Cushman et al., 1993; Pacquit et al., 1993; Hartwell et al., 1996). Phosphorylation of the PEPC protein activates the enzyme leading to decreased inhibition by malate or increased activation by G6P. The K_m and V_{max} values measured for the photosynthetic PEPC isoforms in C_4 plants are higher than those reported for C_3 isoforms (Jiao and Chollet, 1991; Duff and Chollet, 1995).

Gln acts as an amino donor in relatively few reactions but amino groups are transferred from Glu to oxaloacetate and pyruvate to produce Asp and Ala, respectively (see Ireland, 1997, for review). In photorespiration, amino groups are transferred to glyoxylate to produce Gly and, subsequently, Ser. PEP and erythrose-4-P are the C skeletons used for the synthesis of aromatic amino acids (Phe, Trp, Tyr). Aspartate is the starting-point for the synthesis of Asn, Thr, Lys and Met (Ireland, 1997). C skeletons for the branched-chain amino acids come from pyruvate (Val, Leu) or 2-oxobutyrate (Ile) and acetyl CoA. Ribose-5-P is used for His synthesis and 2-OG is required for Arg synthesis. In almost all of these pathways the immediate amino donor is Glu or Asp (Heldt, 1997).

B. Effects of Nitrate Assimilation on Photosynthesis, Sucrose and Starch Synthesis

While the relative composition of the photosynthetic apparatus is largely unaffected by N supply (Evans, 1983; Evans and Terashima, 1987), the availability of nitrogen plays a crucial role in determining overall photosynthetic capacity (Evans and Terashima, 1987; Heckathorn et al., 1997), since about three-quarters of the N in leaves is contained in chloroplast proteins

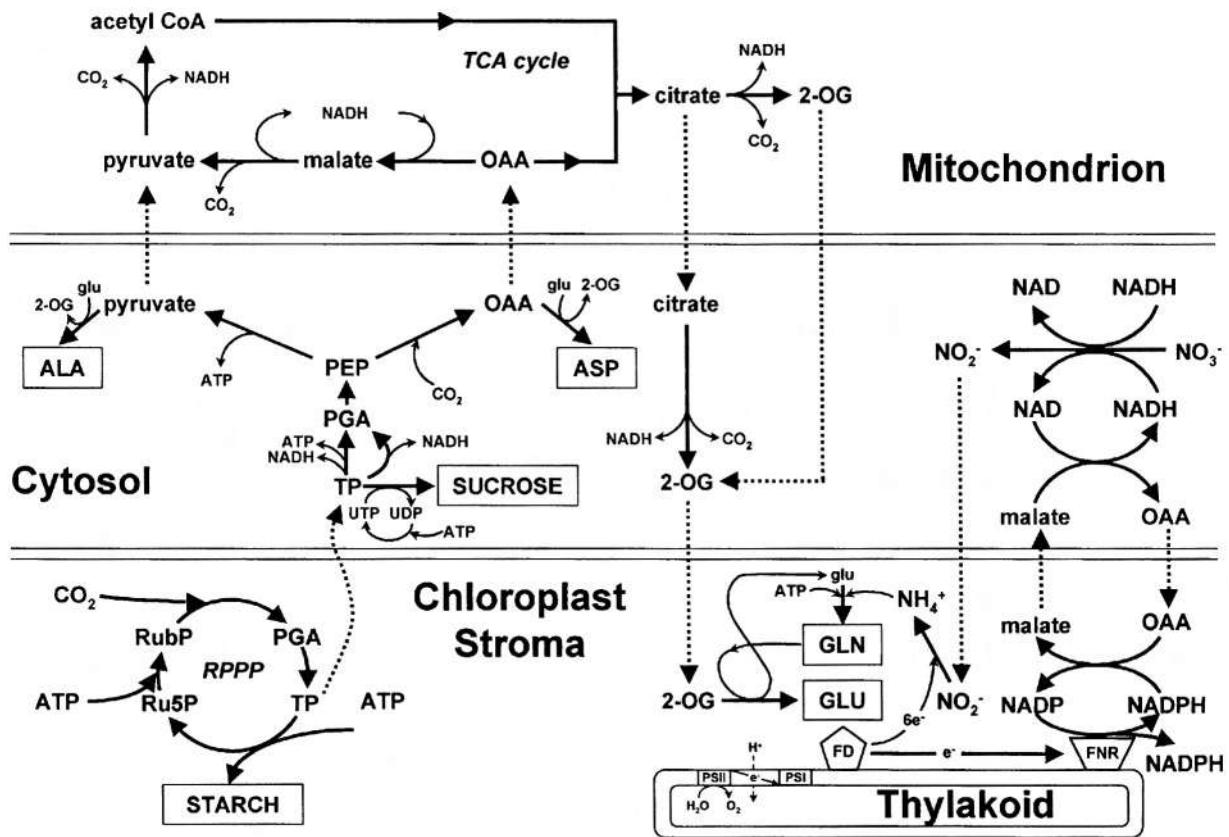


Fig. 2. Metabolic scheme showing the shortest metabolic route for the production of 2-oxoglutarate (2-OG) from triose phosphate (TP) and the subsequent utilization of 2-OG for amino acid biosynthesis.

(Evans, 1989). The availability of N also affects leaf growth (Makino et al., 1984), shoot to root ratios (Scheible et al., 1997a) and the capacity of plants to recover from drought (Heckathorn et al., 1997; Ferrario-Méry et al., 1998).

The assimilation of N in leaves leads to a drop in the assimilatory quotient (net CO_2 fixed per net O_2 evolved). This is due to a stimulation of photosynthetic O_2 evolution as a result of nitrite reduction (de la Torre, 1991) but concurrent respiratory O_2 uptake may partially offset this increase. Unregulated competition for reducing power and ATP between the processes of carbon and nitrogen assimilation is not observed in the leaves of higher plants. Nitrate assimilation does not inhibit CO_2 assimilation in leaves but it changes the partitioning of carbon, decreasing the rates of sucrose and starch synthesis and stimulating the flow of carbon through the pathways of organic acid biosynthesis. This coordination is achieved by a network of controls that regulate the activities of enzymes regulating the

flux through these pathways.

Appropriate provision of triose phosphate in the required stoichiometries for sucrose synthesis and for the formation of oxoacids for primary N assimilation into amino acids requires precise coordination between the pathways of C and N assimilation in plants. Many lines of evidence point to numerous steps of reciprocal control at the levels of gene transcription, mRNA stability and translation, as well as post-transcriptional regulation. Increased nitrate assimilation, for example, leads to increased synthesis of organic acids (Kaiser and Forster, 1989; Foyer and Ferrario, 1994), and decreased sucrose and starch synthesis (Waring et al., 1985; Fichtner and Schulze, 1992). Glutamine, glutamate, aspartate, 2-oxoglutarate and malate accumulation are modified following addition of either nitrate or ammonium to the alga *Selenastrum minutum* and carbon flow into organic acids is stimulated by coordinated activation of pyruvate kinase and PEPC (Huppe and Turpin, 1994; Turpin et al., 1997). In higher plants, there is

evidence for allosteric regulation of these enzymes in response to N supply (Champigny and Foyer, 1992; Turpin et al., 1997). Transgenic plants lacking the primary enzyme of CO₂ fixation, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), have low NR activity and accumulate more nitrate but contain less glutamine and other amino acids than untransformed controls (Stitt and Schulze, 1994). Such studies indicate that low rates of photosynthesis are accompanied by low rates of nitrate assimilation and vice versa.

The activities of several enzymes in the cytosol, including NR, SPS and PEPC, are modulated in response to changes in photosynthesis. In the light, photosynthesis becomes active and signals are transmitted from the chloroplast to the cytosol so that NR, SPS and PEPC are activated within a few minutes (see Section II). NR and SPS are activated by dephosphorylation and PEPC is activated by phosphorylation. The activation of the enzymes increases their capacities and modulates flux through sucrose synthesis, nitrate assimilation and organic acid synthesis (Champigny and Foyer, 1992). Coordinate regulation of PEPC (Foyer et al., 1994; Duff and Chollet, 1995; Li et al., 1996) and SPS (Champigny et al., 1992; Huber and Huber, 1996) has been suggested to be achieved via regulation by protein phosphorylation (see Section II). SPS, NR and PEPC are not always regulated in parallel, however, indicating that the metabolic and environmental factors that modulate the activities of these enzymes are not strictly identical.

In higher plants and in algae N assimilation stimulates respiratory metabolism in both the light and dark thereby directing carbon from starch and sucrose biosynthesis to respiration and amino acid biosynthesis. This activation of respiration results in inhibition of photosynthesis in algae, but not in higher plants. This is caused by redirection of carbon and reducing power to capture limited resources. The assimilation of NO₃⁻ in both light and dark activates the oxidative pentose phosphate pathway providing reducing power for nitrate reduction and C skeletons for amino acid synthesis (Vanlerberghe, 1991; Huppe et al., 1992). In algal systems glucose 6-phosphate dehydrogenase (G6PDH), which catalyzes the first step of this pathway and is regulated by reversible oxidation/reduction by the ferredoxin-thioredoxin system, is modified in the presence of nitrate. At limiting NO₃⁻ light-dependent reduction of chloroplastic G6PDH inhibits the oxidative pentose

phosphate pathway during photosynthetic carbon assimilation. The addition of nitrate causes rapid oxidation and activation of G6PDH in algae, stimulating carbon flow through the oxidative pentose phosphate pathway (Huppe et al., 1994). In contrast, the assimilation of ammonium activates glycolysis and the TCA cycle but not G6PDH in algae (Huppe et al., 1992).

C. Effects of Sucrose on Nitrogen Assimilation

Sucrose and 2-OG have been shown to be signal compounds that communicate and integrate information on metabolic status to ensure the appropriate balance of supply and demand between source and sink reactions. Sucrose and other signals arising from primary carbon metabolism directly influence photoassimilate production and utilization through the control of gene expression. Current concepts implicate hexokinase activity in sugar sensing (Chapter 10, Graham and Martin; Jang et al., 1997) but other components at the plasmamembrane such as two sugar sensors Snf3p and Rgt2p are also involved (Ozcan et al., 1996; Smeekens and Rook, 1997).

Signals from carbon metabolism regulate nitrate assimilation and amino acid synthesis in response to changes in the availability of carbohydrate (Morcuende et al., 1998). The abundance of transcripts for several enzymes involved in nitrogen metabolism is modified by sugars. For example, feeding sucrose and glucose to leaves increased NR transcript abundance (Cheng et al., 1992; Vincentz et al., 1993) and decreased the asparagine synthetase transcript abundance (Lam et al., 1994, 1996). Sugars also lead to increased activation of NR (Kaiser and Spill, 1991; Kaiser and Huber, 1994). Sucrose acts in concert with nitrate (and antagonistically to Gln) to increase NR activity (Morcuende et al., 1998). Although sugars contribute to the increase of *nia* transcript, NR protein, and post-transcriptional activation of NR after illumination of mature leaves, the abundance of *nia* transcripts and of NR protein have been found to decrease as the photoperiod progresses, even though carbohydrates continue to accumulate in the leaf (Galangau et al., 1988; Scheible et al., 1997b). In tobacco, the abundance of NR gene (*nia*) transcripts is maximal at dawn, decreasing throughout the day, whereas in maize, NR transcripts are maximal 1 to 2 h into the photoperiod (Huber et al., 1994). These changes probably reflect the complex

interplay of modulation by nitrate (Cheng et al., 1986), sucrose (Cheng et al., 1992; Vincentz et al., 1993) and Gln (Vaucheret et al., 1990; Deng et al., 1991; Vincentz et al., 1993; Hoff et al., 1994; Dzuibany et al., 1998) which stimulate and inhibit transcription of *nia*. Dzuibany et al. (1998) have argued against a role for Gln as an effective repressor of *nia2* transcript accumulation in *Arabidopsis*. Recent studies with transformed tobacco expressing an antisense Fd-GOGAT construct suggest that 2-oxoglutarate acts antagonistically to Gln, hence Gln will only inhibit NR transcription when 2-oxoglutarate is low (see Section V). Studies of transgenic tobacco plants overexpressing *nia* under the control of a constitutive promoter indicate that the decay is due to post-transcriptional regulation (Vincentz and Caboche, 1991; Ferrario et al., 1995) and not just a consequence of decreased *nia* transcription rate. Sucrose and glucose act antagonistically on NR activation (Kaiser and Huber, 1994; Scheible et al., 1997b).

Nitrate and sugars modulate translation of NR mRNA and affect the stability of NR protein. NR protein content and NR activity are generally higher in the light than in the dark providing strong evidence that post-transcriptional controls are important; neither correlate with transcript abundance (Scheible et al., 1997b). Studies of transgenic plants over-expressing a modified NR that is not susceptible to phosphorylation, together with observations on NR phosphorylation state and NR stability (Kaiser and Huber, 1997), indicate that phosphorylation regulates NR degradation as well as NR activity. Increased expression of PEPC, cytosolic pyruvate kinase, citrate synthase and the NADP-dependent isocitrate dehydrogenase has been observed following addition of nitrate to N-deficient plants (Scheible et al., 1997c). This is considered necessary to allow increased synthesis of organic acids, while decreased ADP-glucose pyrophosphorylase activity inhibits starch synthesis in these conditions (Scheible et al., 1997c).

D. Covalent Modification and Allosteric Control

Protein phosphorylation is generally recognized as being an important process controlling many aspects of signal transduction, development and membrane transport, but in the area of metabolism, there are still relatively few enzymes known to be regulated by this mechanism. Most of the currently recognized enzymes are localized in the plant cell cytoplasm,

where they are involved in major pathways of C- and N-metabolism. The relevant enzymes, and the pathways in which they function, are shown in simplified fashion in Fig. 3. With many of the enzymes, their phosphorylation status (and hence enzymatic activity) is modulated in leaves by light/dark transitions. Enzymes controlled in this manner include SPS, PEPC, and NR (for reviews see Huber et al., 1994; Huber and Kaiser 1996; Chollet et al., 1996). In addition, recent evidence suggests that the activity of the plasma membrane H⁺-ATPase may also be regulated by phosphorylation in a complex process that also involves 14-3-3 proteins (Moorhead et al., 1996).

E. Analysis of C/N Metabolism in Mutants and Transformed Plants

Transformed plants and mutants have been widely used to study the pathways of carbon, nitrogen and ammonium assimilation in plants. While it is not possible to provide an exhaustive list of the results of such studies to date, Table 1 attempts to summarize the major findings of key studies involving SPS, PEPC, NR, GS and GOGAT. Such studies have yielded interesting and occasionally surprising results. Early studies with mutants deficient in NR suggested that this enzyme was present in vast excess of that required to support growth and development. Protein content and growth were not decreased in barley mutants with only 10% of NR activity measured in the wild type controls (Warner and Kleinhofs, 1981; Warner and Huffaker, 1989). Similar results were obtained in *Arabidopsis* mutants (Wilkinson and Crawford, 1993). *Nicotiana plumbaginifolia* and *N. tabacum* mutants with 40–50% of the wild type NR activity were also unaltered in phenotype to the wild-type controls (Müller and Mendel, 1989; Vaucheret et al., 1990; Vincentz and Caboche 1991; Dorbe et al., 1992; Scheible et al., 1997a). Only when the evidence for post-transcriptional and post-translational regulation of NR was presented was this situation clarified. Regulated changes in the activation state of the enzyme may be used to compensate for decreases in maximal extractable enzyme activity, to maintain the appropriate balance between C and N acquisition in the plant (Scheible et al., 1997b). Changes in NR expression together with regulation of NR activity, allowing assimilation to occur for a longer period each day, were found to offset the effects of decreased N assimilation capacity (Scheible

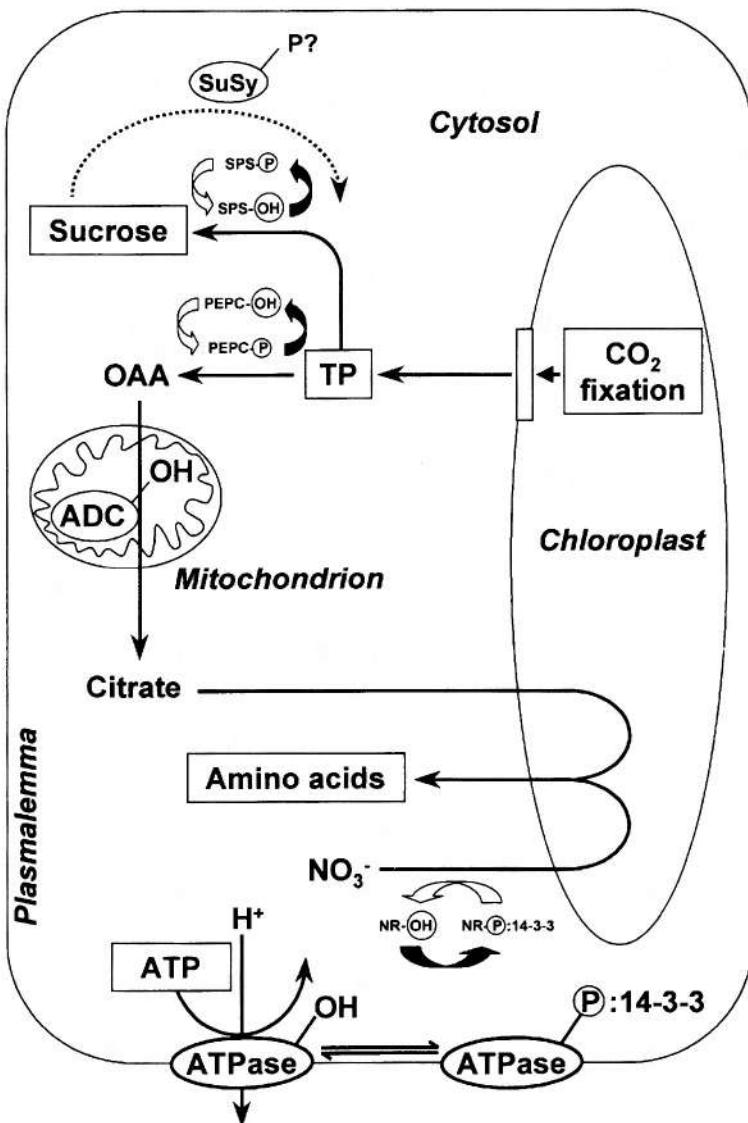


Fig. 3. Scheme showing key phosphorylated enzymes associated with the cytosol of plant cells: sucrose phosphate synthetase (SPS), phosphoenolpyruvate carboxylase (PEPC), the plasmamembrane bound ATPase and nitrate reductase (NR).

et al., 1997b). Transformed plants with decreased NR activity have also been used to demonstrate a role for nitrate in the regulation of root-shoot allocation (Scheible et al., 1997a) and in the modulation of carbon partitioning (Scheible et al., 1997c).

II. Sucrose-P Synthase

High SPS activities are found in leaves where they

are subject to complex regulatory controls involving: i) molecular genetic regulation of gene expression and steady state enzyme protein contents), ii) allosteric regulation involving Glc-6-P activation and Pi inhibition, and iii) protein phosphorylation. Many different mechanisms work in parallel to adjust SPS protein content as well as the catalytic activity of the enzyme protein. Phosphorylation of SPS was originally characterized as the mechanism underlying light/dark modulation of SPS activity (Huber and Huber, 1992). More recently phosphorylation of SPS

Table 1. A summary of some of the results obtained in various studies on mutants and transformed plants modified in nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPC), glutamine synthetase (GS) and glutamate synthase (GOGAT)

Reference	Type of mutation or transformation	Plant material	Consequences on C, N metabolism
NR			
Scheible et al., 1997a	mutant deficient for 1 or 2 <i>nia</i> gene	<i>N. tabacum</i>	Modification in diurnal regulation of NR mRNA transcription post-translational modification and turnover of NR
Vaucheret et al., 1990	tobacco <i>nia2</i> gene	<i>N. plumbaginifolia</i> mutant deficient in NR	Functional complementation of NR but low level of NR activity
Vincentz and Caboche, 1991	35S <i>nia2</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	Increase in NR activity and mRNA
Dorbe et al., 1992	tomato <i>nia</i> gene	<i>N. plumbaginifolia</i> mutant deficient in NR	NR activity restored and regulated but low level of NR
Foyer et al., 1993	a) 35S <i>nia2</i> b) tobacco <i>nia2</i> gene	<i>N. plumbaginifolia</i> mutant deficient in NR	a) Increase in NR activity; increase in amino acid; no change in starch and soluble sugar b) Low NR activity; decrease in leaf protein, Chl, amino acid; increase in starch and sucrose
Foyer et al., 1994	35S <i>nia2</i> or tobacco <i>nia2</i> gene	<i>N. plumbaginifolia</i> mutant deficient in NR	Maximum rate of photosynthesis modified in low NR transformant; unchanged in high NR expressor
Quilleré et al., 1994	35S <i>nia</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	Increase in NR activity; decrease in NO_3^- content; increase in Gln and malate contents
Nussaume et al., 1995	35S deleted <i>nia2</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	Suppression of post-transcriptional regulation of NR by light increase in Gln and Asn contents
Ferrario et al., 1995	35S <i>nia2</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	NR activity photosynthesis and biomass decreased by low nitrogen supply
Ferrario et al., 1996	35S <i>nia2</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	NR activation state is not influenced by NO_3^-
Navarro et al., 1996	35S <i>nia2</i>	<i>Chlamydomonas</i>	Inactivation of NR in absence of NO_3^-
Ferrario-Méry et al., 1997	35S <i>nia2</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	Increase in NR activity; increase in Gln level and high C/N ratio maintained in high CO_2 conditions
Ferrario-Méry et al., 1998	35S <i>nia2</i>	<i>N. plumbaginifolia</i> mutant deficient in NR	Delay in the decrease in NR activity due to drought
Scheible et al., 1997b	a) mutant deficient for 1 or 2 gene b) tobacco <i>nia2</i> gene	a) <i>N. tabacum</i> b) <i>N. tabacum</i> mutant deficient in NR	Varying levels of NR accumulation in NO_3^- but low amino acid and protein contents and increased shoot to root allocation correlated to a decreased sugar allocation to roots
Scheible et al., 1997c	tobacco <i>nia2</i> gene	<i>N. tabacum</i> mutant deficient in NR	Varying levels of NR accumulation of, NO_3^- and induction of NR, NiR, GS1 GOGAT, PEPC, ICDH and repression of ADPGlc PPase

Table 1. (Continued)

Reference	Type of mutation or transformation	Plant material	Consequences on C, N metabolism
PEPC			
Tagu et al., 1991	C ₄ Sorghum PEPC	<i>N. tabacum</i>	Expression in leaves but not in roots
Hudspeth et al., 1992	a) Cab C ₄ maize PEPC b) C ₄ maize PEPC	<i>N. tabacum</i>	a) Expression in mesophyll cells; increase in PEPC activity and malate content; no change in photosynthesis b) Aberrant transcription initiation; light regulation maintained
Cushman et al., 1993	CAM <i>Mesembryanthemum</i> PEPC promoter-GUS	<i>N. tabacum</i>	Role of 5' untranslated region in salt stress inducibility
Matsuoka et al., 1994	C ₄ maize promoter-GUS	rice	Light regulation maintained
Kogami et al., 1994	35S c ₄ maize PEPC	<i>N. tabacum</i>	Increase in PEPC; decrease in growth rate and Chl content
Stokhaus et al., 1994	C ₄ <i>Flaveria</i> promoter-GUS	<i>N. tabacum</i>	Role of 5' untranslated region in C ₄ specific expression
Gehlen et al., 1996	a) bacterial PEPC b) antisense bacterial PEPC	potato	a) Decrease in growth rate; increase in malate; no change in photosynthesis b) No change in photosynthesis
Pathirana et al., 1997	nodule alfalfa PEPC promoter-GUS	alfalfa	Role of 5' untranslated region in nodule specific expression
GS/GOGAT			
Eckes et al., 1989	35S alfalfa GS1	<i>N. tabacum</i>	Increase in GS, decrease in NH ₄ ⁺ ; no change in amino acid composition
Hirel et al., 1992	35S soybean GS1	<i>N. tabacum</i>	Increase in GS; induction of mRNA for a native cytosolic GS
Temple et al., 1993	35S alfalfa GS a) sense construct b) antisense construct	<i>N. tabacum</i>	a) Increase in GS1 b) Decrease in GS1 and GS2; decreases in PEPC and hydroxypyruvate reductase
Temple et al., 1994	phloem specific promoter-antisense GS1	alfalfa	Low viability
Vincentz et al., 1997	35S soybean GS1	<i>Lotus corniculatus</i>	Increase in GS; increase in aa, NH ₄ ⁺ due to early senescence; decrease in carbohydrates in roots
Temple and Sengupta-Gopalan, 1997	nodule or vascular specific promoter-alfalfa GS1 (differing in 3' untranslated region)	alfalfa	Decrease in GS in nodule; increase in GS in stem

Somerville et al., 1980	Fd-GOGAT deficient mutant	<i>Arabidopsis</i>	a) In high CO ₂ ; no changes in Rubisco, GS, GDH and in photosynthesis b) In air; increase in NH ₄ ⁺ , Gln, organic acids; decreases in Glu and amino acid; decrease in photosynthesis
Kendall et al., 1986	Fd-GOGAT deficient mutant	barley	a) In high CO ₂ ; no change in photosynthesis b) In air; increase in malate, Gln, Asn and NH ₄ ⁺ ; decreases in Asp, Ala, Glu, Gly and Ser; decrease in photosynthesis; ¹⁴ C incorporation increases into sugars P, glycollate and malate and decreased into Ser, Gly, glycinate and sucrose
Blackwell et al., 1988a	Fd-GOGAT and/or GS deficient mutant	barley	Increase in NH ₄ ⁺ in air; decrease in photosynthesis in air; reduced ability to incorporate ¹⁴ C derived from ¹⁴ CO ₂ into sucrose in air
Blackwell et al., 1987 Wallsgrove et al., 1987 Matson et al., 1997	GS2 deficient mutant	barley	Viable only in non-photorespiratory conditions; increase in NH ₄ ⁺ in air; inhibition in photosynthesis in air
Morris et al., 1989	Fd-GOGAT deficient mutant	<i>Arabidopsis</i>	In air; accumulation of NH ₄ ⁺ ; inhibition of photosynthesis is probably due to the depletion of amino donors for glyoxalate
Coschigano et al., 1998	Fd-GOGAT deficient mutant	<i>Arabidopsis</i>	The two Fd-GOGAT genes have distinct roles in photorespiration and primary nitrogen assimilation
Joy et al., 1992	Fd-GOGAT or GS deficient mutant	barley	a) In high CO ₂ ; organic nitrogen exported (Gln) from roots to shoots in the xylem sap is reduced in GS deficient mutants and enhanced in the Fd-GOGAT mutant (¹⁵ N feeding experiments); ¹⁵ N supplied to excised leaves is accumulated in Gln but at very low level in other amino acid in Fd-GOGAT deficient mutants
Hausler et al., 1994a	a) Fd-GOGAT with reduced activities (63–75%) b) GS mutants with reduced activities (47–97%)	barley	Growth is not severely impaired in air a) in air; no change in Rubisco, prot Chl ammonium increase; amino acid decrease and increase in Ser/Gly b) in air; decrease in Rubisco, prot, Chl, NO ₃ ⁻ ; no change in ammonium and amino acid but Gln increase Glu and ASP decrease Ser/Gly increase
Hausler et al., 1994b	a) Fd-GOGAT with reduced activities (63–75%) b) GS mutants with reduced activities (47–97%)	barley	a) In air; electron requirement per CO ₂ assimilated is decreased b) In air; CO ₂ assimilation is reduced
Hausler et al., 1996	a) Fd-GOGAT with reduced activities (63–75%) b) GS mutants with reduced activities (47–97%)	barley	a) In air; serine:glyoxylate aminotransferase decreased Glutamate and alanine: glyoxylate aminotransferase increased Negative correlation between the activation state of Rubisco and glyoxylate content

Table 1. (Continued)

Reference	Type of mutation or transformation	Plant material	Consequences on C, N metabolism
Lea et al., 1992	Fd-GOGAT deficient mutant	pea	a) In air; increase in Gln; decrease in Asn and other amino acid b) In high CO ₂ or in air, ¹⁵ N Gln feeding experiments leads to very low ¹⁵ N incorporation in other amino acids
Edwards et al., 1990	pea chloroplast GS2 promoter-GUS pea cytosolic GS3A promoter-GUS	<i>N. tabacum</i>	Cell specific expression of GS2 in leaves and GS3A in phloem
Miao et al., 1991	soybean cytosolic GS15 promoter-GUS	<i>Lotus corniculatus</i> <i>N. tabacum</i>	Root specific expression inducibility by NH ₄ ⁺ in <i>Lotus</i> but not in tobacco roots
Brears et al., 1991	a) pea cytosolic GS3A promoter-GUS b) 5' deleted GS3A promoter-GUS	<i>N. tabacum</i> alfalfa	Expression in phloem and in root nodule not altered; identification of a nuclear DNA binding protein
Cock et al., 1992	bean plastidic GS2 5' deleted promoter-GUS	<i>N. tabacum</i>	Tissue specific and light regulation of GS2 related to an essential region for promoter
Tjaden and Coruzzi, 1994	pea chloroplastic GS2-AT rich clement deleted promoter-GUS		Decrease in GUS activity and isolation of a novel DNA binding protein

has also been implicated in the activation of the enzyme that occurs when leaf tissue is subjected to osmotic stress (Toroser and Huber, 1997).

A. Regulatory Phosphorylation Sites

SPS is phosphorylated on multiple seryl residues, only one of which is involved in the dark-inactivation of the enzyme (Huber and Huber, 1992). The phosphorylated residue was subsequently shown to be Serine-158, and this residue is conserved among species (Huber and Huber, 1996). Phosphorylation of Ser-158 reduces SPS activity by altering affinities for substrates and effectors, without affecting maximum catalytic activity. Recent evidence suggests that there is a second regulatory phosphorylation site at Ser-424, which is phosphorylated when leaf tissue is subjected to osmotic stress (Toroser and Huber, 1997). This site is also conserved among species. Phosphorylation of Ser-424 may activate the enzyme by antagonizing the inhibitory effect of Ser-158 phosphorylation, and thereby allow sucrose synthesis to occur when it would otherwise be restricted.

B. Sucrose-P Synthase-Kinase

Ion-exchange chromatography of spinach leaf proteins resolves several peaks of protein kinase activity that will phosphorylate a synthetic peptide based on the amino acid sequence surrounding Ser-158 (McMichael et al., 1995; Douglas et al., 1996). However, not all of the kinases that will phosphorylate the synthetic peptide are capable of phosphorylating the regulatory site in the native protein. One of the major protein kinase activities that phosphorylates Ser-158 in the native protein has been designated Peak III (or **PK_{III}**) and tends to co-purify with SPS. **PK_{III}** is **Ca²⁺-independent**, has a native Mr of ~150,000 (McMichael et al., 1995), and a catalytic subunit of ~ 60 kDa that is recognized by antibodies against RKIN1, a plant SNF1 homolog (Douglas et al., 1996). The SNF1 family of protein kinases was named for the sucrose non-fermenting kinase of yeast, and subsequently, kinases with similar catalytic sub-units were identified in mammalian and plant systems (Halford and Hardie, 1998). A characteristic property of the SNF1-like kinases is that they are often regulated by phosphorylation, and this has been demonstrated for **PK_{III}** (Douglas et al., 1996). Dephosphorylation/inactivation of **PK_{III}** is catalyzed by PP2C in vitro but details of the phosphorylation/

activation reaction are lacking. If a distinct kinase is involved, then a phosphorylation cascade would be involved that could be subject to regulation. It is not known at present whether the phosphorylation state, and hence activity, of **PK_{III}** varies in vivo. If so, this could also contribute directly to the control of SPS activation state.

One factor that is thought to regulate the **PK_{III}**-catalyzed phosphorylation/inactivation of SPS are metabolites, in particular Glc-6-P (Weiner et al., 1992). The Glc-6-P could act by binding to SPS at its allosteric site, or by directly affecting the protein kinase (**PK_{III}**). The limited evidence to date suggests that direct effects on the kinase may be involved, although some effect of binding to SPS as well may also contribute (McMichael et al., 1995). Regulation of **PK_{III}** by Glc-6-P could involve one of the putative regulatory subunits thought to associate with the ~60-kDa catalytic subunit to produce the native oligomeric protein.

C. Sucrose-P Synthase-Protein Phosphatase

Phospho-Ser-158-SPS can be dephosphorylated and activated in vitro by the catalytic subunit of protein phosphatase (PP) PP2A and not PP1 (Weiner, 1997). However, in vivo PP2As likely occur as oligomeric proteins, with the ~35-kDa catalytic subunit associated with one or more regulatory sub-units. One of the interesting regulatory properties of the PP2As that act on SPS is inhibition by Pi. Interestingly, the activity and regulatory properties of SPS-PP2A appear to change with light/dark treatment of leaves; the enzyme from illuminated leaves has a slightly higher activity and several-fold reduced sensitivity to Pi inhibition. The molecular basis for the shift in properties is not known, but involves cytoplasmic protein synthesis as the light-activation of SPS can be inhibited by cycloheximide (Weiner et al., 1992), and interconversion of the native enzyme between a dimeric and trimeric form (Weiner, 1997).

The action of PP2As on SPS may also be influenced by amino acids. It has been observed that the light activation of SPS in detached leaves can be reduced by feeding amino acids via the transpiration stream (Huber et al., 1996). The effect was not specific for a given amino acid, suggesting that it may be a rather general response. Inhibition of SPS-PP2A activity by amino acids has been observed in vitro, but relatively high concentrations (10 to 50 mM) were required which raises the possibility that the effect

may not be of physiological significance. However, amino acids are found at relatively high concentrations in the cytosol of leaf cells (Reins et al., 1994). Interestingly, changes in tissue amino acid content often parallel changes in SPS activation state, as would be expected if they inhibit the requisite PP2A(s). For example, in spinach leaves, accumulation of amino acids during the photoperiod tends to parallel the progressive inactivation of SPS that occurs. Another example involves *Vicia faba* seed development. Cotyledon SPS is partially inactivated (presumably by phosphorylation) during the storage phase (40 to 50 days after fertilization) and this generally corresponded with an accumulation of free amino acids (Weber et al., 1996). Furthermore, in vitro culture of *V. faba* cotyledons in a high hexose medium increased the activation state of SPS and decreased the level of free amino acids compared to cultures on a high sucrose medium. Thus, accumulation of free amino acids (presumably in excess of some optimum level) has been correlated with inactivation of SPS in several different tissues. Inhibition of SPS-PP2As by amino acids could be responsible.

D. Manipulation of Sucrose-P-Synthase Activity in Transformed Plants

Attempts to overexpress spinach SPS in potato have been largely thwarted by co-suppression phenomena. Similarly, replacement of serine residue 162, modifies the light/dark regulation of SPS but does not enhance total foliar SPS activity. In contrast, overexpression of a maize SPS cDNA in tomato or *Arabidopsis* leaves leads to substantial increases in total foliar SPS activity in both the light and dark. In the following studies SPS overexpression did not lead to a change in maximal extractable foliar NR activity or the C/N ratio in comparison to the controls when plants were grown in air or with CO₂ enrichment.

Overexpression of a maize SPS cDNA under the control of the promoter for the small sub-unit of Rubisco (rbcS; Worrell et al., 1991), increased foliar SPS activity between 300–600%. SPS activity was also increased in the roots of the rbcS transformants (Galtier et al., 1993). Increased foliar SPS activity was also observed when the maize leaf SPS cDNA was expressed under the control of the CaMV 35S promoter (Ferrario-Méry et al., 1997) or when the rbcS-SPS construct was expressed in *Arabidopsis* (Signora et al., 1998). Overexpression of an SPS

from a C₄ plant, maize, hence resulted in substantial increases in foliar SPS activity and an increased capacity for sucrose synthesis in the transformed C₃ plants (Worrell et al., 1991; Galtier et al., 1993, 1995; Micallef et al., 1995; Signora et al., 1998). The marked increase in the maximum extractable foliar SPS activities in these transformants may be due to incomplete regulation of the introduced SPS protein by protein phosphorylation. Unlike the native tomato or *Arabidopsis* SPS forms, the introduced maize enzyme did not appear to be inactivated in darkness (Worrell et al., 1991; Galtier et al., 1993, 1995; Signora et al., 1998).

While Micallef et al. (1995) and Signora et al. (1998) found no statistically significant differences in photosynthesis between the untransformed controls and the transformants in air, Galtier et al. (1993, 1995) found that photosynthetic CO₂ assimilation was increased in leaves of the transformed tomato plants in air relative to controls. The light and CO₂-saturated rates of photosynthesis were increased by as much as 20% relative to the untransformed controls (Galtier et al., 1993, 1995). When plants are exposed to CO₂ enrichment, CO₂ assimilation can exceed end-product (triose phosphate) utilization (Stitt, 1986). The increased capacity for sucrose synthesis afforded by SPS overexpression can alleviate this feedback inhibition (Signora et al., 1998).

There was a strong positive correlation between foliar SPS activity and the ratio of sucrose to starch in the leaves when plants overexpressing SPS were grown in air or with CO₂ enrichment (Galtier et al., 1993, 1995; Signora et al., 1998). Improved rates of photosynthesis at elevated CO₂ and modification of foliar carbon partitioning in favor of sucrose, may therefore be regarded as common features of SPS overexpression (Galtier et al., 1993, 1995; Micallef et al., 1995; Signora et al., 1998). While SPS overexpression did not affect the yield of tomato fruit, it affected both the import and turnover of sucrose in the fruit of the transformed plants (Nguyen-Quoc et al., 1999).

III. Phosphoenolpyruvate Carboxylase

PEPC plays an important role in C₄ and CAM plants as the primary carboxylating enzyme of photosynthesis and in C₃ plants functions broadly in pH regulation (the 'pH stat'), stomatal opening and nitrogen assimilation. The enzyme is generally

regulated by feedback inhibition by malate and allosteric activation by Glc-6-P, and these effects are often most pronounced at suboptimal pH (Chollet et al., 1996). Phosphorylation activates PEPC (Chapter 18, Fig. 3, Furbank et al.) by modulating sensitivity to metabolic effectors, in particular, inhibition by malate. Given that the N-terminal phosphorylation domain is strictly conserved, it is quite likely that protein phosphorylation is an important mechanism regulating higher plant PEPC, including the C_3 leaf enzyme (Li et al., 1996).

A. Regulation of Phosphoenolpyruvate Carboxylase-Kinase Activity

It is thought that PEPC is phosphorylated by one or more highly specific protein kinases, that are Ca^{2+} -independent and composed of 30- to 39-kDa polypeptides. The PEPC-kinase(s) are induced by light in C_3 leaves, and the induction is blocked by cycloheximide, as has been extensively studied in C_4 leaves and other systems. Studies with isolated C_4 mesophyll cells have identified a complex light-signal transduction pathway that involves increased cytosolic pH and $[Ca^{2+}]$. In situ, the mechanism of C_3 PEPC-kinase upregulation appears to differ from that of C_4 systems by an apparent requirement for Gln.

B. Manipulation of Phosphoenolpyruvate Carboxylase Activity in Transformed Plants

The possibility of transferring characteristics of the C_4 isoforms to C_3 plants have been evaluated using hybridization techniques involving C_3 and C_4 plants and also by direct genetic manipulation. Expression of C_4 type PEPC cDNAs from maize in a C_3 plant, tobacco, have been achieved using either constitutive expression under the control of the 35S promoter (35S-PEPC transformants; Kogami et al., 1994) or expression specific to photosynthetic tissues the *N. plumbaginifolia cab* (chlorophyll *a/b* binding protein) promoter (Hudspeth et al., 1992). Aberrant transcription initiation was observed when expression was under the control of the native promoter of the maize PEPC gene but expression of PEPC transcripts remained light-regulated (Hudspeth et al., 1992). When the *cab* gene promoter was used, expression of the transgene was observed only in the mesophyll cells. Total foliar PEPC activity in the transformants was twice that of the untransformed plants. In

addition, two novel forms of PEPC with different kinetic properties (low and high K_m values for PEPC) were identified in the transformants (Hudspeth et al., 1992). The transformants had higher foliar malate contents than the untransformed controls but photosynthesis was comparable in all plants. An increase in total extractable foliar PEPC activity and two forms of PEPC were also observed in the 35S-PEPC transformants (Kogami et al., 1994) but in this case phenotypic effects were observed in the transformed plants (which had a lower growth rate and less chlorophyll).

When a bacterial PEPC cDNA was expressed in potato the growth rates of the transformed plants was decreased (Gehlen et al., 1996) without any changes in photosynthetic rate. In this case malate accumulated in the leaves but no other changes were reported. When the PEPC cDNA was expressed in the antisense orientation malate accumulation in the leaves was decreased but no changes in photosynthesis or growth rate were observed (Gehlen et al., 1996).

PEPC is encoded by a gene family, each member of which exhibits differential expression in leaf mesophyll (C_3 plants), bundle sheath (C_4 plants), roots or nodules. Several studies have therefore attempted to identify the main regions of the promoter determining tissue specificity and light inducibility. The expression of the complete coding sequence of a C_4 type PEPC gene from *Sorghum vulgare* flanked by the 5' upstream sequence and the 3' sequence, was obtained in transformed tobacco plants (Tagu et al., 1991). This study showed that a promoter from a monocotyledonous species was effective in a dicotyledonous plant and also allowed correct excision of introns in the gene. Moreover, transgene mRNA was not detected in roots of the transformants, suggesting a role for the 5' region of the promoter in tissue-specific expression. Evidence that the 5' untranslated region of the PEPC gene is of great importance in nodule-specific expression (Pathirana et al., 1997), in salt-stress inducibility in CAM plants (Cushman et al., 1993) and in the C_4 -specific expression (Stockhaus et al., 1994) was provided by expression of a GUS reporter gene fused to different PEPC promoter regions in transformed tobacco, alfalfa and rice. Similar homology between light-regulated expression was observed in C_3 and C_4 plants (Matsuoka et al., 1994).

Site-directed mutagenesis of recombinant *Sorghum* PEPC was used to study the role of the highly conserved Ser residue which is involved in reversible

phosphorylation/dephosphorylation reactions controlling light/dark modulation of enzyme activity. The results obtained from such studies are in agreement with those obtained in other studies on structure/function and phylogenetic relationships. These data suggest that the differences between PEPC activities in plants arise from differential control of gene expression rather than variations in the primary structure of the isoforms.

IV. Nitrate Reductase

Control of NR by phosphorylation is a two-step process. The inactivation of NR by covalent modification involves: i) phosphorylation of Ser-543; and ii) binding of a 14-3-3 inhibitor protein to phospho-NR in the presence of a divalent cation. The regulatory phosphorylation site, Ser-543, is contained in the hinge 1 region that connects the heme and molybdenum-cofactor domains. Phosphorylation of Ser-543 alone has no effect on NR activity but rather completes the motif required for binding of the 14-3-3 protein (Huber et al., 1996; Moorhead et al., 1996). The amino acid sequence surrounding the phosphorylation site (R-T-A-phosphoS⁵⁴³-T-P) matches closely the consensus motif for 14-3-3 protein binding (R-S-X-phosphoS-X-P) originally identified by Muslin et al., (1996). The 14-3-3 proteins are small acidic proteins that are ubiquitous among eukaryotes and are generally highly conserved. In plants they are present in small gene families, and by analogy to animal systems, are thought to function in regulation of a wide array of regulatory and signaling pathways. In addition to their role in NR regulation, they apparently also serve as the fusicoccin receptor and may also function in the regulation of the plasma membrane H⁺-ATPase.

A. Nitrate Reductase Protein Kinases and Protein Phosphatase

Spinach leaves contain several protein kinases that can phosphorylate a synthetic peptide based on Ser-543 and can also phosphorylate this residue in the native protein. Two of the enzymes are Ca²⁺-dependent (Bachmann et al., 1996; Douglas et al., 1996) and thus, cytosolic [Ca²⁺] may be involved in regulating NR phosphorylation. In addition, other potential regulators of the NR-kinase(s) are metabolic intermediates such as DHAP, Glc-6-P, and Fru-1,6-

P₂ (Bachmann et al., 1996). Inhibition of NR-kinase activity by metabolites may be one of the components of the light-signal transduction pathway that mediates the light activation of NR. It is not known whether the metabolites interact with the kinase(s) and/or the target protein.

The endogenous protein phosphatase(s) that dephosphorylate and activate phospho-NR are thought to belong to the PP2A family, but little is known about the native enzymes that function in vivo. As discussed above, the 14-3-3 inhibitor protein that inactivates phospho-NR binds directly to the regulatory phosphorylation site (Ser-543). As a result, accessibility of the phospho-serine to phosphatases is reduced (Bachmann et al., 1996). This may have two important consequences. First, it may explain why the rate of NR activation in vivo is often slower than the rate of inactivation. Second, it may explain how chelation of Mg²⁺ or addition of Pi or salt, stimulate NR activation in vitro. It appears that Mg²⁺ binding to the 14-3-3 protein is required in order for the inhibitor protein to bind to phospho-NR (Athwal et al., 1998). The binding of the 14-3-3 protein is also affected by high ionic strength or Pi. Conditions that weaken binding would be expected to increase accessibility of phosphoSer-543 to phosphatases and thereby stimulate the rate of dephosphorylation.

B. Nitrate Reductase Hysteresis

Hysteresis refers to slow changes (e.g. in protein conformation) that affect the catalytic activity of an enzyme. Hysteretic behavior of NR was first described with purified NR in vitro by Lillo and Ruoff (1992) in reference to a time-dependent activation by pyridine nucleotides. It was subsequently shown that NR extracted from leaves placed in the dark for several hours had lower activity than when extracted from illuminated leaves, even when assays were performed in the absence of Mg²⁺ to eliminate inactivation by phosphorylation. The surprising observation was that NR activity in the dark extracts could be increased to the light activity by preincubation of extracts at 25 °C with one or more ‘activators,’ including Pi, AMP and pyridine nucleotides (Huber and Huber, 1995). The increase in activity was not blocked by protein phosphatase inhibitors indicating that dephosphorylation of Ser-543 was not involved. Rather, the activation may reflect the hysteretic behavior of the enzyme. Recent results suggest that it is only the phosphorylated form of NR that can adopt

the conformation with reduced activity, as assayed in the absence of Mg^{2+} (Kaiser and Huber, 1997). Although not demonstrated experimentally, the postulated less active conformation of NR may involve a tightened binding of the 14-3-3 inhibitor protein that is semi-stable in the absence of Mg^{2+} .

Conversion to the active conformation (hysteresis) *in vitro* is stimulated by a variety of ‘activator compounds.’ The process also seems to occur *in vivo* and may also involve effectors. In addition to activators, amino acids may serve as negative effectors that can block the activation *in situ*. Thus, feeding amino acids to excised leaves via the transpiration stream largely prevented the increase in NR activity that can be observed when assays are performed in the absence of Mg^{2+} . Thus, amino acids may effectively reduce NR activity *in vivo* by affecting the hysteretic behavior of the enzyme. It should also be noted that high concentrations of amino acids are also known to reduce NR gene expression (see Section I), and would likely restrict de novo NR protein synthesis.

C. Manipulation of Nitrate Reductase Activity in Transformed Plants

When the NR-deficient mutant of *N. plumbaginifolia* was transformed with a full-length tobacco NR cDNA fused to the CaMV 35S promoter, the transformed plants were phenotypically comparable to the untransformed controls (Vincentz and Caboche, 1991; Foyer et al., 1993, 1994). Constitutive NR expression caused a two-fold increase in maximal extractable NR activity, a decrease in the foliar NO_3^- content and an increase in the total amino acid (largely due to an increase in the glutamine pool) contents without any changes in total N, soluble sugars or starch (Foyer et al., 1994; Quilleré et al., 1994). The observed increase in the glutamine to glutamate ratio and the glutamine to sucrose ratio in the 35S-NR plants had no apparent effect on extractable activities of other enzymes such as SPS or PEPC, or on photosynthesis (Foyer et al., 1994). Tobacco plants transformed with a 35S-NR construct showed an increase in the $^{15}NO_3^-$ assimilation rate when supplied with 1 mM $^{15}NO_3^-$ (Gojon and Touraine, 1995). The increased efficiency in NO_3^- reduction was found to be partially negated in this case by decreased NO_3^- uptake by the roots. Consequently, the rate of amino acid synthesis was unchanged. Photosynthesis and growth were both inhibited

similarly by growth at low N in both 35S-NR and untransformed controls. NR activity decreased in untransformed controls and in the 35S-NR plants as a result of N limitation (Ferrario et al., 1995), suggesting that NR turnover is increased by N-deficiency (Ferrario et al., 1995). Nitrate is required to stabilize the NR protein since NR protein levels and NR activity were found to decrease within 24 h of N-deprivation (Galangau et al., 1988).

Pronounced diurnal changes are observed in the nitrate metabolism of source leaves when plants are provided with an adequate nitrogen supply (Hoff et al., 1994; Scheible et al., 1997b). When plants are exposed to CO_2 enrichment, they can become deficient in essential nutrients such as nitrogen if root growth is constrained, for example. In tobacco CO_2 enrichment was found to have different effects on NR activity, according to the age of the plants studied (Geiger et al., 1998). In older plants CO_2 enrichment led to a modified diurnal rhythm in foliar NR activity whereas in young seedlings elevated CO_2 caused larger increase in NR activity and NR activation state. CO_2 -dependent increases in glutamine, glutamate and in minor amino acids were also observed (Geiger et al., 1998). These studies suggest that the diurnal regulation of NR activity is modified in enhanced CO_2 .

Leaf NO_3^- contents decreased considerably in both 35S-NR transformants and untransformed controls when these plants were transferred from air to conditions of CO_2 enrichment (Ferrario-Méry et al., 1997). This effect was accompanied by decreases in foliar NR activity, mRNA levels and amino acid pools (Ferrario-Méry et al., 1997). Since NR expression in the 35S-NR transformants is no longer responsive to nitrate supply, this suggests that NR protein and NR message turnover are influenced by NO_3^- and/or amino acid levels (Ferrario-Méry et al., 1997). This phenomenon appears to be specific for NR in these conditions since the activities of other enzymes (SPS, PEPC, GS) were not modified (Ferrario-Méry et al., 1997). In hydroponically-grown plants NR activity, NO_3^- and amino acid levels were not decreased following CO_2 enrichment. NR mRNA was decreased, however, and glutamine was increased in all plants grown at high CO_2 even though NR activity and NO_3^- levels were similar to those of plants grown in air. The NR activation state did not vary in response to CO_2 enrichment suggesting that the phosphorylation/dephosphorylation state of the protein was not responsive to CO_2 enrichment

(Ferrario-Méry et al., 1997). High CO₂-induced increases in biomass were similar in all plants and the C/N ratio was increased to the same extent suggesting that NR activity is not limiting N assimilation in tobacco at high CO₂ conditions.

NR activity declined within 48 h of the onset of water stress in the untransformed controls but not in the 35S-NR plants (Ferrario-Méry et al., 1998). NR message levels declined more slowly than NR activity in the untransformed plants and appeared to be more stable than NR protein in drought conditions. NR mRNA levels increased in the 35S-NR plants following water deprivation. Rehydration after three days of water stress restored the leaf NR activity of the untransformed controls to a level similar to that of the water-replete plants within 24 h. NR message levels increased more slowly than NR activity. The NR activation state was not modified by water stress, suggesting that increases in NR activity following water stress in tobacco are due to decreased turnover of the NR protein. This may involve increased translation of existing NR mRNA since there was a delay in NR mRNA loss compared to that in NR activity as a result of water deprivation. Water stress is accompanied by a decrease in leaf NO₃⁻ and amino acids, and an increase in foliar hexoses (Foyer et al., 1998; Ferrario-Méry et al., 1998). These compounds are considered to be involved in the regulation of NR protein turnover. Different strategies may be employed to limit N assimilation in response to water deprivation in different plant species, for example in maize leaves subjected to water deprivation NR message levels decreased as quickly as the NR activity, whereas in tobacco there was a lag between the decrease of NR protein and NR message levels (Foyer et al., 1998).

Transformation of the NR-deficient mutant with a construct composed of a full-length tobacco NR cDNA with an internal deletion of 168 pb in the 5' end under the control of 35S CaMV promoter has produced transformants which not only lack transcriptional regulation but also post-transcriptional regulation since the phosphorylation of the NR protein is impaired (Nussaume et al., 1995). The NR protein contains three highly conserved functional domains (FAD, heme, and molybdenum cofactor [MoCo]; Mendel and Schwarz, 1999). These are involved in the transfer of electrons from NADH to NO₃⁻. The N-terminal region is not particularly well conserved and its function remains equivocal. The presence of acidic residues in the N terminal regions could provide

sites of electrostatic interaction with other proteins. An internal deletion of 56 amino acids in the N terminal MoCo domain of the NR protein (Δ NR) was performed to determine whether this region is involved in the post-translational regulation of the NR protein, for example, in proteolysis, inactivation by protein phosphorylation, or association with the NR protein inhibitor. The resultant transformants contained substantial NR activity and displayed normal growth characteristics comparable to the untransformed controls but, similar to the 35S-NR transformants, the Δ NR gene was not regulated at the transcriptional level. In addition foliar NR activity was no longer regulated by light at the post-transcriptional level, the activation state of the enzyme remaining constant during light/dark transitions. The NR protein was always in the activated form (Nussaume et al., 1995). Interestingly, the Δ NR deletion does not include the serine residue involved in protein phosphorylation. This site occurs in the hinge region of the NR protein which separates the molybdenum cofactor and the heme domains in spinach (Ser-543) and in *Arabidopsis* (Ser-534; Bachmann et al., 1996; Su et al., 1996). Whether phosphorylation of the protein still occurs in the Δ NR protein or whether linkage to the NR protein inhibitor is impaired by the N terminal deletion remains unresolved. Diurnal fluctuations in total foliar amino acid pools, particularly Gln, which are observed in the untransformed plants, are damped in the Δ NR plants. Experiments using ¹⁵N incorporation to determine nitrogen assimilation rates have not yet been performed. It is unclear whether constitutively active NR will lead to increased rates of N assimilation; assimilation at night may be limited, for instance, by reducing power availability in the absence of photosynthetic electron transport.

Such studies demonstrate the complexity of NR regulation at the transcriptional, post-transcriptional and post-translational levels. These regulatory mechanisms act in parallel to determine the overall leaf rate of NO₃⁻ reduction. The metabolic factors acting at each of these steps have not been clearly identified. NO₃⁻, N metabolites such as glutamine, and carbohydrates such as sucrose, are involved in induction of NR gene transcription and may also be involved in turnover of the NR mRNA and NR protein. The relative importance of each of these regulatory devices may vary from species to species; NR is inactivated in the absence of NO₃⁻ in transformed *Chlamydomonas* constitutively expressing NR

(Navarro et al., 1996) and in tobacco (Ferrario et al., 1995). In our experiments we have demonstrated the importance of NO_3^- for the turnover of NR mRNA and NR protein (Ferrario et al., 1995, 1996; Ferrario-Méry et al., 1997). These studies also demonstrate that overexpression of a single component of a metabolic pathway may have a relatively small effect on overall flux if the enzyme activity is highly regulated. Post-transcriptional regulation involving mRNA stability, translation efficiency, and protein stability in enzymes such as NR, have a profound effect on the outcome of experiments involving constitutive or other types of overexpression.

V. Glutamine Synthetase and Glutamate Synthase

Plants modified in GS and GOGAT activities have proved to be valuable tools for studying the control of photosynthetic and photorespiratory C/N interactions (Häusler et al., 1994a,b; Leegood et al., 1995, 1996) as well as to study the effects of metabolic intermediates on gene expression (Dzuibany et al., 1998). Screening procedures designed to isolate photorespiratory mutants in *Ambidopsis*, barley and pea (Somerville, 1986; Blackwell et al., 1988a,b) also identified plants modified in GS and GOGAT activities. These mutants were recognized by their ability to grow with a comparable phenotype to the wild-type under non-photorespiratory conditions of atmospheric CO_2 enrichment but showed severe stress symptoms in air where photorespiration occurs (Blackwell et al., 1988b). These mutants are unable to reassimilate photorespiratory NH_4^+ . When these mutants are exposed to air photosynthetic CO_2 assimilation is inhibited, foliar NH_4^+ increases and Gln rapidly accumulates at the expense of almost all other amino acids. Since metabolism in these mutants has been extensively reviewed elsewhere (Leegood et al., 1995, 1996) the following sections describe results obtained from the analysis of transformed plants only.

Transformed tobacco plants expressing a cytosolic GS cDNA from alfalfa, under the control of the 35S promoter, were obtained by Eckes et al. (1989) and Temple et al. (1993). The abundance of the mRNA derived from the transgene was 10 times higher than that measured in untransformed alfalfa plants. Maximal extractable foliar GS activity were enhanced in the transformants by 10–25% (Temple et al.,

1993) or by 500% (Eckes et al., 1989). The NH_4^+ content of the leaves of the transformants was drastically decreased but the amino acid composition was unchanged (Eckes et al., 1989). In one case a 45% increase in the total soluble protein content of the leaves was observed (Temple et al., 1993). Growth and seed production of the transformed plants were comparable to those of the controls. The transformants were 20-fold more resistant in vitro to the GS inhibitor, phosphinothricin (Eckes et al., 1989).

Overexpression of cytosolic GS in transformed tobacco constitutively expressing cytosolic or chloroplastic GS cDNAs correlated with increased growth whereas in cases where co-suppression was observed growth was inhibited (Lam et al., 1995). Transformation of the legume *Lotus corniculatus* L. to overexpress a soybean cytosolic GS cDNA (Vincent et al., 1997) led to increases in total foliar GS activity of up to 80%. When these transformants were grown on 12 mM NH_4^+ , greater increases in foliar amino acids and ammonium were observed in leaves and roots while decreases in carbohydrates in roots were more pronounced in transformants than in the untransformed controls. Increases in amino acid contents appeared to arise because of modified shoot protein degradation and to an early senescence and floral development in transformed *L. corniculatus*.

A role of cytosolic GS in the vascular tissue in nitrogen assimilation and transport was suggested in transformed alfalfa overexpressing a cytosolic GS gene in the antisense orientation under the control of a phloem specific promoter (Temple et al., 1993). The only transformants which remained viable were those where minimum suppression of GS was achieved. Expression of two alfalfa cytosolic GS cDNAs differing in their 3' untranslated regions and fused to either vascular or nodule-specific promoters from *Arabidopsis thaliana* or soybean in alfalfa resulted in down-regulation of GS in the nodule isoform but increased in the stem (Temple and Sengupta-Gopalan, 1997).

Transformed tobacco plants with decreased GS expression in the phloem and roots contained less proline indicating that GS in the phloem plays a major role in the regulation of proline synthesis. Despite the low nucleotide homology between alfalfa GS1 and tobacco GS2 genes the decrease in GS activity in the transformants was caused by inhibition of the chloroplastic GS2 only. Decreased GS activity was accompanied by a 40% decrease in total soluble protein and decreases in PEPC and hydroxypyruvate

reductase activities. Plants with low GS were comparable to untransformed controls and did not suffer from NH_4^+ accumulation when plants were grown under photorespiratory conditions. Expression of the antisense construct in alfalfa led to a decrease in native alfalfa GS (Temple and Sengupta-Gopalan, 1997).

Tobacco transformants with Fd-GOGAT activities of between 36% and 83% were grown with CO_2 enrichment (4000 ppm) to prevent photorespiration and to avoid toxic NH_4^+ accumulation. In these conditions, the growth rate was similar to that measured in the untransformed controls (Fig. 4). Following transfer to air, the transformants produced chlorotic symptoms on the leaves and development was reduced (Hirel et al., 1997). This suggests that a 60% decrease in Fd-GOGAT activity did not limit primary nitrogen assimilation and confirmed the role of Fd-GOGAT in the recycling of photorespiratory NH_4^+ in agreement with results obtained in studies of photorespiratory mutants deficient in GS (Blackwell et al., 1987; Wallsgrove et al., 1987) or Fd-GOGAT activities (Somerville and Ogren, 1980; Kendall et al., 1986; Lea et al., 1992). These mutants were only viable when grown in CO_2 -enriched atmospheres. Following transfer to air, these mutants showed large increases in the Gln and NH_4^+ contents of the leaves together with decreases in other amino acids and an inhibition of CO_2 assimilation.

Transformed plants with intermediate levels of GOGAT activity allow the study of long-term effects on nitrogen and carbon assimilation, particularly the impact of changes in metabolite contents on the regulation and expression of key enzymes. After four days exposure to photorespiratory conditions, increased foliar NH_4^+ accumulation was observed in the leaves of the transformants with the lowest Fd-GOGAT activity. This was accompanied by large increases in other metabolites, especially Gln and 2-OG. In contrast Glu contents were lower in the transformants than in the untransformed controls. No change in foliar Glu content was observed following the transfer to air, suggesting that an alternative pathway of Glu production was activated in the transformants. Such an alternative may be provided by GDH, whose activity was increased in the transformants. Although this enzyme has a relatively low affinity for NH_4^+ and 2-OG, these properties could be less restricting in the transformants, where levels of these compounds are increased. It is possible that these high levels of

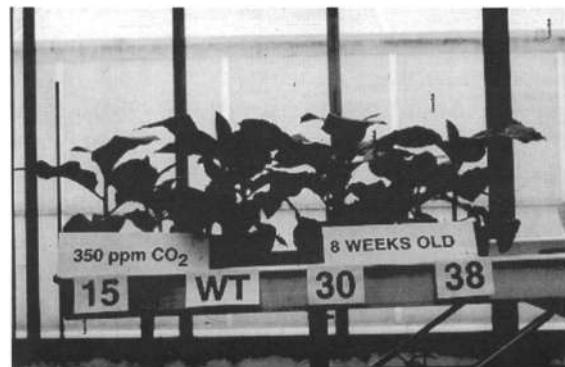


Fig. 4. Phenotypic effects of antisense expression of Fd-GOGAT in transformed tobacco plants grown with CO_2 enrichment (4000 ppm CO_2 ; top) or following transfer to air (350 ppm CO_2 for 2 weeks; bottom). The untransformed controls (WT) are compared with antisense transformants containing 36% (15), 61% (30) and 83% (38) of the Fd-GOGAT activity of the leaves from untransformed controls in both growth conditions.

2-OG and NH_4^+ are, at least partly, responsible for GDH induction. A correlation between low Fd-GOGAT activity and high NR and NiR transcript abundance was observed in transformed leaves but neither foliar NR nor NiR activities were changed. The increases in NR and NiR transcript abundance are surprising since both Gln and NH_4^+ accumulate in the leaves of the transformants. Interestingly, significant correlations were established between the ratio of Gln to 2-OG and the abundance of NR and NiR transcripts, suggesting that 2-OG could prevent the inhibitory effect of glutamine on NR and NiR transcription. The abundance of transcripts for a high affinity NO_3^- transporter and for NR were decreased in the roots of the Fd-GOGAT transformants, even though the only metabolite modification observed in roots was a large increase in NH_4^+ . NH_4^+ or low Fd-GOGAT may thus influence shoot to root interactions. High shoot Gln and NH_4^+ contents may convey information suggesting greater abundance of reduced N in leaves. This would lead to a decrease in N uptake and in primary N assimilation in the roots. Gln also induces increased PEPC activity but PEPC transcript abundance was unchanged. Gln accumulation alone may not elicit an appropriate response and while parallel increases in Gln and 2-OG are probably uncommon in plants, they may be required to elicit appropriate responses in extreme stress situations (Fig. 5).

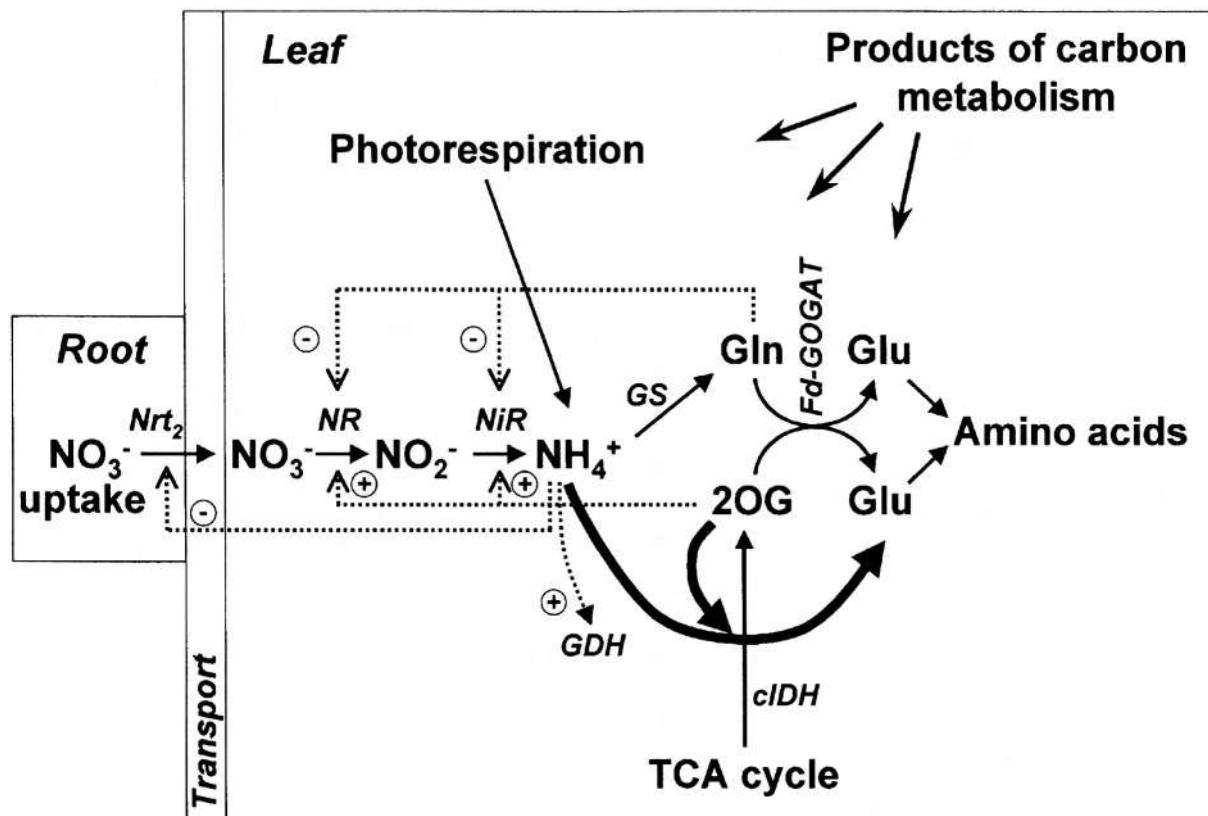


Fig. 5. A diagram illustrating the coordinate regulation of C and N metabolism in antisense Fd-GOGAT tobacco plants involving ammonium, Gln and 2-OG. The scheme shows positive and negative effects of these metabolites on the expression of genes and on enzyme activities (dashed lines).

VI. Plasma Membrane H⁺-ATPase

The plasma membrane H⁺-pumping ATPase functions to generate a pH and electrical gradient that drives ion uptake, and is involved in a variety of processes including nutrient uptake, osmoregulation and pH control. A major recent development is that the activity of the pump may also be controlled by phosphorylation in a mechanism that also involves 14-3-3 proteins.

A. Regulation by Phosphorylation

Recent evidence suggests that the pump is inactivated by phosphorylation and binding of a 14-3-3 protein (Moorhead et al., 1996), which also serves as the fusicoccin (FC) receptor (for review see De Boer, 1997). Activation of the ATPase can be achieved by dephosphorylation catalyzed by PP2A or dissociation of the ATPase: 14-3-3 complex with phosphoSer-

259-Raf-1 peptide (Moorhead et al., 1996), which conforms to the 14-3-3 binding motif (Muslin et al., 1996). The pump can also be activated with FC, and although different models exist for the mechanism of activation, both involve displacement of the 14-3-3 proteins from at least one of its binding sites on the ATPase. Concerted regulation of NR and the H⁺-ATPase (which provides the driving force for nitrate uptake) by a common mechanism involving 14-3-3s, suggests the potential for coordinate regulation but details remain to be elucidated.

VII. Conclusions

C and N assimilation are closely coordinated in leaves; if CO_2 is removed from the air surrounding an illuminated leaf N assimilation ceases soon after photosynthesis is inhibited. Similarly, a strong correlation between maximal extractable NR activity

and ambient photosynthesis was found in tobacco and maize in well-watered and droughted plants (Ferrario-Méry et al., 1998). The coordination of the pathways of carbon and nitrogen assimilation in plants involves the concerted action of a repertoire of signals that allow graded molecular and physiological responses. A large number of studies have concerned the mechanisms controlling the expression of genes encoding the enzymes of N assimilation (NR, NiR, GS and GOGAT) and the regulation of NR activity by protein phosphorylation. In contrast, relatively few signal molecules coordinating the C/N interaction have been identified and fundamental questions regarding mechanism remain unanswered. Sugars (such as sucrose and glucose) and 2-OG are involved in the transcriptional and post-transcriptional regulation of enzymes associated with N assimilation, while nitrate and amino acids modulate the expression and activation state of enzymes of C metabolism. Such metabolites convey information on carbohydrate and nitrogen status throughout the plant to allow rapid and appropriate adaptive responses to environmental and metabolic stimuli. Protein phosphorylation regulates key enzymes of N assimilation, sucrose biosynthesis and the synthesis of organic and amino acids, ensuring coordination of the pathways of C and N assimilation and the anaplerotic pathway which provides carbon skeletons for amino acid biosynthesis (Fig. 3). It is possible that pathways of signal transduction for the regulation of GS similar to that observed in *E. coli*, occur in plants. This involves 4 proteins including PII, which control the transcription of the GS gene (Atkinson et al., 1994).

Nitrate, per se, has been shown to be a metabolic signal that enhances transcription of several genes including those encoding PEPC, cytosolic pyruvate kinase, mitochondrial citrate synthase, and cytosolic isocitrate dehydrogenase (Scheible et al., 1997c). Adequate N-nutrition increases enzymes necessary for nitrate reduction and amino acid biosynthesis and favors enhanced rates of photosynthesis. Nitrate also directly represses expression of the large subunit of ADP-glucose pyrophosphorylase, and this will tend to reduce the partitioning of carbon into starch in N-replete leaves (Fig. 1). N assimilation also increases PEPC activity and decreases SPS activity favoring decreased rates of sucrose synthesis and enhanced C flow through the anaplerotic pathway (Fig. 3).

At least two important C/N interactions, regulated

by protein phosphorylation, in the N-sufficient leaves involve light-activation of enzymes. Firstly, Gln is required for the light activation of PEPC-kinase in C₃ leaves (Champigny and Foyer, 1992). This enzyme phosphorylates and activates PEPC (Li et al., 1996). Thus, PEPC will only be activated when N-nutrition is adequate. Second, light activation of NR (by dephosphorylation of Ser-543) requires photosynthesis and metabolites that inhibit NR-kinase, such as DHAP, are probably involved. Thus, nitrate will only be reduced when C-metabolites are available for incorporation into amino acids.

N-deficiency causes inhibition of photosynthesis and hence the synthesis of carbohydrates. However, low nitrate, per se, is a signal for increased expression of ADPGlc PPase and thereby increases the capacity for starch synthesis. In addition, the accumulation of PGA, that is characteristic of N-deficiency, will increase ADP-glucose production by allosteric activation of ADPGlc PPase (Scheible et al., 1997c). The low levels of amino acids that are found in N-deficient leaves may also have other metabolic effects. Firstly, the light activation PEPC-kinase will be inhibited and hence organic acid synthesis will be reduced. Secondly, foliar amino acids drop to values low enough to restrict protein synthesis, inhibition of SPS activation may also be observed. Thirdly, since N-deficiency reduces photosynthesis, decreased metabolite availability (especially Glc 6-P) and elevated Pi will also contribute to a reduction in SPS activation. Thus, N-deficient leaves will tend to partition more C into starch and less into organic acids, amino acids and sucrose.

A decrease in assimilate export will cause accumulation of assimilates such as sucrose and amino acids in leaves. A build-up of amino acids has been postulated to inhibit SPS-PP(s) and thereby reduce SPS activation, resulting in increased diversion of C into starch. The increases in metabolites in the cytosol observed when SPS is inactivated in the light will inhibit NR-kinase(s) and result in 'hyper-activation' of NR. Thus, nitrate reduction will continue or perhaps even increase in the short-term. In the long-term, nitrate reduction and amino acid biosynthesis cannot continue unabated for at least two reasons. Firstly, amino acid accumulation in leaves will restrict NR gene expression (Vincentz et al., 1993), and thus restrict de novo synthesis of NR enzyme protein. Secondly, amino acid accumulation may prevent the hysteretic activation of NR that is postulated to involve conformational changes (Huber

et al., 1996). Thus, nitrate assimilation will be restricted even in the presence of sufficient nitrate.

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References

- Atkinson MR, Kamberov ES, Weiss RJ and Ninfa AJ (1994) Reversible uridylation of the *Escherichia coli* PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator (NR or NrtC). *J Biol Chem* 269: 28288–28293
- Bachmann M, Huber JL, Liao P-C, Gage DA and Huber SC (1996) The inhibitor protein of phosphorylated nitrate reductase from spinach (*Spinacia oleracea* L.) leaves is a 14-3-3 protein. *FEBS Lett* 387: 127–131
- Blackwell RD, Murray AJS and Lea PJ (1987) Inhibition of photosynthesis in barley with decreased levels of chloroplastic glutamine synthetase activity. *J Exp Bot* 38: 1799–1809
- Blackwell RD, Murray AJS, Lea PJ, Kendall AC, Hall NP, Turner JC and Wallsgrove RM (1988a) The value of mutants unable to carry out photorespiration. *Photosynth Res* 16: 155–176
- Blackwell RD, Murray AJS, Lea PJ and Joy KW (1988b) Photorespiratory amino donors, sucrose synthesis and the induction of CO_2 fixation in barley deficient in glutamine synthetase and glutamate synthase. *J Exp Bot* 39: 845–858
- Brears T, Walker EL and Coruzzi GM (1991) A promoter sequence involved in cell-specific expression of the pea glutamine synthetase gene GS3A in organs of transgenic tobacco and alfalfa. *Plant J* 1: 235–240
- Champigny ML and Foyer CH (1992) Nitrate activation of cytosolic protein kinases diverts photosynthetic carbon from sucrose to amino acid biosynthesis. *Plant Physiol* 100: 7–12
- Champigny ML, Brauer M, Bismuth E, ThiManh C, Siegl G, Van Quy L and Stitt M (1992) The short term effect of NO_3^- and NH_4^+ assimilation on sucrose synthesis in leaves. *J Plant Physiol* 139: 361–368
- Cheng CL, Dewdney J, Kleinhofs A and Goodman HM (1986) Cloning and nitrate induction of nitrate reductase mRNA. *Proc Natl Acad Sci USA* 83: 6825–6828
- Cheng CL, Acedo GN, Christins M and Conkling MA (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. *Proc Natl Acad Sci USA* 89: 1861–1864
- Chollet R, Vidal J and O'Leary MH (1996) Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 273–298
- Cock JM, Hemon P and Cullimore JV (1992) Characterization of the gene encoding the plastid-located glutamine synthetase of *Phaseolus vulgaris*: regulation of β -glucuronidase gene fusions in transgenic tobacco. *Plant Mol Biol* 18: 1141–1149
- Coschigano KT, Melo-Oliveira R, Lim J and Coruzzi GM (1998) *Arabidopsis gls* mutants and distinct Fd-GOGAT genes: Implications for photorespiration and primary nitrogen assimilation. *Plant Cell* 10: 741–752
- Cushman JC, Meiners MS and Bohnert HJ (1993) Expression of a phosphoenolpyruvate carboxylase promoter from *Mesembryanthemum crystallinum* is not salt-inducible in mature transgenic tobacco. *Plant Mol Biol* 21: 561–566
- Daniel-Vedele F, Filleur S and Caboche M (1998) Nitrate transport: A key step in nitrate assimilation. *Curr Opin Plant Biol* 1: 235–239
- De Boer B (1997) Fusococcin—a key to multiple 14-3-3 locks? *Trends Plant Sci* 2: 60–66
- De la Torre A, Delgado B and Lara C (1991) Nitrate-dependent O_2 evolution in intact leaves. *Plant Physiol* 96: 898–901
- Deng M-D, Moureaux T, Cherel I, Boutin JP and Caboche M (1991) Effects of nitrogen metabolites on the regulation and circadian expression of tobacco nitrate reductase. *Plant Physiol Biochem* 29: 239–247
- Dorbe MF, Caboche M, Daniel-Vedele F (1992) The tomato *nia* gene complements a *Nicotiana plumbaginifolia* nitrate reductase deficient mutant and is properly regulated. *Plant Mol Biol* 18: 363–375
- Douglas P, Pigaglio E, Ferrier A, Halford NG and MacKintosh C (1996) Three spinach leaf nitrate reductase- 3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca^{2+} ions. *Biochem J* 325: 101–109
- Duff SMG and Chollet R (1995) In vivo regulation of wheat-leaf phosphoenolpyruvate carboxylase by reversible phosphorylation. *Plant Physiol* 107: 775–782
- Dzuibany C, Haupt S, Fock H, Biehler K, Migge A and Becker TW (1998) Regulation of nitrate reductase transcript levels by glutamine accumulating in the leaves of a ferredoxin-dependent glutamate synthase deficient *gluS* mutant of *Arabidopsis thaliana* and by glutamine provided via the roots. *Planta* 206: 515–522
- Eckes P, Schmitt P, Daub W and Wengenmayer F (1989) Overproduction of alfalfa glutamine synthetase in transgenic tobacco plants. *Mol Gen Genet* 217: 263–268
- Edwards JW, Walker EL and Coruzzi GM (1990) Cell specific expression in transgenic plants reveals non-overlapping roles for chloroplast and cytosolic glutamine synthetase. *Proc Natl Acad Sci USA* 87: 3459–3463
- Evans JR (1983) Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Physiol* 72: 279–302
- Evans JR (1989) Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* 78: 9–19
- Evans JR and Terashima I (1987) Effects of nitrogen nutrition on electron transport components and photosynthesis in spinach. *Aus J Plant Physiol* 14: 59–68
- Ferrario-Méry S, Murchie E, Hirel B, Galtier N, Quick WP and Foyer CH (1997) Manipulation of the pathways of sucrose biosynthesis on nitrogen assimilation in transformed plants to improve photosynthesis and productivity. In: Foyer CH and Quick WP (eds) *A Molecular Approach to Primary Metabolism in Higher Plants*, pp 125–153. Taylor and Francis Publishers, London
- Ferrario-Méry S, Valadier MH and Foyer CH (1996) Short-term modulation of nitrate reductase activity by exogenous nitrate in *Nicotiana plumbaginifolia* and *Zea mays* leaves. *Planta* 199: 366–371

- Ferrario-Méry S, Valadier MH and Foyer CH (1998) Over-expression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. *Plant Physiol* 117: 293–302
- Ferrario S, Valadier M-H, Morot-Gaudry J-F and Foyer CH (1995) Effects of constitutive expression of nitrate reductase in transgenic *Nicotiana plumbaginifolia* L. in response to varying nitrogen supply. *Planta* 196: 288–294
- Fichtner K and Schulze E-D (1992) The effect of nitrogen nutrition on annuals originating from habitats of different nitrogen availability. *Oecologia* 92: 236–241
- Flora LL and Madore MA (1993) Stachyose and mannitol transport in olive (*Olea europaea* L.). *Planta* 189: 484–490
- Foyer CH and Ferrario S (1994) Modulation of carbon and nitrogen metabolism in transgenic plants with a view to improved biomass production. *Biochem Soc Trans* 22: 909–915
- Foyer CH and Galtier N (1996) Source-sink interaction and communication in leaves. In: Samski E, Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops*, pp 311–340. Marcel Dekker Inc., New York, Basel, Hong Kong
- Foyer CH, Lefebvre C, Provot M, Vincentz M and Vaucheret H (1993) Modulation of nitrogen and carbon metabolism in transformed *Nicotiana plumbaginifolia* mutant E23 lines expressing either increased or decreased nitrate reductase activity. In: White E, Kettlewell PS, Parry MA and Ellis RP (eds) *Aspects of Applied Biology*, pp 137–145. Association of Applied Biologists, Wellesbourne, Warwick
- Foyer CH, Noctor G, Lelandais M, Lescure JC, Valadier MH, Boutin JP and Horton P (1994) Short-term effects of nitrate, nitrite and ammonium assimilation on photosynthesis, carbon partitioning and protein phosphorylation in maize. *Plant Physiol* 192: 211–220
- Foyer CH, Valadier M-H, Migge A and Becker TW (1998) Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. *Plant Physiol* 117: 283–292
- Galangau F, Daniel-Vedèle F, Moureaux T, Dorbe MF, Leydecker MT and Caboche M (1988) Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. *Plant Physiol* 88: 383–388
- Galtier N, Foyer CH, Huber J, Voelker TA and Huber SC (1993) Effects of elevated sucrose-phosphate synthase activity on photosynthesis, assimilate partitioning, and growth in tomato (*Lycopersicon esculentum* var UC82B). *Plant Physiol* 101: 535–543
- Galtier N, Foyer CH, Murchie E, Alred R, Quick P, Voelker T, Thépenier C, Laseve G and Betsche T (1995) Effects of light and atmospheric carbon dioxide enrichment on photosynthesis and carbon partitioning in leaves of tomato (*Lycopersicon esculentum*) plants overexpressing sucrose phosphate synthase. *J Exp Bot* 46: 1335–1344
- Gehlen J, Panstruga R, Smets H, Merkelbach S, Kleines M, Porsch P, Fladung M, Becker I, Rademacher T, Häusler RE and Hirsch HJ (1996) Effects of altered phosphoenolpyruvate carboxylase activities on transgenic C₃ plant *Solanum tuberosum*. *Plant Mol Biol* 32(5): 831–848
- Geiger M, Walch-Liu P, Engels C, Harnecker J, Schulze E-D, Ludewig F, Sonnewald U, Scheible W-R and Stitt M (1998) Enhanced carbon dioxide leads to a modified diurnal rhythm of nitrate reductase activity in older plants, and a large stimulation of nitrate reductase activity and higher levels of amino acids in young tobacco plants. *Plant Cell Environ* 21: 253–268
- Gojon A and Touraine B (1995) Effects of NR gene overexpression on ¹⁵NO₃⁻ uptake and reduction in transgenic tobacco, p. 49. 4th Intl Symposium on Inorganic Nitrogen Assimilation, Darmstadt, Germany
- Halford NG and Hardie DG (1998) SNF1-related protein kinases: Global regulators of carbon metabolism in plants? *Plant Mol Biol* 37: 735–748
- Hartwell J, Smith LH, Wilkins MB, Jenkins GI and Nimmo HG (1996) Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. *Plant J* 10: 1071–1078
- Häusler RE, Blackwell RD, Lea PJ and Leegood RC (1994a) Control of photosynthesis in barley leaves with reduced activities of glutamine synthetase or glutamate synthase. I. Plant characteristics and changes in nitrate, ammonium and amino acids. *Planta* 194: 406–417
- Häusler RE, Lea PJ and Leegood RC (1994b) Control of photosynthesis in barley leaves with reduced activities of glutamine synthetase or glutamate synthase. II. Control of electron transport and CO₂ assimilation. *Planta* 194: 418–435
- Häusler RE, Bailey KJ, Lea PJ and Leegood RC (1996) Control of photosynthesis in barley leaves with reduced activities of glutamine synthetase or glutamate synthase. III. Aspects of glyoxylate metabolism and effects of glyoxylate on the activation state of ribulose 1,5-bisphosphate carboxylase-oxygenase. *Planta* 200: 388–396
- Heckathorn SA, De Lucia EH and Zielinski RE (1997) The contribution of drought-related decreases in foliar nitrogen concentration to decreases in photosynthetic capacity during and after drought in prairie grasses. *Physiol Plant* 101: 173–182
- Heldt HW (1997) *Pflanzenbiochemie*, pp 274–306. Spektrum Akademisches Verlag, Heidelberg
- Hirel B, Marsolier MC, Hoarau A, Hoarau J, Brangeon J, Schafer R and Verma DPS (1992) Forcing expression of a soybean root glutamine synthetase gene in tobacco leaves induces a native gene encoding cytosolic enzyme. *Plant Mol Biol* 20: 207–218
- Hirel B, Phillipson B, Murchie E, Suzuki A, Kunz C, Ferrario-Méry S, Limani A, Chaillou S, Deleens E, Brugiére N, Chaumont-Bonnet M, Foyer CH and Morot-Gaudry J-F (1997) Manipulating the pathway of ammonia assimilation in transgenic legumes and non-legumes. *J Plant Nutrition and Soil Science*. Z. Pflanzenernähr Bodenkd 160: 283–290
- Hoff T, Truong H-N and Caboche M (1994) The use of mutants and transgenic plants to study nitrate assimilation. *Plant Cell Environ* 17: 489–506
- Huber JL and Huber SC (1992) Site-specific serine phosphorylation of spinach leaf sucrose-phosphate synthase. *Biochem J* 283: 877–882
- Huber JL, Redinbaugh MG, Huber SC and Campbell WH (1994) Regulation of maize leaf nitrate reductase activity involves both gene expression and protein phosphorylation. *Plant Physiol.* 106: 1667–1674
- Huber SC and Huber JL (1995) Metabolic activators of spinach leaf nitrate reductase: Effects on enzyme activity and dephosphorylation by endogenous phosphatases. *Planta* 196: 180–189
- Huber SC and Huber JL (1996) Role and regulation of sucrose-phosphate synthase in higher plants. *Annu Rev Plant Physiol* 47: 431–444
- Huber SC and Kaiser WM (1996) Regulation of C/N interactions

- in higher plants by protein phosphorylation. In: Verma DPS (ed) Signal Transduction in Plant Growth and Development, pp 87–112, Springer-Verlag, New York
- Huber SC, McMichael RW and Huber JL (1994) Control of plant enzyme activity by reversible protein phosphorylation. *Intl Rev of Cytol* 149: 47–98
- Huber SC, McMichael RW Jr, Bachmann M, Huber JL, Schannon JC, Kang K-K and Paul MJ (1996) Regulation of leaf sucrose-phosphate synthase and nitrate reductase by reversible protein phosphorylation. In: Shewry PR, Halford NG and Hooley R (eds) Protein Phosphorylation in Plants, pp 20–34. Clarendon Press, Oxford
- Hudspeth RL, Grula JW, Kai Z, Edwards GE and Ku MSB (1992) Expression of maize phosphoenolpyruvate carboxylase in transgenic tobacco: Effects on biochemistry and physiology. *Plant Physiol* 98: 458–464
- Huppe HC and Turpin DH (1994) Integration of carbon and nitrogen metabolism in plant and algal cells. *Annu Rev Plant Physiol Plant Mol Biol* 45: 577–607
- Huppe HC, Vanlerberghe GC and Turpin DH (1992) Evidence for activation of the oxidative pentose phosphate pathway during photosynthetic assimilation of NO_3^- but not NH_4^+ by a green algae. *Plant Physiol* 100: 2096–2099
- Huppe HC, Farr TJ and Turpin DH (1994) Coordination of chloroplastic metabolism in N limited *Chlamydomonas reinhardtii* by redox modulation. II. Redox modulation activates the oxidative pentose phosphate pathway during photosynthetic nitrate assimilation. *Plant Physiol* 105: 1043–1048
- Ireland R (1997) Amino acid and ureide metabolism. In: Dennis DT, Turpin DH, Lefebvre DD and Layzell DB (eds) *Plant Metabolism*, pp 509–524. Addison Wesley Longman Limited, Harlow
- Jang JC, Leon P, Zhou L and Sheen J (1997) Hexokinase as a sugar sensor in higher plants. *Plant Cell* 9: 5–19
- Jiao J-A and Chollet R (1991) Posttranslational regulation of phosphoenolpyruvate carboxylase in C_4 and Crassulacean acid metabolism plants. *Plant Physiol* 95: 981–985
- Joy KW, Blackwell RD and Lea PJ (1992) Assimilation of nitrogen in mutants lacking enzymes of the glutamate synthase cycle. *J Exp Bot* 43: 139–145
- Kaiser WM and Forster J (1989) Low CO_2 prevents nitrate reduction in leaves. *Plant Physiol* 91: 970–974
- Kaiser WM and Huber SC (1994) Post-translational regulation of nitrate reductase in higher plants. *Plant Physiol.* 106: 817–821
- Kaiser WM and Huber SC (1997) Correlation between apparent phosphorylation state of nitrate reductase (NR), NR hysteresis and degradation of NR protein. *J Exp Bot* 48: 1367–74
- Kaiser WM and Spill D (1991) Rapid modulation of spinach leaf nitrate reductase by photosynthesis, II: In vitro modulation by ATP and AMP. *Plant Physiol.* 96: 368–375
- Kendall AC, Walls-grove RM, Hall NP, Turner JC and Lea PJ (1986) Carbon and nitrogen metabolism in barley (*Hordeum vulgare* L.) mutants lacking ferredoxin-dependent glutamate synthase. *Planta* 168: 316–323
- Kogami H, Shono M, Koike T, Yanagisawa S, Izui K, Sentoku N, Tanifuji S, Uchimiya H and Toki S (1994) Molecular and physiological evaluation of transgenic tobacco plants expressing a maize phosphoenolpyruvate carboxylase gene under the control of the cauliflower mosaic virus 35S promoter. *Transgenic Res* 3: 287–296
- Krömer S (1995) Respiration during photosynthesis. *Ann Rev Plant Physiol Plant Mol Biol* 46: 45–70
- Krömer S and Heldt HW (1991) On the role of mitochondrial oxidative phosphorylation in photosynthesis metabolism as studied by the effect of oligomycin on photosynthesis in protoplasts and leaves of barley (*Hordeum vulgare*). *Plant Physiol* 95: 1270–1276
- Krömer S, Stitt M and Heldt HW (1988) Mitochondrial oxidative phosphorylation participating in photosynthetic metabolism of a leaf cell. *FEBS Lett* 226: 352–356
- Lam H-M, Coschigano K, Oliveira I, Melo-Oliveira R and Coruzzi G (1996) The molecular genetics of nitrogen assimilation into amino acids in higher plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 569–593
- Lam HM, Peng SS-Y and Coruzzi GM (1994) Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. *Plant Physiol* 106: 1347–1357
- Lam H-M, Coschigano K, Schultz C, Melo-Oliveira R, Tjaden G, Oliveira I, Ngai N, Hsieh M-H and Coruzzi G (1995) Use of *Arabidopsis* mutants and genes to study amide amino acid biosynthesis. *Plant Cell* 7: 887–898
- Lea PJ and Miflin BJ (1974) An alternative route for nitrogen assimilation in higher plants. *Nature* 251: 614–616
- Lea PJ, Blackwell RD and Joy KW (1992) Ammonia assimilation. In: Mengel K and Pillbeam DJ (eds) *Nitrogen Metabolism in Plants*, pp 153–186. Clarendon Press, Oxford
- Leegood RC, Lea PJ, Adcock MD and Hausler E (1995) The regulation and control of photorespiration. *J Exp Bot* 46: 1397–1414
- Leegood RC, Lea PJ and Hausler RE (1996) Use of barley mutants to study the control of photorespiratory metabolism. *Biochem Soc Trans* 24: 757–762
- Li B, Zhang X-Q and Chollet R (1996) Phosphoenolpyruvate carboxylase kinase in tobacco leaves is activated by light in a similar but not identical way as in maize. *Plant Physiol* 111: 497–505
- Lillo C and Ruoff P (1992) Hysteretic behavior of nitrate reductase. Evidence of an allosteric binding site for reduced pyridine nucleotide. *J Biol Chem* 267: 13456–13459
- Loescher WH and Everard JD (1996) Sugar alcohol metabolism in sinks and sources. In: Zamski E and Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships*, pp 185–207. Marcel Dekker, New York
- Makino A, Mae T and Ohira K (1984) Changes in photosynthetic capacity in rice leaves from emergence through senescence. Analysis of ribulose-1, 5-bisphosphate carboxylase and leaf conductance. *Plant Cell Physiol* 25: 511–521
- Matsuoka M, Kyosuka J, Shimamoto K and Kano-Murakami Y (1994) The promoters of two carboxylases in a C_4 plant (maize) direct cell-specific, light-regulated expression in a C_3 plant (rice). *Plant J* 6: 311–319
- McMichael RW Jr, Bachmann M and Huber SC (1995) Spinach leaf sucrose-phosphate synthase and nitrate reductase are phosphorylated/ inactivated by multiple protein kinases in vitro. *Plant Physiol* 108: 1077–1082
- Mendel RR and Schwarz G (1999) Molybdoenzymes and molybdenum cofactor in plants. *Crit Rev in Plant Sci* 18: 33–69
- Miao GH, Hirel B, Marsolier MC, Ridge RW and Verma DPS (1991) Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. *Plant Cell* 3: 11–22

- Micallef BJ, Haskins KA, Vanderveer PJ, Roh KS, Shewmaker CK and Sharkey TD (1995) Altered photosynthesis, flowering, and fruiting in transgenic tomato plants that have an increased capacity for sucrose synthesis. *Planta* 196: 327–334
- Millar AH and Day DA (1996) Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. *FEBS Lett* 398: 155–158
- Millar AH and Day DA (1997) Alternative solutions to radical problems. *Trends Plant Sci* 8: 289–290
- Moorhead G, Douglas P, Morrice N, Scarabel M, Aitken A and MacKintosh C (1996) Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. *Curr Biol* 6: 1104–1113
- Morcuende R, Krapp A, Hurry V and Stitt M (1998) Sucrose-feeding leads to increased rates of nitrate assimilation, increased rates of α -oxoglutarate synthesis and increased synthesis of a wide spectrum of amino acids in tobacco leaves. *Planta* 206: 394–409
- Morris PF, Layzell DB and Canvin DT (1989) Photorespiratory ammonia does not inhibit photosynthesis in glutamate synthase mutants of *Arabidopsis*. *Plant Physiol* 89: 498–500
- Müller A and Mendel R (1989) Biochemical and somatic cell genetics of nitrate reduction in *Nicotiana*. In: Wray JL and Kinghorn JL (eds) Molecular and Genetic Aspects of Nitrate Assimilation, pp 166–185. Oxford Science Publications, Oxford
- Muslin AJ, Tanner JW, Allen PM and Shaw AS (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* 84: 889–897
- Navarro MT, Preito R, Fernandez E and Galvan A (1996) Constitutive expression of nitrate reductase changes the regulation of nitrate and nitrite transporters in *Chlamydomonas reinhardtii*. *Plant J* 9: 819–827
- Nguyen-Quoc B, N'tchobo H, Foyer CH and Yelle S (1999) Overexpression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit. *J Exp Bot* 50: 785–791
- Noctor G and Foyer CH (1998) A re-evaluation of the ATP:NADPH budget during C_3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity? *J Exp Bot* 49: 1895–1908
- Nusumme L, Vincentz M, Meyer C, Boutin J-P and Caboche M (1995) Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. *Plant Cell* 7: 611–621
- O'Leary MH (1982) Phosphoenolpyruvate carboxylase: An enzymologist's view. *Annu Rev Plant Physiol* 33: 297–315
- Ozcan S, Dover J, Rosenwald AG, Wolfi S and Johnston M (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for the induction of gene expression. *Proc Natl Acad Sci, USA* 93: 12428–12432
- Pace GH, Volk RJ and Jackson WA (1990) Nitrate reduction in response to CO_2 -limited photosynthesis. Relationship to carbohydrate supply and nitrate reductase activity in maize seedlings. *Plant Physiol* 92: 286–292
- Pacquet V, Santi S, Cretin C, Bui VL, Vidal J and Gadad P (1993) Production and properties of recombinant C_3 -type phosphoenolpyruvate carboxylase from *Sorghum vulgare*: In vitro phosphorylation by leaf and root PyrPC protein serine kinases. *Biochem Biophys Res Comm* 197: 1415–1423
- Pathirana MS, Samac DA, Roeven R, Yoshioka H, Vance CP and Gantt JS (1997) Analyses of phosphoenolpyruvate carboxylase gene structure and expression in alfalfa nodules. *Plant J* 12(2): 293–304
- Quilleré I, Dufossé C, Roux Y, Foyer CH, Caboche M and Morot-Gaudry JF (1994) The effects of the deregulation of NR gene expression on growth and nitrogen metabolism of winter-grown *Nicotiana plumbaginifolia*. *J Exp Bot* 45: 1205–1212
- Reins B, Lohaus G, Winter H and Heldt HW (1994) Production and diurnal utilization of assimilates in leaves of spinach (*Spinacia oleracea* L.) and barley (*Hordeum vulgare* L.). *Planta* 192: 497–501
- Rennenberg H, Kreutzer K, Papen H and Weber P (1998) Consequences of high loads of nitrogen for spruce (*Picea abies*) and beech (*Fagus sylvatica*) forests. *New Phytol* 139: 71–86
- Scheible W-R, Lauerer M, Schulze E-D, Caboche M and Stitt M (1997a) Accumulation of nitrate in the shoots acts as a signal to regulate shoot to root allocation in tobacco. *Plant J* 11: 671–691
- Scheible W-R, González-Fontes A, Morcuende R, Lauerer M, Geiger M, Glaab J, Gojon A, Schulze E-D, Caboche M and Stitt M (1997b) Tobacco mutants with a decreased number of functional nia-genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase. *Planta* 203: 304–319
- Scheible W-R, González-Fontes A, Lauerer M, Müller-Röber B, Caboche M and Stitt M (1997c) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* 9: 783–798
- Signora L, Galtier N, Skøt L, Lucas H and Foyer CH (1998) Overexpression of sucrose phosphate synthase in *Arabidopsis thaliana* results in increased foliar sucrose/starch ratios and favours decreased foliar carbohydrate accumulation in plants after prolonged growth with CO_2 enrichment. *J Exp Bot* 49: 669–680
- Smeekens S and Rook F (1997) Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiol* 115: 7–13
- Somerville CR (1986) Analysis of photosynthesis and photorespiration using mutants of higher plants and algae. *Annu Rev Plant Physiol* 37: 467–507
- Somerville CR and Ogren WL (1980) Inhibition of photosynthesis in *Arabidopsis* mutants lacking leaf glutamate synthase activity. *Nature* 286: 257–260
- Stitt M (1986) Limitation of photosynthesis by carbon metabolism. I. Evidence for excess electron transport capacity in leaves carrying out photosynthesis in saturating light and CO_2 . *Plant Physiol* 81: 1115–1122
- Stitt M and Schulze E-D. (1994) Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant Cell Environ* 17: 518–552
- Stockhaus J, Poetsch W, Steinmuller K and Westhoff P (1994) Evolution of the C_4 phosphoenolpyruvate carboxylase promoter of the C_4 dicot *Flaveria trinervia*: An expression analysis in the C_3 plant tobacco. *Mol Gen Genet* 245: 286–293
- Stöhr C, Tischner R and Ward M R (1993) Characterisation of the plasmamembrane-bound nitrate reductase in *Chlorella saccharophila* (Krüger) Nadson. *Planta* 191: 79–85
- Stöhr C and Ullrich WR (1997) A succinate-oxidising nitrate reductase is located at the plasmamembrane of plant roots. *Planta* 203: 129–132
- Su W, Huber SC and Crawford NM (1996) Identification in vitro of a post-transcriptional regulatory site in the hinge 1 region of *Arabidopsis* nitrate reductase. *Plant Cell* 8: 519–527
- Tagu D, Cretin C, Bergounioux C, Lepiniec L and Gadad P (1991) Transcription of a *sorghum* phosphoenolpyruvate

- carboxylase gene in transgenic tobacco leaves: Maturation of monocot PRE-mRNA by dicot cells. *Plant Cell Reports* 9: 688–690
- Temple SJ, Knight TJ, Unkefer PJ and Sengupta-Gopalan C (1993) Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: Molecular and biochemical analysis. *Mol Gen Genet* 236: 315–325
- Temple SJ and Sengupta-Gopalan C (1997) Manipulating amino acid biosynthesis. In: Foyer CH and Quick WP (eds) *Molecular approach to Primary Metabolism in Higher Plants*, pp 155–177, Taylor and Francis, London
- Tjaden G and Coruzzi GM (1994) A novel AT-rich DNA binding protein that combines an HMG I like DNA binding domain with a putative transcription domain. *Plant Cell* 6: 107–118
- Toroser D and Huber SC (1997) Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase in spinach (*Spinacia oleracea* L.) leaves. *Plant Physiol* 114: 947–955
- Turpin DH, Weger HG and Huppe HC (1997) Interactions between photosynthesis, respiration and nitrogen assimilation. In: Dennis DT, Turpin DH, Lefebvre DD and Layzeel DB (eds) *Plant Metabolism*, pp 509–524, Longman, Singapore
- Vanlerberghe GC, Huppe HC, Vlossak KDM and Turpin DH (1991) Activation of respiration to support dark NO_3^- and NH_4^+ assimilation in the green alga *Selenastrum minutum*. *Plant Physiol* 99: 495–500
- Vaucheret H, Chabaud M, Kronenberger J and Caboche M (1990) Functional complementation of tobacco and *Nicotiana plumbaginifolia* nitrate reductase deficient mutants by transformation with the wild-type alleles of the tobacco structural genes. *Mol Gen Genet* 220: 468–474
- Vincent R, Fraisier V, Chaillou S, Limami MA, Deleens E, Phillipson B, Douat C, Boutin J-P and Hirel B (1997) Overexpression of a soybean gene encoding cytosolic glutamine synthetase in shoots of transgenic *Lotus corniculatus* L. plants triggers changes in ammonium assimilation and plant development. *Planta* 201: 424–433
- Vincentz M and Caboche M (1991) Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia* plants. *EMBO J* 10: 1027–1035
- Vincentz M, Moureaux T, Leydecker M-T, Vauchere H and Caboche M (1993) Regulation of nitrate and nitrite reductase expression in *Nicotiana plumbaginifolia* leaves by nitrogen and carbon metabolites. *Plant J* 3: 315–324
- Walls-grove RM, Turner JC, Hall NP, Kendall AC and Bright SWJ (1987) Barley mutants lacking chloroplast glutamine synthetase—biochemical and genetic analysis. *Plant Physiol* 83: 155–158
- Waring RH, McDonald AJS, Larsson S, Ericsson T, Wiren A, Arwidsson E, Ericsson A and Lohammar T (1985) Differences in chemical composition of plants grown at constant relative growth rates with stable mineral nutrition. *Oecologia* 66: 157–160
- Warner RL and Huffaker RC (1989) Nitrate transport is independent of NADH and NAD(P)H nitrate reductases in barley seedlings. *Plant Physiol* 91: 947–953
- Warner RL and Kleinhofs A (1981) Nitrate utilization by nitrate reductase-deficient barley mutants. *Plant Physiol* 67: 740–743
- Weber H, Buchner P, Borisjuk L and Wobus U (1996) Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: Expression patterns, metabolic regulation and implications for seed development. *Plant J* 9: 841–850
- Weiner H (1997) Protein phosphatase 2A in spinach leaves: A sensor for the metabolic phosphate and sugar status? In: Gadal P, Kreis M, Dron M, Brulfert J, Bergounioux D and Vidal J (eds) *Protein Phosphorylation in Plants*, pp 136–139, University of Paris-Sud, Orsay, France
- Weiner H, McMichael RW Jr and Huber SC (1992) Identification of factors regulating the phosphorylation status of sucrose-phosphate synthase in vivo. *Plant Physiol* 99: 1435–1442
- Wilkinson JQ and Crawford NM (1993) Identification and characterisation of a chlorate-resistant mutant of *Arabidopsis thaliana* with mutations in both nitrate reductase structural genes *NIA1* and *NIA2*. *Mol Gen Genet* 239: 289–297
- Worrell AC, Bruneau JM, Summerfelt K, Boersig M and Voelker TA (1991) Expression of a maize sucrose-phosphate synthase in tomato alters leaf carbohydrate partitioning. *Plant Cell* 3: 1121–1130

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Chapter 9

Starch Metabolism in Leaves

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Summary

Starch is a carbohydrate reserve used by many higher plants. Transitory starch is synthesized in the chloroplasts of higher plants both as an overflow for newly-assimilated carbon and as a reserve of carbohydrate for periods of darkness when photosynthesis is not possible. Starch is present in the chloroplast as insoluble granules consisting of two types of glucose polymer: amylopectin and amylose. The pathway of synthesis has been well defined and involves the action of phosphoglucomutase, ADPglucose pyrophosphorylase, starch synthases, and branching enzymes. However, there are multiple isoforms of starch synthases and branching enzymes and the extent to which different isoforms play distinct roles in determining starch structure is not clear. The simultaneous action of degradative enzymes may also play a role in determining the structure of starch during its synthesis. It is likely that starch synthesis is regulated through the allosteric control of ADPglucose pyrophosphorylase. Many enzymes have been implicated in the process of transitory starch degradation although few have been confirmed to play a role in vivo. It is not clear whether the initial attack on the starch granules is hydrolytic—catalyzed by amylases—or phosphorolytic—catalyzed by starch phosphorylase. The route by which sugar is released from shorter soluble glucan chains also remains unclear. There is physiological evidence that the process of starch degradation is subject to regulation. However, given the uncertainty about the mechanism of starch degradation, it is not possible to develop hypotheses about its regulation. The products of degradation may be exported from the chloroplast as sugars or as triose phosphate, but there is evidence that sucrose synthesis in the cytosol at night is supported by the export from the chloroplast of sugars rather than triose phosphate.

I. Introduction

Starch is the most abundant carbohydrate reserve in plants: it has been estimated that in excess of 10^9 tonnes are produced annually (Galliard, 1987). Two types of starch can be distinguished according to function: reserve starch and transitory starch. Reserve starch accumulates in organs of perennation and dispersal, and is localized in non-green plastids, called amyloplasts (Martin and Smith, 1995). Transitory starch is formed during photosynthesis in the chloroplasts of many higher plants, and serves as an important store of carbohydrate for periods of darkness when photosynthesis is not possible. In this chapter we will discuss the occurrence, importance, structure, synthesis, and degradation of transitory starch. We will pay particular attention to the physiological significance of transitory starch and discuss current ideas on how starch metabolism is regulated at the biochemical level.

II. The Occurrence and Function of Transitory Starch

The pattern of starch accumulation in leaves is often

Abbreviations: 3-PGA – 3-phosphoglycerate; AGPase – ADPglucose pyrophosphorylase; GBSSI – granule-bound starch synthase I; PGM–phosphoglucomutase; P_i –inorganic phosphate; SSI – starch synthase I; SSII – starch synthase II; SSIII – starch synthase III; TPT – triose-phosphate translocator

portrayed as a strong diurnal rhythm, in which starch is synthesized in relatively large amounts during the day and almost entirely degraded during the night. This is certainly the case in some well-studied experimental systems. Leaves of pea grown under an 18-h photoperiod, *Arabidopsis* grown under a 12-h photoperiod and spinach grown under a 9-h photoperiod all degraded 85% or more of the accumulated starch in the subsequent dark period (Stitt et al., 1978; Gerhardt et al., 1987; Lin et al., 1988b). However, the extent of starch accumulation and the magnitude of the diurnal change vary considerably. First, different species allocate very different proportions of their newly-assimilated carbon to starch. Many species do not accumulate starch in their chloroplasts under normal growth conditions. Sucrose or—in the Gramineae—fructans are stored in the vacuoles of mesophyll cells in these species. In other species, starch synthesis can account for anything from a few percent to a very substantial proportion of the newly-assimilated carbon. For example, leaves of annual meadow grass (*Poa annua* L.) partitioned most of their assimilated carbon into sucrose and fructans, and less than 2% into starch (Borland and Farrar, 1988) whereas in leaves of sugar beet (*Beta vulgaris* L.) 40% of assimilated carbon was partitioned into starch (Li et al., 1992b). Second, the extent of the diurnal change in starch content changes as the leaf ages in some species but not in others. The starch content of tobacco, soybean and cotton leaves increases as leaves age, and the

magnitude of the diurnal change decreases (Matheson and Wheatley, 1962, 1963; Chang, 1979; Franceschi and Giaquinta, 1983), but there is little change in the diurnal pattern of starch accumulation and degradation through development in *Arabidopsis* leaves (Trethewey and ap Rees, 1994b). Third, the extent of starch accumulation may vary from one cell type to another within a single leaf. The parenchymatous bundle sheath of barley leaves contains four distinct types of photosynthetic cell, each of which displays different patterns of starch accumulation and degradation (Williams et al., 1989). The relative amounts of starch in the two palisade layers and the spongy mesophyll layer of soybean leaves changed considerably during the photoperiod (Franceschi and Giaquinta, 1983). Fourth, starch content and the extent of the diurnal change are highly dependent upon the conditions in which the plant is grown—for example, the length of the photoperiod, the photon flux density and the night temperature (Hewitt et al., 1985; Fondy et al., 1989). Deficiencies of nitrogen and potassium and elevated levels of carbon dioxide usually result in increased accumulation of starch in leaves (Rufty et al., 1988; Farrar and Williams, 1991; Paul and Stitt, 1993). Attack by fungal and viral pathogens may also lead to accumulation of starch. This accumulation may be specifically in or around infected cells (Scholes and Farrar, 1987; Técsi et al., 1994), or generally throughout the leaves of an infected plant (Técsi et al., 1992).

The importance of transitory starch in those plants in which it accumulates is demonstrated by the phenotypes of mutant and transgenic plants in which accumulation is prevented or its magnitude is altered. Mutations in *Arabidopsis* and tobacco that eliminate activity of plastidial phosphoglucomutase [PGM; tobacco NS458, NS514, Hanson and McHale (1988); *Arabidopsis* TC-7, Caspar et al. (1986)] or ADPglucose pyrophosphorylase (AGPase; *adg2* mutant of *Arabidopsis*, Lin et al., 1988b) prevent accumulation of starch in the leaves. Growth of these mutants is indistinguishable from that of wild-type plants under long photoperiods. In the tobacco mutants, sucrose is synthesized and exported during the light period at a higher rate than in wild-type plants, compensating for the lack of sucrose synthesis from starch at night (Huber and Hanson, 1992; Geiger et al., 1995). However, when grown with long dark periods this compensatory effect is not adequate to support growth rates comparable with those of wild-type plants: all of the mutants exhibit reduced growth rates (Caspar et al., 1986; Lin et al., 1988b; Hanson and McHale,

1988; Schulze et al., 1991; Huber and Hanson, 1992). Indeed, a mutant of *Arabidopsis* lacking plastidial phosphoglucomutase also exhibited a late-flowering phenotype in days shorter than 16 hours (Corbesier et al., 1998). Transgenic plants in which the extent of accumulation of transitory starch has been altered also show compensatory changes in the diurnal pattern of sucrose export from the leaf. Potato plants in which the activity of the triose-phosphate translocator (TPT) in the chloroplast envelope has been reduced through the expression of antisense RNA have increased rates of transitory starch synthesis and reduced rates of sucrose synthesis and export during the day. This is compensated for by high rates of starch degradation leading to increased sucrose synthesis and export at night (Heineke et al., 1994). Potato plants in which the synthesis of transitory starch is decreased through expression of an antisense RNA for AGPase display increased rates of sucrose synthesis and export during the day, compensating for the reduction in sucrose synthesis from starch at night (Leidreiter et al., 1995).

Consideration of conditions which promote the accumulation of starch in leaves has given rise to two opinions about its function. First, transitory starch synthesis may occur primarily as an overflow for newly-assimilated carbon when assimilation exceeds the demand for sucrose (Stitt and Quick, 1989). The sequestration of carbon as insoluble starch allows high rates of assimilation to be maintained when the capacity to store and to export sucrose has been reached. Recent studies on transgenic plants with reduced rates of transitory starch synthesis have demonstrated a positive correlation between the capacity for starch synthesis and the rate of photosynthesis at elevated carbon dioxide concentrations (Ludewig et al., 1998). In intact leaves of some species [for example pea (Stitt et al., 1978), sugar beet (Fondy and Geiger, 1982) and maize (Kalt-Torres and Huber, 1987)] the rate of starch synthesis is low in relation to that of sucrose during the early part of the day and rises later, in parallel with a rise in the sucrose content of the leaf. Further experimental support for the idea of transitory starch as an overflow is provided by the general observation that restriction of the export of carbon from the chloroplast or from the leaf leads to increased rates of starch synthesis. For example, starch synthesis is increased in transgenic potato plants in which activity of the TPT or a sucrose-proton translocator involved in phloem loading has been reduced (Heineke et al., 1994, Kühn et al., 1996), and in plants in which

phloem loading has been impaired by expression of high activities of invertase in the apoplast (von Schaewen et al., 1990). Starch synthesis is also increased in leaves of *Clarkia* mutants with decreased activity of cytosolic phosphoglucoisomerase and hence a reduced rate of sucrose synthesis (Neuhaus et al., 1989). In spinach leaf discs the rate of starch relative to sucrose synthesis increased as the sucrose content of the discs increased (Stitt et al., 1984).

Second, transitory starch may provide a source of carbon for growth during the following night. This view is supported by the compensatory changes in the pattern of sucrose synthesis and export that occur in mutants unable to synthesize transitory starch, and by the fact that growth of these plants is restricted under short-day regimes (see above). The importance of transitory starch to the growth of the plant is clearly dependent upon the general growth conditions: an inability to synthesize transitory starch is likely to restrict growth to a greater extent in conditions when carbohydrate supply is limiting growth (for example in high nitrogen) than in conditions where some other factor is more important in limiting growth (Schulze et al., 1991). The pattern of starch synthesis in several species is also not consistent with a role simply as an overflow mechanism. For example, starch synthesis can occur concurrently with sucrose synthesis at low rates of photosynthesis. In *Clarkia xantiana* Gray (Onagraceae) rates of starch and sucrose synthesis were approximately equal under both saturating and limiting light, even though the rate of photosynthesis was three times greater in saturating than in limiting light (Kruckeberg et al., 1989). The ratio of sucrose to starch synthesis in *Phaseolus* was constant over a wide range of light intensities and carbon dioxide concentrations except where the rate of photosynthesis was very low, when sucrose synthesis was preferentially maintained (Sharkey et al., 1985). In a number of species [soybean, spinach, maize, pangola (*Digitaria decumbens* Stent.), sugarbeet (Chatterton and Silvius, 1981; Jablonski and Geiger, 1987), and potato (Lorenzen and Ewing, 1992)] the proportion of newly-assimilated carbon that is partitioned to starch varies inversely with the length of the photoperiod. These observations, and extensive studies of the allocation of newly-assimilated carbon to starch in leaves of sugar beet, have led Geiger and colleagues to suggest that partitioning to starch may be regulated by an endogenous circadian rhythm which ensures that the amount of starch synthesized during the day is

commensurate with the demand for carbon during the following night (Li et al., 1992b).

Overall, it seems likely that transitory starch is synthesized both as an overflow for newly-assimilated carbon and as a source of carbon during the night. The relative importance of these two roles depends on the species and the growth conditions. The ways in which the partitioning of newly-assimilated carbon into starch may be regulated are discussed in Section IV.B.

III. The Structure of Transitory Starch

Very little is known about the composition and structure of the starch granules in chloroplasts: almost all research on starch structure has been on the starches of non-photosynthetic storage organs. We will summarise the present understanding of storage starches and discuss whether this picture is likely to be applicable to transitory starch.

Storage starch granules vary enormously between species in size and shape but are similar in important features of composition and structure. They consist of two distinct types of glucose polymer. About 20–30% of their mass is made up of amylose, a predominantly linear molecule consisting of between 500 and 10,000 $\alpha(1\rightarrow 4)$ linked glucose residues. The remaining 70–80% of the granule is amylopectin, a polymer consisting of relatively short chains of $\alpha(1\rightarrow 4)$ linked glucose residues, joined by $\alpha(1\rightarrow 6)$ linkages.

Microscopical studies and X-ray diffraction and scatter analyses indicate that granules of storage starches are semi-crystalline, containing concentric rings of alternating crystalline and amorphous lamellae with a repeat distance of 9 nm (French, 1984; Imbert et al., 1991; Jenkins et al., 1993; Fig. 1). This level of organization is a function of amylopectin rather than amylose molecules since it occurs in the storage starches of both wild-type plants and mutant plants unable to synthesize amylose. The existence of a 9-nm repeat has been explained by the fact that the distribution of chain lengths within the amylopectin molecule is polymodal, with maxima at chain lengths of approximately 12–16, about 40 and about 70 glucose residues (French, 1984; Hizukuri, 1986). It is believed that the amylopectin chains of 12–16 glucose residues are organised into clusters. Chains of 40 glucose residues span two clusters, and chains of 70 glucose residues span three clusters. Within clusters, adjacent chains form double

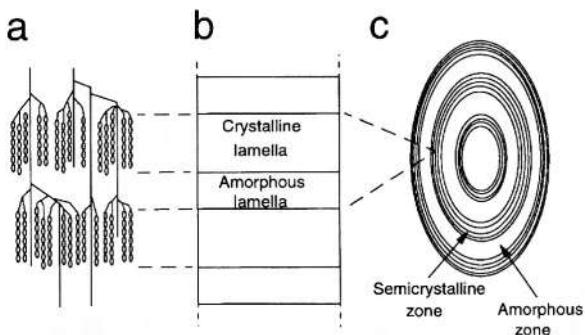


Fig. 1. Organization of starch polymers within a granule of storage starch. (a) 'Cluster' arrangement of chains in an amylopectin molecule. Adjacent chains within a cluster form double helices. The free (non-reducing) ends of the chains point towards the periphery of the granule. (b) Crystalline lamellae, formed by regular packing of the double helices in the clusters, alternating with amorphous lamellae representing inter-cluster regions with high concentrations of $\alpha(1,6)$ linkages. The periodicity of the crystalline-amorphous repeat is 9 nm. (c) Cross-section of a starch granule, showing alternating concentric zones ('growth rings') of semi-crystalline material—consisting of the alternating crystalline and amorphous lamellae shown in B—and amorphous material. The zones of semi-crystalline material are several hundred nm in width.

helices which pack together in ordered arrays, giving rise to the crystalline lamellae (Fig. 1). The amorphous lamellae are the zones in which a large number of $\alpha(1\text{-}6)$ linkages occur, hence few double helices are formed (Hizukuri, 1986; Jenkins et al., 1993). The crystalline and amorphous lamellae form concentric zones, usually several hundreds of nm wide, within the starch granule. These semi-crystalline zones alternate with amorphous zones in which the arrangement of the amylopectin molecules is not understood (Fig. 1). It is thought that one semi-crystalline and one amorphous zone are laid down per day—this repeat structure is often termed a 'growth ring' (Buttrose, 1960, 1962, 1963)—but definitive evidence that this is the case is lacking.

The transitory starch granules of chloroplasts are generally discoid in shape and less than five microns across (Badenhuizen, 1969). Where it has been studied, considerable variation has been found in the size of transitory granules within a single leaf. For example, most of the granules in a sunflower leaf were 0.2 to 2.5 microns across, but about 3% of the granules were 4 to 5.5 microns across (Radwan and Stocking, 1957). In *Pellionia* leaves granules in the spongy mesophyll were much larger than those in the palisade layer (Weier, 1936). There is general

agreement that transitory starch granules contain both branched polymers of high molecular mass and relatively unbranched polymers of lower molecular mass, corresponding approximately to the amylopectin and amylose components of storage starch. The ratio of amylose to amylopectin is lower in transitory than in storage starch, and the size and chemical structure of both of these components probably differs between transitory and storage starch. Around 16% or less of the starch of leaves of tobacco, sunflower, rice, pea and potato is amylose, whereas this value is almost invariably 20% or more for storage starches (Radwan and Stocking, 1957; Hovenkamp-Hermelink et al., 1988; Taira et al., 1991; Matheson, 1996; Tomlinson et al., 1997). The amylose content of the transitory starches of tobacco and cotton leaves increases as the starch content of the leaves increases through development (Chang, 1979; Matheson, 1996). The amylose of transitory starch is reported to be of lower molecular mass and more branched than that of storage starches (Radwan and Stocking, 1957; Matheson, 1996). The amylopectin of transitory starch has a polymodal distribution of chain lengths which approximates to that of storage starches (Matheson, 1996). However, a detailed study of the distribution of lengths of the shorter chains of amylopectins from the transitory and storage starches of pea revealed considerable differences between the two (Tomlinson et al., 1997). The amylopectin of the transitory starch had a very strong polymodal distribution of chain lengths in the range of 5–40 glucose residues, with maxima at lengths of 12, 15 and about 21 glucose residues. This pattern was observed regardless of the age and growth conditions of the plants. It may be a common feature of transitory starches: a similar distribution is observed for the amylopectin of the transitory starch of *Arabidopsis* (Zeeman et al., 1998a).

Increases in the starch content of leaves during the light period are generally accompanied by decreases in the ratio of amylose to amylopectin, and in the molecular mass of amylopectin (Chang, 1979; Matheson, 1996; Tomlinson, 1996). Taken together with the developmental changes in the composition of leaf starches described above, this may indicate that the diurnal changes in starch content occur primarily in an amylopectin fraction of relatively low molecular mass which is preferentially synthesized during the day and preferentially degraded at night. Amylose, and amylopectin of higher molecular mass, appear to accumulate gradually as the leaf ages.

Very little is known about the organization of amylose and amylopectin within granules of transitory starch. In general it appears that these granules lack the relatively high level of crystalline order seen in storage starches (Radwan and Stocking, 1957; Steup et al., 1983). However, like the starch of storage organs, the starch of pea leaves is at least in part semi-crystalline with a lamellar repeat of 9 nm (Waigh, 1997). The starch of tobacco leaves contained amorphous and semi-crystalline zones analogous to the growth rings of storage starches (Buttrose, 1963), and the starch of spinach leaves was reported to consist of an amorphous outer 'mantle' and an inner, more crystalline core. The mantle was more readily degraded during the night than the core (Beck, 1985). The mantle may represent the amylopectin of relatively low molecular mass that is preferentially turned over on a diurnal basis. The core, and the material organised into growth rings in tobacco starch, may represent material that accumulates as the basal level of starch in these leaves increases with leaf age. The material in transitory starches that is not turned over on a diurnal basis may thus resemble storage starch in that it contains amylopectin molecules of high molecular mass and considerable amounts of amylose and it is probably semi-crystalline.

IV. The Synthesis of Transitory Starch

A. Pathway

Starch in chloroplasts is synthesized from fructose 6-phosphate derived from the reductive pentose phosphate pathway. Fructose 6-phosphate is converted to glucose 6-phosphate and then to glucose 1-phosphate via the enzymes phosphoglucomutase and PGM (Fig. 2). The substrate for the synthesis of the starch polymers, ADPglucose, is synthesized from glucose 1-phosphate and ATP via the enzyme AGPase (EC 2.7.7.27) in a reaction which also produces pyrophosphate (Fig. 3). This reaction is rendered effectively irreversible in vivo by the presence in the chloroplast of an alkaline inorganic pyrophosphatase, which hydrolyzes the pyrophosphate produced to phosphate (Weiner et al., 1987).

Evidence that this pathway is exclusively responsible for starch synthesis in leaves comes from mutant plants with reduced or undetectable activities in leaves of either plastidial PGM or AGPase. Both classes of mutant are either unable to synthesize

transitory starch or have a severely restricted capacity to do so. For example, peas carrying a mutation at the *rug3* locus (Harrison et al., 1997), tobacco (Hanson and McHale, 1988, see above) and the *Arabidopsis* mutant TC7 (Caspar et al., 1986) all lack activity of the plastidial isoform of PGM, and are unable to synthesize transitory starch. *Arabidopsis* plants carrying mutations at the *adg2* and *adg1* loci have 5% of wild-type and undetectable activities of AGPase, respectively (Lin et al., 1988b,c). *adg1* mutants are unable to make transitory starch, and *adg2* mutants accumulate only 40% of the amount of starch in leaves of wild-type plants.

ADPglucose is the substrate for starch synthase, which adds glucose residues to the non-reducing ends of glucan chains via $\alpha(1\text{-}4)$ linkages. The $\alpha(1\text{-}6)$ linkages that create the branch points in the starch polymers are introduced by starch-branched enzyme (SBE), which transfers linear glucans from the end

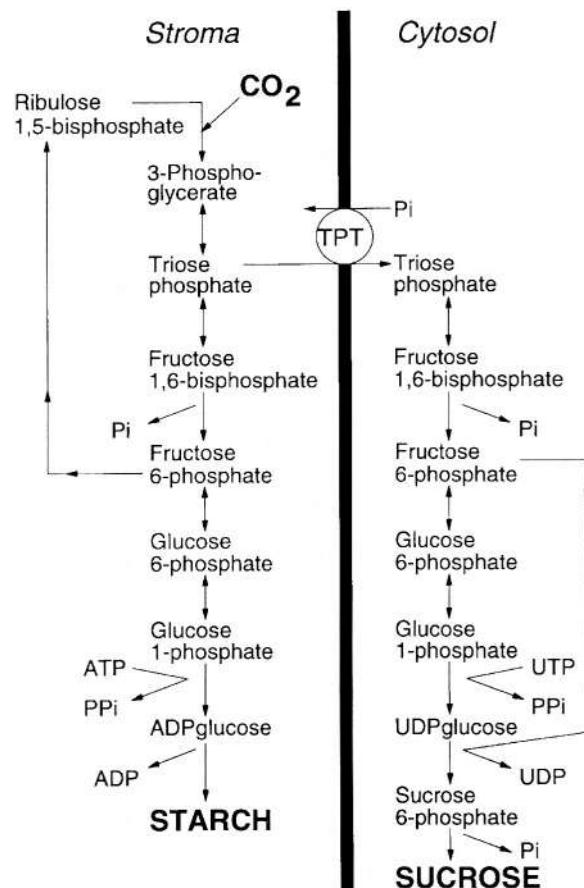


Fig. 2. Pathways of starch and sucrose metabolism in leaves. TPT, triose-phosphate translocator.

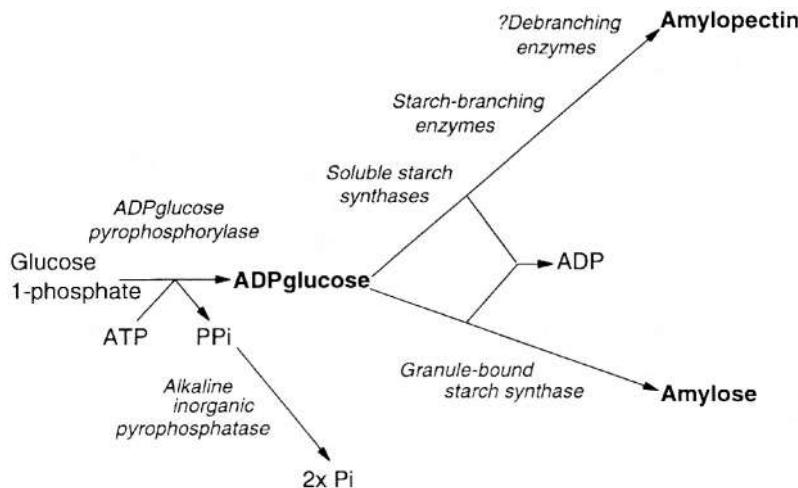


Fig. 3. Pathway of starch synthesis in leaves. PP_i, inorganic pyrophosphate; Pi, inorganic phosphate.

of a linear chain to the side of the same or an adjacent chain (Fig. 3).

The present understanding of the nature and properties of AGPase, starch synthase and SBE in leaves, and the roles of starch synthase and SBE in determining starch structure, are considered below.

1. ADPglucose Pyrophosphorylase

The activity of AGPase in leaves is potentially subject to regulation both allosterically by metabolites, and at the levels of transcription and translation. Our description will concentrate on aspects of its regulation that are of relevance to its role in the regulation of starch synthesis (Section IV.B).

AGPase from leaves is allosterically regulated by a number of metabolites central to chloroplast metabolism. It is activated most strongly by 3-phosphoglycerate (3-PGA): for example the enzymes from spinach and barley leaves are activated about 25-fold and 13-fold with $A_{0.5}$ values of $30 \mu\text{M}$ and $5 \mu\text{M}$, respectively (Morell et al., 1988; Kleczkowski et al., 1993)—but fructose 6-phosphate, fructose 1,6-bisphosphate, phosphoenolpyruvate and 2-phosphoglycerate also activate the enzyme from some sources (Sanwal et al., 1968). Inorganic phosphate (Pi) is a potent inhibitor, with $I_{0.5}$ values of $45-64 \mu\text{M}$ and $25 \mu\text{M}$ for the enzymes from spinach and barley leaves respectively. The presence of 3-PGA weakens and alters the nature of the inhibition by Pi, causing the Pi inhibition curve to change from hyperbolic to sigmoidal (Copeland and Preiss, 1981; Kleczkowski et al., 1993). The activity of the enzyme is thus

extremely sensitive to changes in the ratio of 3-PGA to Pi, and this has important consequences for its regulation in the chloroplast (see Section IV.B).

AGPase is a heterotetramer of two types of subunit—known as large and small subunits—that are related in sequence (Smith-White and Preiss, 1992). The precise roles of the two subunits in determining the properties of the enzyme are not yet fully understood, and both appear to be capable of binding substrates and effectors. Studies of the catalytic properties of large and small subunits expressed separately and together in the bacterium *Escherichia coli* are consistent with the view that the small subunits form a basic catalytic unit, the regulatory properties of which are modified by association with a large subunit (Ballicora et al., 1995; Preiss and Sivak, 1996).

Many species of plant contain small multigene families encoding one or both of the subunits. The small subunit is evolutionarily highly conserved while the large subunit shows much more divergence of sequence between species, and between members of multigene families within a species. Analysis of transcripts indicates that members of large and small subunit families are differentially expressed between organs, and during the development of individual organs (Krishnan et al., 1986; Olive et al., 1989; Prioul et al., 1994; La Cognata et al., 1995; Weber et al., 1995).

The expression of subunit genes has been studied in most detail in potato. One gene encoding a small subunit and two different genes encoding large subunits are expressed in potato leaves. Transcript

from the large-subunit gene *agpS2* was more abundant in young sink leaves than in source leaves, and was weakly induced by incubation of detached leaves in sucrose (La Cognata et al., 1995). Only small amounts of transcript from the second large-subunit gene, *agpS*, were detectable in attached leaves, but levels increased dramatically upon incubation of detached leaves with sucrose (Müller-Röber et al., 1990). The *agpS* and *agpS2* genes appear to be expressed in different tissues within the leaf. A promoter- β -glucuronidase fusion system indicated that the *agpS* gene is expressed in stomata and the starch sheath surrounding the main veins, but apparently not in the mesophyll cells (Müller-Röber et al., 1994). It seems likely that the *agpS2* gene is expressed in mesophyll cells.

Amounts of protein of AGPase subunits in potato leaves do not reflect directly the amounts of transcript, implying that there is strong post-transcriptional regulation of expression. Whereas levels of small-subunit transcript increased during the photoperiod, and levels of both small and large subunit transcripts increased in response to incubation with sucrose, there were no corresponding changes in amounts of the proteins or in AGPase activity (Nakata and Okita, 1995). Activity and amounts stayed constant or actually fell both during the photoperiod and in response to incubation with sucrose.

The significance of this regulatory complexity is not yet clear. It is not known whether the transcriptional and post-transcriptional regulation of expression actually leads to changes in the subunit composition of AGPase in the mesophyll cells of the leaf during development, on a diurnal basis, or in response to carbohydrate status or environmental conditions. If the subunit composition does change, this may lead to changes in the regulatory properties of the enzyme. It is likely that AGPases with different subunit compositions will be differently sensitive to allosteric activation and inhibition (Ballicora et al., 1995).

AGPase is generally accepted to be an exclusively plastidial enzyme, and there is compelling evidence for some plant organs that this is the case (ap Rees, 1995). However, recent work has demonstrated that endosperms of barley and maize have distinct plastidial and extra-plastidial forms of the enzyme (Shannon et al., 1998; Denyer et al., 1996b; Thorbjørnsen et al., 1996). In both tissues most of the AGPase activity is contributed by the extra-plastidial form, and in maize endosperm analysis of

a mutant (*brittle2*) lacking the extra-plastidial activity indicates that it provides much of the ADPglucose for starch synthesis in wild-type endosperm (Denyer et al., 1996b; Thorbjørnsen et al., 1996). There is at present no evidence that leaves contain an extra-plastidial AGPase (ap Rees, 1995). In leaves of barley, immunological studies suggested that the AGPase was attributable to chloroplastic proteins likely to be the same as those responsible for the minor, plastidial activity in the endosperm (Thorbjørnsen et al., 1996).

2. Starch Synthase

The starch synthase activity of plant organs is composed of several, distinct isoforms, each encoded by a different gene. Some of these isoforms are soluble, ie in the stroma of the plastid, some are tightly bound to the starch granule, and some are present in both of these locations. For a few starch-synthesising storage organs, most or all of the isoforms have been identified. However, their kinetic and regulatory properties are poorly understood, and *in vitro* studies have shed little light on their roles in the synthesis of the starch polymers. Recent studies of mutant and transgenic plants with altered isoform compositions are starting to elucidate these roles. Although some of the first descriptions of starch synthases were made on the enzyme from leaves (Frydman and Cardini, 1964; Doi et al., 1965) studies of starch synthase in leaves have been less extensive and less detailed than those in storage organs. We will summarise the present understanding of the enzyme in storage organs, then examine the extent to which this picture is applicable to leaves.

Starch-synthesising storage organs of wild-type plants all contain a highly-conserved class of isoform of starch synthase that is tightly bound to the starch granule. This class is known as granule-bound starch synthase I (GBSSI; Smith et al., 1997). Analysis of mutant and transgenic plants in which GBSSI activity has been reduced or eliminated (for example the *waxy* mutants of cereals, the *lam* mutant of pea, the *amf* mutant of potato, and potatoes expressing antisense RNA for GBSSI; Denyer et al., 1995a and reviewed in Smith and Martin, 1993; Smith et al., 1997) provides evidence consistent with the idea that GBSSI is specifically responsible for the synthesis of the amylose component of the starch (Fig. 3). These mutants have a normal starch content, but the starch consists almost entirely of amylopectin. Exactly how GBSSI is able to synthesize an almost linear glucan

in the same compartment and at the same time as the synthesis of a highly branched glucan is unclear. One possibility is that it is buried within the starch granule in such a way that it is accessible to its substrates—ADPglucose and glucans of low molecular mass—but its product is not accessible to SBEs (Denyer et al., 1993, 1996a). It is also not clear whether GBSSI contributes to the elongation of amylopectin chains *in vivo*. It is certainly capable of incorporating glucose residues from ADPglucose into long chains of amylopectin in isolated starch granules (Denyer et al., 1996a).

Other isoforms of starch synthase—those responsible for amylopectin synthesis (Fig. 3)—are less well understood. Three classes, distinguished by features of their predicted amino-acid sequences and/or their molecular mass and antigenic properties, have been identified so far. Starch synthase I (SSI) has been identified in potato and the endosperms of rice and maize (Baba et al., 1993; Abel et al., 1996; Knight et al., 1998), SSII in potato, pea embryo and cereal endosperms (Denyer and Smith, 1992; Dry et al., 1992; Denyer et al., 1995b; Edwards et al., 1995; Hylton et al., 1996; Harn et al., 1998), and SSIII in potato, pea embryo and maize endosperm (Abel et al., 1996; Marshall et al., 1996; Craig et al., 1998; Gao et al., 1998). It is likely that these three classes are of widespread occurrence, and between them they may account for starch synthase activity other than that accounted for by GBSSI. However, their relative importances differ from one type of storage organ to another. For example, SSII accounts for 60–70% of the soluble activity in pea embryos (Denyer and Smith, 1992; Edwards et al., 1996), and SSIII was a minor component of the activity (Craig et al., 1998). A mutation that eliminates SSII (at the *rug5* locus) dramatically reduces the average chain length of amylopectin in the embryo, indicating that this isoform plays a specific role in determining amylopectin structure (Craig et al., 1998). In contrast, SSII accounts for about 10% and SSIII for 85% of the soluble starch synthase activity in a potato tuber (Edwards et al., 1995; Abel et al., 1996; Marshall et al., 1996). Large reductions in SSII activity in the tuber, achieved through expression of antisense RNA, had very little effect on amylopectin structure (Edwards et al., 1995; Marshall et al., 1996). It is thus not possible at present to make generalizations about the nature and specific roles of isoforms of starch synthase other than GBSSI.

There is good evidence that leaves, like storage

organs, contain multiple isoforms of starch synthase, and that these are both soluble and granule bound. Multiple, soluble forms of the enzyme have been resolved by ion-exchange chromatography and gel electrophoresis from spinach, rice and maize leaves (Ozbun et al., 1971, 1972; Hawker et al., 1974; Dang and Boyer, 1988, 1989; Tacke et al., 1991; Yamanouchi and Nakamura, 1992), and granule-bound activity has been reported for starch from, for example, geranium and rice leaves (Frydman and Cardini, 1967; Taira et al., 1991). These reports have generally not identified the proteins actually responsible for the activities, hence it is not possible to determine unequivocally the nature and the number of gene products that contribute to the activity. Nonetheless, there is evidence that the isoforms of starch synthase present in leaves of cereals differ from those in the storage organs of the same plant. For example, Taira et al (1991) reported that the granule-bound activity and the amylose content of the starch of rice leaves were unaffected by the waxy mutation that eliminates GBSSI and amylose from the endosperm. Leaf starch contained no proteins antigenically related to the GBSSI of the endosperm. In maize, the chromatographic behaviour and kinetic properties of the soluble starch synthase activity of leaves and developing endosperm differed considerably, leading to the suggestion that leaves contained only one of the two isoforms distinguished biochemically in endosperm (Dang and Boyer, 1988; 1989).

Detailed study of pea revealed that both the granule-bound and soluble starch synthase activities of leaves are attributable to isoforms different from those in the starch-storing embryo. Leaf starch contains an isoform of starch synthase (GBSS Ib) closely related in kinetic and antigenic properties and in N-terminal sequence to the GBSSI class, but encoded by a different gene from the GBSSI of the embryo (Denyer et al., 1995a, 1997; Tomlinson et al., 1998). Most of the soluble activity in the leaf is contributed by an isoform antigenically related to the SSIII class. Although a minor isoform of the SSIII class is also present in the embryo (Craig et al., 1998) it is not clear whether the leaf and the embryo SSIII-like isoforms are the same gene product. The SSII isoform that contributes 60–70% of the soluble activity of the embryo contributes only about 10% of the soluble activity of the leaf (Tomlinson et al., 1998).

The precise contributions of different isoforms of starch synthase to the synthesis of amylopectin in transitory starches has not been assessed. Very few

isoforms have been positively identified in leaves, and no mutations affecting them have been described. It is nonetheless possible that at least some of the differences in the structure and organization of amylopectin molecules between transitory and storage starches are caused by differences in the isoform composition of the starch synthases responsible for the synthesis of these two sorts of starch. However, other factors are also likely to contribute to the differences in the structure and organization of amylopectin molecules. The structure of storage starches is determined over many days, during which time the relative levels of expression of different isoforms of both starch synthase and starch-branched enzyme probably change (Martin and Smith, 1995). Transitory starch, in contrast, is mostly the immediate product of the isoforms of starch synthase and starch branching enzyme present in the chloroplast during the preceding photoperiod. The structure of any starch present at the start of the photoperiod will be strongly influenced by the actions of starch-degrading enzymes during the previous night.

The isoforms of starch synthase responsible for the synthesis of amylose in leaves are not well understood. In potato leaves, amylose synthesis is almost certainly a function of the same GBSSI isoform of starch synthase responsible for amylose synthesis in the tuber. A mutation in the gene encoding GBSSI eliminates amylose from both tuber and leaf starches (Jacobsen et al., 1989). However, as discussed above, leaf amylose is not synthesized by the GBSSI isoform of the storage organ in pea and rice. The *lam* mutation that eliminates amylose from the starch of pea embryo has no effect on the amylose content of leaf starch. A second isoform of the GBSSI class, GBSSIIb, may be responsible for amylose synthesis in pea leaves (Denyer et al., 1997), but definitive proof that this is the case is lacking. It is possible that the amylose of the leaf is at least in part a product of isoforms of starch synthase other than those of the GBSSI class.

3. Starch-Branching Enzyme

Like starch synthase, SBE exists as multiple isoforms that are much better described for storage organs than for leaves. The isoforms of storage organs fall into two distinct classes (known as A and B), distinguished by features of their amino-acid sequences (Burton et al., 1995; Martin and Smith,

1995). All of the storage organs so far examined—including those of rice, pea, maize and potato—possess both A and B isoforms, and these appear to account for all of the activity of SBE in the organ (Smith et al., 1997). In the endosperm of maize, two different A isoforms as well as a B isoform are present (isoforms IIa and IIb; Gao et al., 1997; Boyer and Preiss, 1978a,b) and rice endosperm also appears to contain multiple forms of the two types of isoform (Mizuno et al., 1992, 1993; Yamanouchi and Nakamura, 1992). The A and B isoforms have distinct properties. Detailed studies of these isoforms from maize, both *in vitro* and when expressed in *Escherichia coli*, show that the A isoform (maize isoform II) prefers more highly-branched substrates and preferentially transfers shorter branches than the B isoform (maize isoform I; Guan and Preiss, 1993; Takeda et al., 1993; Guan et al., 1995). More limited studies on the A and B isoforms of other species indicate that the properties of the maize isoforms define general differences between the A and B classes (Smith, 1988; Morell et al., 1997).

There is evidence from pea, rice and maize that leaves also contain A and B isoforms. The isoforms of pea leaves are antigenically related to those of the embryo, and the effects of a mutation that eliminates the A isoform (at the *r* locus; Bhattacharyya et al., 1990; Burton et al., 1995) combined with the results of immunoprecipitation experiments provide good evidence that the same A and B isoforms account for all of the SBE activity of both the leaf and the embryo (Tomlinson et al., 1997). The two main forms of SBE purified from maize and rice leaves have kinetic, antigenic and chromatographic properties similar to the A and B isoforms of the endosperm (Dang and Boyer, 1988; Yamanouchi and Nakamura, 1992) although the relative contributions of the A and B isoforms to the total SBE activity probably differs between leaf and endosperm. Biochemical evidence, and the effects on leaf and endosperm SBE of mutations (at the *amylose-extender* loci) that lie in genes encoding an A isoform (maize isoform IIb) indicate that leaves of maize and rice may contain a different A isoform from the endosperms of these species (Boyer and Preiss, 1978a,b; Dang and Boyer, 1988, 1989; Yamanouchi and Nakamura, 1992; Mizuno et al., 1993; Fisher et al., 1996; Gao et al., 1996).

The fact that starch-synthesising organs have two isoforms of SBE with different properties has led to speculation that the two isoforms create branches of

different lengths or at different intervals along the amylopectin chains during amylopectin synthesis in vivo. For example, it has been suggested that the A isoform creates the chains of 12–16 glucose units that lie within the amylopectin clusters and the B isoform creates the longer chains that span clusters (Takeda et al., 1993; Preiss and Sivak, 1996). Studies of the effects of the *r* mutation that eliminates the A isoform in peas (Bhattacharyya et al., 1990) show that this idea requires refinement. Although the mutation increases the average chain length of amylopectin in both embryos and leaves, it has little effect on the polymodal distribution of chain lengths. The amylopectin of mutant leaves—containing only the B isoform—has a distribution of chain lengths from 5 to 40 glucoses that is virtually indistinguishable from the wild-type (Tomlinson et al., 1997). The extent to which the A and B isoforms play distinct roles in amylopectin synthesis in vivo remains unclear.

4. Debranching enzyme

Studies of mutants of cereals and *Chlamydomonas* that are deficient in debranching enzyme activity have given rise to the proposal that these enzymes, as well as SBEs, are important in determining the branching pattern of amylopectin. The developing endosperms of *sugary1* maize and rice accumulate a highly-branched, water-soluble glucan, phytoglycogen, in place of much of their starch. Both endosperms have reduced activities of debranching enzyme, and the gene at the *sugary1* (*sul*) locus of maize has been shown to encode a debranching enzyme of the isoamylase class (Pan and Nelson, 1984; Doehlert et al., 1993; James et al., 1995; Nakamura et al., 1996). Mutations at the *sta7* locus of *Chlamydomonas* cause the production of phytoglycogen instead of starch, and are associated with the loss of a debranching enzyme (Mouille et al., 1996). It is proposed on the basis of these phenotypes that debranching enzyme may act together with starch-branched enzyme to determine the degree of branching of amylopectin in a broad range of starch-synthesising species. Ball and colleagues (Ball et al., 1996) put forward a model for the synthesis of amylopectin in which trimming by debranching enzyme of a highly-branched, ‘pre-amylopectin’, synthesized by starch synthases and SBEs, is an essential step. It has proved difficult to test the model rigorously, in part because of lack of detailed knowledge of the occurrence and properties

of debranching enzymes and in part because the *sul* mutations have multiple pleiotropic effects on enzymes of starch synthesis in the endosperm. Although the mutation in maize lies in an isoamylase gene, the mutant endosperm has lower activity of a second class of debranching enzyme, pullulanase (also called limit-dextrinase or R enzyme), and alterations in activities of several other enzymes (Doehlert et al., 1993; Singletary et al., 1997). It is thus not possible to attribute the accumulation of phytoglycogen in endosperms unambiguously to the loss of isoamylase activity.

New light has been shed on the involvement of isoamylase in starch synthesis in general, and in starch synthesis in leaves in particular, by the discovery of an *Arabidopsis* mutant that contains phytoglycogen as well as starch in its leaves. This mutant (*dbe1*) is analogous to the *sul* mutants in that isoamylase activity is dramatically reduced or absent in its leaves, and there is evidence that the mutation lies in an isoamylase gene (Zeeman et al., 1998a). Unlike the *sul* mutants, the *dbe1* mutant is not deficient in pullulanase activity, and there are no major effects on other enzymes of starch synthesis. Phytoglycogen accumulates in mutant leaves at the same time and in the same chloroplasts as starch containing amylopectin of essentially identical structure to that in wild-type leaves (Zeeman et al., 1998a).

This phenotype is not obviously compatible with the trimming model proposed by Ball and colleagues. The simplest expectation from the model is that lack of debranching enzyme would give phytoglycogen only, and reduced debranching activity would give a single type of polymer with a structure intermediate between that of phytoglycogen and amylopectin. To account for the simultaneous accumulation of phytoglycogen and amylopectin in chloroplasts of the *dbe1* mutant, Zeeman et al. (1998a) propose that isoamylase is not directly involved in amylopectin synthesis, but rather plays a role in preventing elaboration by starch synthase and SBE of small, soluble glucans in the stroma. They argue that such glucans—for example maltose and maltotriose—can potentially be elongated by starch synthase and then branched by SBE. In the normal plastid, this elaboration of soluble glucan is prevented by a suite of stromal starch-metabolising enzymes such as amylases, phosphorylase and isoamylase. In an isoamylase-deficient mutant, branched soluble glucan can be synthesized in the stroma. Its synthesis

competes with that of amylopectin at the surface of the granule for the starch synthase and SBE available in the plastid, resulting in a reduction in the rate of amylopectin synthesis and the accumulation of phytoglycogen.

Although the *dbe1* mutant illustrates that isoamylase plays an important role in carbohydrate metabolism in the chloroplast during starch synthesis, we emphasize that both the trimming model and the alternative model proposed by Zeeman and colleagues to explain the nature of its involvement remain speculative. Much further work is required to elucidate the precise role of the enzyme. The possible role of debranching enzyme in starch degradation is discussed in Section VA.2 below.

B. Regulation of Starch Synthesis

The fact that AGPase is allosterically regulated by 3-PGA and Pi has led to the idea that changes in the levels of these metabolites in the chloroplast may be the primary means by which transitory starch synthesis is regulated. It is argued that when the rate of photosynthesis is low relative to the demand for sucrose, the ratio of 3-PGA to Pi will be low, hence AGPase will be inhibited and the flux of carbon through the pathway of starch synthesis will be relatively restricted. Newly assimilated carbon will thus be preferentially diverted to sucrose biosynthesis (see Fig. 2 for pathways involved). Conversely, when the rate of photosynthesis is high relative to the demand for sucrose, the ratio of 3-PGA to Pi will be high, AGPase will be activated, and the flux through the pathway of starch synthesis will be relatively high. Thus newly-assimilated carbon will be partitioned more towards starch under conditions of sucrose sufficiency. This hypothesis is attractive because it would be consistent with a role for starch synthesis as an overflow as has been demonstrated in many physiological studies (Section II). It is less clear how this mechanism would ensure that starch synthesis was sufficient to store enough carbohydrate for the following night period as has been observed in several plant species (Section II). There could be additional complexity such as diurnal changes in the activation status of sucrose-phosphate synthase (Huber and Huber, 1992) that ensure a minimum production of starch during the photoperiod.

There are several lines of evidence for the hypothesis that regulation of starch synthesis *in vivo* proceeds primarily through changes in the 3-PGA to

P_i ratio. First, there are good theoretical reasons for believing that the ratio of 3-PGA to P_i in the chloroplast will change as the ratio of assimilation to sucrose synthesis changes. When the rate of assimilation exceeds the rate of sucrose synthesis, import of P_i and export of triose phosphate from the chloroplast via the TPT is expected to be restricted, leading to an increase in the 3-PGA to P_i ratio in the chloroplast. Conversely, when the rate of assimilation is lower than the rate of sucrose synthesis then the high rate of generation of P_i in the cytosol from sucrose synthesis is expected to result in an increased export of triose phosphate from the chloroplast and a relatively low 3-PGA to P_i ratio in the stroma (Stitt and Quick, 1989). Reports of stromal phosphate concentrations, particularly the reduction in stromal phosphate concentration observed following feedback limitation of photosynthesis (Sharkey and Vanderveer, 1989) are consistent with these models. Further support is provided by the strong evidence that signal metabolite fructose 2,6-bisphosphate is involved in linking the biosynthesis of sucrose in the cytosol to the 3-PGA to P_i ratio in the chloroplast (see Stitt, 1990 for a review). This is discussed in accompanying chapters. We wish only to highlight the link between sucrose sufficiency and the 3-PGA to P_i ratio. Studies of spinach leaves show that towards the end of the photoperiod, when the assimilation rate exceeds the rate of sucrose biosynthesis, there is a restriction in sucrose-phosphate synthase activity and a consequent rise in the hexose phosphate levels (Gerhardt et al., 1987). The rise in fructose 6-phosphate stimulates fructose 6-phosphate 2-kinase and inhibits fructose 2,6-bisphosphatase (Stitt, 1990). The subsequent increase in fructose 2,6-bisphosphate inhibits fructose 1,6-bisphosphatase, which in turn leads to an increase in 3-PGA and triose phosphate in the cytosol. The increased concentration of phosphorylated compounds in the cytosol probably leads to a restriction in the availability of P_i in the chloroplast (Stitt and Quick, 1989). When P_i becomes limiting in the chloroplast, the stromal ATP concentration falls, and the rate of reduction of 3-PGA to triose phosphate is decreased (Heldt et al., 1977; Walker and Sivak, 1986), thus leading to a rise in the stromal 3-PGA to P_i ratio and a stimulation of starch synthesis. Recently, further evidence for the importance of fructose 2,6-bisphosphate has come from studies with transgenic tobacco plants that contain elevated levels of this signal metabolite (Scott et al., 1995; Scott and Kruger, 1995). The authors

found an increased partitioning of carbohydrate towards starch in plants with elevated fructose 2,6-bisphosphate levels and proposed that this switch in partitioning was mediated through the 3-PGA to P_i ratio.

Second, studies with isolated chloroplasts confirm that changes in the ratio of 3-PGA to P_i can alter the rate of starch synthesis. A decrease in the supply of P_i to isolated chloroplasts caused an increase in the partitioning of assimilated carbon into starch. It was proposed that the low concentration of P_i led to a decrease in the ATP/ADP ratio and hence to a restriction of 3-PGA reduction. An increase in the 3-PGA to P_i ratio in the chloroplast would then lead to an activation of AGPase and starch synthesis (Heldt et al., 1977; Robinson and Walker, 1979).

Third, circumstantial evidence for the importance of the 3-PGA to P_i ratio in regulating starch synthesis *in vivo* has come from studies where the activity of the TPT has been altered through genetic manipulation. The TPT is the major protein in the chloroplast inner envelope and catalyzes a strict 1:1 counter-exchange of substrates: it has similar affinities for triose phosphates, 3-PGA and P_i (Fliege et al., 1978; Flügge and Heldt, 1991). It therefore plays a central role in the regulation of the 3-PGA to P_i ratio and the communication of this ratio between cytosol and stroma. Transgenic lines of potato (Riesmeier et al., 1993) and tobacco (Barnes et al., 1994) have been generated in which TPT activity is reduced through the expression of antisense RNA. A 20–30% reduction in TPT activity in potato led to an increase in leaf starch by a factor of 300% while in tobacco an 80% reduction in activity led to an increase in leaf starch of only 10%. In both cases it would be expected that restricted TPT activity would increase the stromal 3-PGA to P_i ratio and hence stimulate starch synthesis. The fact that the extent of the increase in starch in response to a decrease in TPT activity is different in potato and tobacco indicates that factors other than simply the 3-PGA to P_i ratio are likely to be involved in regulating starch synthesis in some species.

Fourth, the importance of the AGPase in determining the rate of starch synthesis has been clearly shown by mutant and transgenic plants in which the activity of AGPase is reduced. A reduction of 50% in AGPase activity in a mutant *Arabidopsis* led to 23 and 39% reductions in starch synthesis under low and high light, respectively (Neuhaus and Stitt, 1990). Antisense inhibition of AGPase in potato using a leaf-specific promoter revealed a good positive

correlation between the AGPase activity and the accumulation of starch in the leaf (Leidreiter et al., 1995).

The case for the modulation of starch synthesis through the allosteric regulation of AGPase by the 3-PGA to P_i ratio is convincing. However, it should not be assumed that the AGPase is the only site at which control is exerted over the rate of starch synthesis. The development of a sound theoretical approach to the analysis of metabolic fluxes (Kacser and Burns, 1973; ap Rees and Hill, 1994; Kacser et al., 1995) has led to an increased realization that every step in an enzymic sequence contributes to some extent to the determination of flux. The role of each enzyme in controlling flux can be summarised as a flux control coefficient which is the fractional change in flux given by a fractional change in this enzyme activity. The larger the flux control coefficient of a particular enzyme, the more important this enzyme is in controlling the flux. By definition the flux control coefficients of a linear sequence of reactions without branch points sum to 1. It should therefore be the aim of a biochemist to determine all the flux control coefficients in a given pathway, although such an approach is beset by practical problems and has not yet been achieved for an entire pathway in plants (ap Rees and Hill, 1994). In the case of transitory starch synthesis some initial estimates of flux control coefficients have been made using data from a pea mutant deficient in starch-branched enzyme, *Arabidopsis* mutants deficient in plastidial PGM and AGPase, and a *Clarkia xantiana* mutant defective in plastidial phosphoglucoisomerase (Kruckeberg et al., 1989; Neuhaus et al., 1989; Neuhaus and Stitt, 1990; Smith et al., 1990). The calculated flux control coefficients indicate that AGPase dominates the control of starch synthesis in both high and low light. The flux control coefficients of phosphoglucoisomerase, PGM and SBE in high light were significantly greater than in low light. This analysis must be regarded as preliminary and incomplete because it is an amalgamation of data from different species and does not include an estimate of the flux control coefficients for the starch synthases. However, it is valuable in indicating that the balance of control in the pathway can be different according to the environmental conditions. Despite the technical problems associated with the determination of flux control coefficients in plants, it is to be hoped that further progress will be made in this direction in the future.

V. The Degradation of Transitory Starch

A. Enzymes Implicated in the Degradation of Transitory Starch

Despite extensive investigation, the precise nature of the pathway of transitory starch degradation remains unclear. Many enzyme activities that could contribute to degradative pathways have been described and characterised, and cDNAs encoding some of these enzymes have been cloned. However, the precise function of many of degradative enzymes *in vivo* is not known, and in several cases it is not clear whether the degradative activities are localised in the chloroplast. The possible roles of the degradative enzymes in starch degradation are summarised in Fig. 4. In this section, the enzymes will be described and evidence that they may be involved in transitory starch degradation will be discussed.

1. Amylase

Amylases are widespread in higher plants. They can be divided into two types: endoamylases which can catalyze the hydrolysis of the $\alpha(1\rightarrow 4)$ linkages at any point within amylose and amylopectin except immediately adjacent to $\alpha(1\rightarrow 6)$ branch points, and exoamylases which cleave maltose groups only from the non-reducing end of $\alpha(1\rightarrow 4)$ glucan chains. In plants the endoamylase and exoamylase activities are normally called α -amylase (EC 3.2.1.1) and β -amylase (EC 3.2.1.2), respectively.

a. α -amylase

Multiple isoforms of α -amylase are present in the leaves of many species of plant. Although at least some of these isoforms are not present in the chloroplast, others are chloroplastic and could therefore be involved in transitory starch degradation. Leaves of sugar beet, for example, contain five forms of α -amylase separable on zymograms, of which one is extrachloroplastic and four are exclusively or at least partly chloroplastic (Li et al., 1992a). Reports of the nature of the chloroplastic α -amylases differ from one species to another. Those of mesophyll and bundle-sheath cells of pearl millet (*Pennisetum americanum*) leaves (Vally and Sharma, 1995) are reported to be of the heat-stable, calcium dependent type extensively characterised from cereal endosperms (Briggs, 1967). Calcium-dependent α -

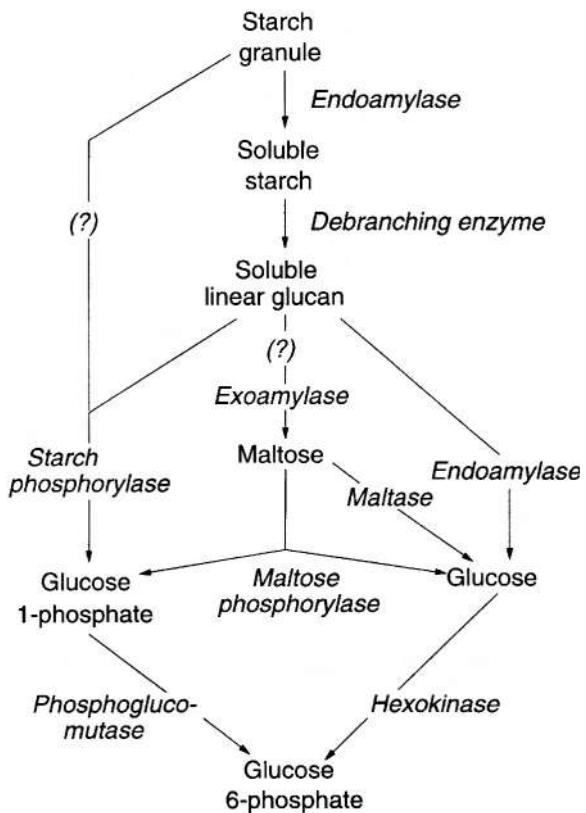


Fig. 4. Enzymes and intermediates implicated in the degradation of transitory starch.

amylases have also been reported from the leaves of sugar beet (Li et al., 1992a) and poplar (Witt and Sauter, 1996), but the enzyme was extrachloroplastic in the former case and localization studies were not performed in the latter case. The α -amylase of spinach chloroplasts, in contrast, is reported to be of the heat-labile, calcium-independent type first described from *Arum* spadix (Okita et al., 1979; Bulfin and ap Rees, 1978). Activity of α -amylase was not detected in studies of the chloroplasts of pea (Stitt et al., 1978) and barley (Jacobsen et al., 1986) leaves. However, in these cases the assays were conducted under conditions in which only heat-stable, calcium dependent α -amylases would be detected. The possibility that heat-labile, calcium-independent α -amylases are of widespread occurrence in chloroplasts requires further investigation.

There are two recent reports of amylases from *Arabidopsis* leaves which may be involved in chloroplastic starch degradation. First, activity of an isoform of endoamylase with a pH optimum of 7 is reported to vary diurnally in a manner consistent

with a role in starch degradation at night (Kakefuda and Preiss, 1997). However, it has not been established whether this activity is in the chloroplast. Second, evidence for the involvement of a different endoamylase in starch degradation has been provided by the discovery of a mutant (*sex4*) which apparently specifically lacks one isoform of the enzyme. The mutant is deficient in starch degradation at night (Zeeman et al., 1998b). Although this endoamylase has been shown to be chloroplastic, its activity does not vary on a diurnal basis. The possible roles of these two endoamylases are discussed further in Sections VB and VC below.

b. β -amylase

β -amylase is present in leaves, and is often discussed in the context of starch degradation in plants. However, it now seems unlikely that this enzyme plays a role in degradation of transitory starch. Rigorously-conducted cell-fractionation studies have shown that the enzyme is not associated with the chloroplasts in pea leaves (Stitt et al., 1978), and that it is associated with the vacuole rather than the chloroplast in protoplasts derived from pea and wheat leaves (Ziegler and Beck, 1986). A report that β -amylase is present in pea chloroplasts (Kakefuda et al., 1986) may be based on preparations of chloroplasts with significant vacuolar contamination. Extensive studies of the β -amylase of *Arabidopsis* leaves—where this enzyme accounts for over 80% of the total amylolytic activity—suggest that it is not chloroplastic but is likely to be vacuolar and perhaps localised within the vascular bundle (Lin et al., 1988a; Munroe et al., 1991; Wang et al., 1995).

2. Debranching enzyme

Debranching enzymes catalyze the hydrolysis of $\alpha(1\rightarrow 6)$ linkages in starch. Two classes of these enzymes have been described from higher plants. Pullulanase [also called limit dextrinase or R-enzyme: amylopectin 6-glucanohydrolase (EC 3.2.1.41)] can hydrolyze the yeast glucan pullulan in addition to starch polymers, whereas isoamylase (EC 3.2.1.68) cannot. Pullulanases have been shown to be present in the leaves of several species of plant (sugar beet, Li et al., 1992a; pea, Kakefuda et al., 1986; spinach, Ludwig et al., 1984, *Vicia faba*: Ghiena et al., 1993). Both extrachloroplastic and chloroplastic isoforms occur in pea leaves (Kakefuda et al., 1986), and

chloroplasts of spinach (Ludwig et al., 1984) and *Vicia faba* (Ghiena et al., 1993) contain pullulanase activity. Isoamylases have been reported from kernels of maize (Manners and Rowe, 1969; Doehlert and Knutson, 1991), pea embryos (Zhu et al., 1998), leaves of *Arabidopsis* (Zeeman et al., 1998a) and potato tubers (Ishizaki et al., 1978, 1983). Possible roles for debranching enzymes in the synthesis of starch are discussed in Section IV.A.4 above.

3. α -glucosidase

α -glucosidase (EC 3.2.1.20), also called maltase, hydrolyzes maltose and malto-oligosaccharides to glucose. α -glucosidases often display wide substrate preferences, which may differ considerably from one source of the enzyme to another. For example, some are specific for $\alpha(1\rightarrow 4)$ linkages and short malto-oligosaccharides, whereas others will hydrolyze $\alpha(1\rightarrow 6)$ and other α -glucosidic linkages in larger substrates. Thus, depending on their characteristics, these enzymes could act solely on the products of amylolytic degradation of starch to ensure their complete conversion to glucose, or could play a wider role in starch degradation by attacking larger, $\alpha(1\rightarrow 4)$, $\alpha(1\rightarrow 6)$ glucans.

There are conflicting reports on the occurrence and subcellular localization of α -glucosidase in leaves. Activity was not detected in studies of pea leaves (Kakefuda et al., 1986) and chloroplasts of pea (Kruger and ap Rees, 1983a) and spinach (Okita et al., 1979). However, Beers et al. (1990) identified two apoplastic and one chloroplastic isoform from pea seedlings. The chloroplastic isoform had a pH optimum of 7.0 and a K_m for maltose of 7.2 mM (Sun et al., 1995). Beers and colleagues suggested that failure to detect α -glucosidase: activity in previous studies was due to inappropriate extraction and assay conditions. The potential contribution of this enzyme to starch degradation in leaves generally thus remains to be established.

4. Maltose Phosphorylase

Little is known about the occurrence of maltose phosphorylase (EC 2.4.1.8) in higher plants. The enzyme is reported to be present in pea chloroplasts (Kruger and ap Rees, 1983b), but we know of no other information on its occurrence in leaves. Maltose phosphorylase catalyzes the interconversion of maltose with glucose 1-phosphate and glucose, and

could potentially contribute to the conversion to sugars of the products of amylolytic degradation of starch.

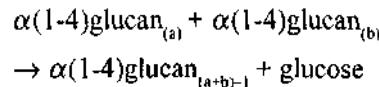
5. Starch Phosphorylase

Starch phosphorylase (α -glucan phosphorylase, EC 2.4.1.1) is of wide occurrence in higher plants. The enzyme catalyzes the phosphorolytic cleavage of a glucose residue from the non-reducing end of a glucan chain, to form glucose 1-phosphate. Plastidial and extraplastidial isoforms of the enzyme (referred to as type I and type II phosphorylases) have been demonstrated in many plant organs, including leaves. The properties and primary sequences of the two types are distinct and highly conserved (Steup and Schächtele, 1981; Fukui et al., 1987; Steup, 1988; Duwenig et al., 1998). In potato, in which the enzyme has been very extensively studied (Shimomura et al., 1982; Brisson et al., 1989; Nakano et al., 1989; Steup, 1990; Mori et al., 1991; Sonnewald et al., 1995), there are two plastidial isoforms, encoded by the *pho 1a* and *pho 1b* genes. The Ph_a product accounts for most of the plastidial activity in the tuber, and the Ph_b product for most of the activity in chloroplasts. Two plastidial isoforms have also been identified in pea (Steup and Latzko, 1979).

Plastidial isoforms of phosphorylase, including those of chloroplasts, prefer malto-oligosaccharides to larger, branched glucans as substrates (Fukui et al., 1987; Steup, 1988). The smallest malto-oligosaccharide that will act as a substrate is maltotetraose (Steup and Schächtele, 1981). There is reason to believe that the reaction catalyzed by the enzyme in the chloroplast is effectively irreversible in the direction of glucose 1-phosphate synthesis. Kruger and ap Rees (1983b) found that the apparent equilibrium constant (0.3–0.7) was very different from the mass action ratio of glucose 1-phosphate:P_i in the chloroplast (7.9×10^{-3}) calculated from estimates of stromal volume and metabolite contents in spinach leaves (Wirtz et al., 1980). These properties are consistent with a potential role for the enzyme in the conversion to hexose phosphate of the products of amylolytic degradation of starch. Partially-purified starch phosphorylase from pea chloroplasts exhibits some regulatory properties that could be of relevance to a role in the degradation of transitory starch at night. Its activity is twice as great at pH 7.2 as at pH 8.0, and it is inhibited by 60% in the presence of 4 mM ADPglucose (Kruger and ap Rees, 1983b).

6. D-Enzyme

D-enzyme (**4**- α -D-glucanotransferase: EC 2.4.1.25) catalyzes a ‘disproportionating’ reaction:



The enzyme has been studied in relatively few plant organs, but has been shown to be chloroplastic in pea (Kakefuda et al., 1986) and *Arabidopsis* (Lin and Preiss, 1988) leaves. The cDNA for D-enzyme from potato tubers encodes a putative plastid transit peptide (Takaha et al., 1993). D-enzyme could thus potentially convert short glucans generated by degradation of transitory starch into longer substrates more suitable for amylases and phosphorylase. A recent study of the properties of a D-enzyme from potato following its expression in *E. coli* reveals that the enzyme can also catalyze the formation of cyclic glucans from amylose (Takaha et al., 1996). The existence of such cyclic glucans in plants has not been demonstrated. Large reductions in the activity of D-enzyme brought about by the expression of antisense RNA in potatoes reduced the growth rate of the plant (Takaha et al., 1998), but the precise role played by the enzyme in the metabolism of starch *in vivo* remains to be established.

7. Hexokinase

The entry into intermediary metabolism of glucose released from starch requires the formation of glucose 6-phosphate via a hexokinase (EC 2.7.1.1). There is continuing debate about whether this phosphorylation occurs within the chloroplast or in the cytosol. The ability of chloroplasts from several species of plant, including pea and spinach, to metabolise exogenously-supplied glucose suggests a chloroplastic location for hexokinase (Stitt, 1984). However, cell-fractionation experiments indicated that hexokinase activity is outside the chloroplast envelope in pea and spinach (Stitt, 1984; Schnarrenberger, 1990). It seems likely that hexokinase is actually located on the chloroplast envelope, but definitive proof that this is the case is lacking.

8. R1 Protein

A novel protein implicated in the process of starch degradation in leaves has recently been identified in

potato (Lorbeth et al., 1998). The protein (named R1) was first identified from the matrix of tuber starch granules. An immuno-screening approach led to the isolation of a cDNA encoding a large protein of 1464 amino acids with no obvious sequence similarity to any other known protein. No enzymatic activity could be assigned to the protein expressed from the cDNA. Expression of antisense RNA for R1 in potatoes had several important effects on the nature and content of starch in the plant. First, there was a reduction in the phosphate content of tuber starch although the amount of starch in the tuber was not affected. Second, leaves contained more starch than those of untransformed controls: complete mobilization of starch during extended periods of darkness was not achieved. Third, a significant reduction in cold sweetening in cold-stored tubers was found in conjunction with an enhanced retention of starch after two months of storage. It is difficult to deduce the precise role of R1 from these complex phenotypic effects. However, expression of the R1 protein in *E. coli* led to a significant increase in the level of phosphorylation of the glycogen made by the bacterium. The authors proposed that the primary function of R1 is in starch phosphorylation, and that a lowering of the phosphate content of the starch of the transgenic potatoes leads by an unknown secondary mechanism to reduced susceptibility to degradation (Lorbeth et al., 1998). Further investigation, including the development of an assay, will be required to resolve the role of R1.

B. The Pathway of Transitory Starch Degradation *in vivo*

There is considerable confusion over which of the above enzymes are important in the degradation of transitory starch. The enzymes can be grouped into pathways around a hydrolytic or a phosphorolytic cleavage of $\alpha(1\rightarrow 4)$ linkages, but it is not clear whether one of these two pathways operates *in vivo* or whether both occur simultaneously. We shall consider the evidence available for transitory starch about the nature of the initial attack on the starch granule, and the fate of products of this initial attack.

There is some evidence that both starch phosphorylase and hydrolytic enzymes may be able to attack intact starch granules from leaves. Endoamylases from several sources and a putative chloroplastic α -glucosidase from pea leaves have been reported to attack isolated starch granules (Stitt

and Steup, 1985; Li et al., 1992a; Sun et al., 1995; Witt and Sauter, 1995). Starch phosphorylase from pea chloroplasts (Kruger and ap Rees, 1983b) and poplar wood (Witt and Sauter, 1995) was also reported to attack intact starch granules, but Steup et al. (1983) were unable to demonstrate degradation of starch granules by this enzyme. All of these *in vitro* experiments on the degradation of starch granules must be interpreted with caution since they are prone to several problems. First, the properties of the degradative enzymes may be altered during purification. Second, the properties of the starch granules may be altered during isolation. In particular, newly-synthesized, less crystalline material—which would be the substrate for degradative enzymes *in vivo*—may be lost. Third, some studies have used granules from a different plant source from the degradative enzymes, or have not taken care to isolate granules at the end of a photoperiod when degradation normally commences *in vivo*. Finally, the possibility that isolated granules may be contaminated with degradative enzymes has not been considered in all studies.

The only direct evidence for the involvement of a specific starch-degrading enzyme in starch degradation in leaves comes from studies of the *sex4* mutant of *Arabidopsis*. This mutant is deficient in starch degradation at night. Although some degradation occurs, the rate is much lower than in wild-type leaves, and as a result starch accumulates to high levels as the leaf develops (Zeeman et al., 1998b). The mutant lacks one, chloroplastic isoform of endoamylase (termed A2), identified by native gel electrophoresis of extracts of wild-type leaves. Other enzymes of starch synthesis and degradation, including debranching enzymes, phosphorylase and amylases, are apparently unaffected by the mutation. Although the gene at the *sex4* locus has not been identified, it is likely that the reduced rate of starch degradation in *sex4* leaves is directly attributable to the loss of the A2 endoamylase. The properties of this enzyme have not yet been established.

Studies with isolated chloroplasts have provided some evidence that the hydrolytic and phosphorolytic pathways can both operate in the degradation of transitory starch. Stitt and ap Rees (1980) labeled the starch in chloroplasts isolated from pea shoots by incubating the chloroplasts with ^{14}C -glucose in the light. During the subsequent dark period, ^{14}C lost from the starch appeared predominantly in maltose but also in sugar phosphates and 3-PGA. The situation

was similar in experiments with chloroplasts isolated from spinach leaves: ^{14}C lost during degradation of ^{14}C starch was found in glucose, maltose, 3-PGA, triose phosphates and CO_2 (Stitt and Heldt, 1981). The conclusion that both phosphorolytic and hydrolytic pathways are operating in these chloroplasts must, however, be treated with caution. It is theoretically possible that the products of apparent hydrolytic degradation are in fact synthesized from the products of phosphorolytic degradation (Kruger and ap Rees 1983a). In experiments with pea chloroplasts, the presence of maltose phosphorylase and the dependence of maltose synthesis on exogenous P_i led to the suggestion that maltose was synthesized from glucose 1-phosphate formed via phosphorolytic degradation of starch (Kruger and ap Rees, 1983a). Taken as a whole, therefore, experiments with isolated starch granules and chloroplasts have thus far failed to define the pathway of starch degradation.

C. Regulation of the Degradation of Transitory Starch

Given the uncertainty over the pathway of starch degradation, it is impossible to draw any firm conclusions about the regulation of the process. Net degradation occurs predominantly during the night, although some studies have reported that net starch degradation can also occur at the beginning and end of the photoperiod (spinach, Servaites et al., 1989; sugar beet and *Phaseolus vulgaris*, Fondy et al., 1989). One possible model is that the rate of starch degradation in leaves is constant, and that changes in the amount of starch are brought about solely by changes in the rate of synthesis (Preiss, 1982). However, observations about the timing and rate of degradation of transitory starch indicate this is not the case, and that the process must be subject to tight regulation. First, radioactive labeling experiments have provided no evidence for starch turnover in illuminated leaves during the normal photoperiod (pea, Kruger et al., 1983; sugar beet, Li et al., 1992b). Second, the rate of degradation is subject to variation during the dark period. Whereas degradation of transitory starch can occur immediately following the light/dark transition in some species (eg. pea, Stitt et al., 1978), in others there is a lag at the beginning of the dark period before degradation commences. In studies of spinach (Stitt et al., 1978) and soybean (Mullen and Koller, 1988a) leaves,

sucrose content started to fall immediately after the light/dark transition but there was a short lag before starch degradation occurred. In barley leaves (Gordon et al., 1980), starch degradation did not occur until sucrose content fell below a threshold value, nine hours after the start of the dark period. A correlation between the starch content of the leaf and the rate of degradation in the dark has been observed in studies of soybeans grown under different light intensities (Mullen and Koller, 1988b), and mutant *Arabidopsis* lines with differing starch contents (mutants with reduced AGPase, Lin et al., 1988b,c). Taken as a whole these experiments suggest that the rate of starch degradation in leaves may be sensitive to the diurnal cycle, the starch content, and the demand for sucrose in the dark.

The only obvious point at which starch degradation could be regulated is at the reaction catalyzed by starch phosphorylase. The pH optimum, the sensitivity to inhibition by ADPglucose and the dependence upon P_i of starch phosphorylase would be expected to result in an increase in its activity after a light-dark transition, when stromal pH becomes more alkaline, P_i concentration probably rises and the ADPglucose concentration falls (Kruger and ap Rees, 1983b). The potential importance of the effects of P_i concentration on the activity of starch phosphorylase is demonstrated by experiments with isolated chloroplasts, in which the rate of phosphorolytic starch degradation is sensitive to the exogenous P_i concentration (pea, Stitt and ap Rees, 1980; Kruger and ap Rees, 1983b; spinach, Stitt and Heldt, 1981). The dependence of starch phosphorylase activity on P_i concentration provides a means by which starch degradation in the leaf may be linked to the demand for sucrose. It is believed that the fall in sucrose content at the onset of the dark period leads to an increase in cytosolic and stromal P_i concentration. This would be expected to lead to a higher activity of starch phosphorylase, and hence a greater rate of starch degradation (Stitt et al., 1985).

Although the effects of P_i concentration on starch phosphorylase may contribute to the regulation of starch degradation, it is clear that other mechanisms must also operate. First, although the activity of starch phosphorylase in the chloroplasts of some species is sufficient to account for the rate of starch degradation (eg. pea, Stitt et al., 1978), in others it is not. In *Arabidopsis* leaves, only 4% of the starch phosphorylase activity is plastidial, and this plastidial activity is probably lower than the rate of starch

degradation in the dark (Lin et al., 1988a). Second, removal of exogenous P_i from isolated spinach chloroplasts in the dark results in a shift from phosphorolytic to apparently amylolytic starch degradation and has little effect on the total rate of degradation (Stitt and Heldt, 1981). Third, a regulatory mechanism based on changes that occur during a light-dark transition cannot explain those circumstances in which starch is degraded in the light.

It seems probable that amylolytic enzymes as well as starch phosphorylase must play a role in the regulation of transitory starch degradation. As discussed above, there is good evidence that amylolytic activities occur inside chloroplasts and that, at least in *Arabidopsis*, they are required for starch degradation. However, apart from a sharp pH optimum around pH 6 (Beck and Ziegler, 1989), there are few reports of properties of chloroplastic amylases that suggest how their activity might be modulated *in vivo*. There are reports that the total amylolytic activity in chloroplasts of spinach (Pongratz and Beck, 1978) and *Vicia faba* (Ghieni et al., 1993) is subject to diurnal variation, but these measurements must be treated with caution because the possibility of diurnal variation in the recovery of activity during the extraction procedure was not ruled out. The extractable activity of a specific isoform of endoamylase with a pH optimum of 7 has recently been shown to vary on a diurnal basis in *Arabidopsis* leaves (Section VA.1). Activity – detected on native gels – was seen only in leaves harvested in the dark, whereas activity of other starch-hydrolysing enzymes did not vary on a diurnal basis (Kakefuda and Preiss, 1997). This amylase has not been shown to be chloroplastic, and its involvement in starch degradation remains to be established.

D. Export of the Products of Starch Degradation from Chloroplasts

The synthesis of sucrose is by far the major fate for the carbon released by degradation of transitory starch. For example, in sugar beet leaves the rates of starch breakdown and sucrose synthesis at night have been estimated as 0.23 and **0.16 $\mu\text{g C cm}^{-2}$ per min** respectively (Fondy and Geiger, 1982). Transitory starch degradation at night must be precisely integrated with cytosolic metabolism, and in particular with sucrose biosynthesis. The nature of the export mechanisms for carbon from the chloroplast are of central importance to this

integration. Recent experiments indicate that the triose phosphate translocator (TPT)—responsible for the export of carbon from the chloroplast during the day—is not the main route of export of carbon produced by the degradation of transitory starch at night. Reduction of the activity of the TPT through the expression of antisense RNA in potato plants results, as expected, in increased accumulation of transitory starch during the day, but also causes increased mobilization of transitory starch and export of sucrose from the leaves at night (Riesmeier et al., 1993; Heineke et al., 1994). In tobacco plants with reduced activities of the TPT, starch degradation is promoted not only at night but also at the end of the light period. Although leaves of transgenic plants contained three times more starch than those of control plants after six hours of the light period, the starch content of the transgenic leaves actually fell towards the end of the light period and transgenic and control leaves contained similarly low amounts of starch by the end of the dark period (Häusler et al., 1998). It seems very unlikely from these results that the products of starch degradation are exported via the TPT.

The results described above strongly indicate that sucrose synthesis at night is supported by export from the chloroplast of metabolites other than triose phosphate. Three further observations lend support to this idea. First, mutants of *Flaveria linearis* with very low activities of cytosolic fructose bisphosphatase (Sharkey et al., 1992) and transgenic potato plants with large reductions in cytosolic fructose bisphosphatase brought about by the expression of antisense RNA (Zrenner et al., 1996) partitioned more of their photosynthate into starch and less into sucrose during the day than did wild-type plants. This result is consistent with a restricted ability to convert triose phosphate to sucrose in the mutant and transgenic plants. However, during the night the rate of starch mobilization in leaves of mutant and transgenic plants was twice as great as in wild-type plants, indicating that carbon exported from the chloroplast at night was not in the form of triose phosphates. Second, radioactive feeding experiments with wild-type *Arabidopsis* leaves showed that there is a net glycolytic flux at night: if carbon export from the chloroplast were at the level of triose phosphates there would have to be a net gluconeogenic flux (Trethewey et al., 1994b). Third, leaves of several species have a high concentration of fructose 2,6-bisphosphate at night (Stitt et al., 1985; Usuda et al.,

1987; Servaites et al., 1989; Stitt, 1990; Scott and Kruger, 1994). This should ensure that the cytosolic fructose 1,6-bisphosphatase is substantially inhibited, and thus prevent a gluconeogenic flux from triose phosphate to sucrose. These experiments suggest strongly that carbon for sucrose synthesis must be exported from the chloroplast as a sugar or a sugar phosphate.

A direct demonstration of a TPT-independent route for export from the chloroplast has been provided by investigations into an *Arabidopsis* mutant, *sex1*, that has severely reduced rates of starch degradation (TC265; Caspar et al., 1991; Trethewey and ap Rees, 1994a,b). The mutation results in a four- to five-fold accumulation of starch, and a reduced growth rate. The mutant contained appreciable activities of all of the enzymes putatively involved in starch degradation (Section V.A; Caspar et al., 1991; R. Trethewey, unpublished), but physiological analysis clearly indicated that it was defective in the conversion of starch to sucrose at night (Trethewey and ap Rees, 1994a,b). Uptake studies on intact chloroplasts isolated from mutant and wild type plants revealed that the mutant was deficient in glucose uptake (Trethewey et al., 1994a). This work suggested strongly that the products of starch degradation in *Arabidopsis* chloroplasts are exported via a glucose transporter. Evidence that this may also be the case in tobacco leaves comes from transgenic plants with reduced activity of the TPT. As described above, these plants show increased rates of starch degradation at night and at the end of the light period. This phenomenon is accompanied by a three-fold increase relative to control plants in the capacity for glucose transport by isolated chloroplasts (Häusler et al., 1998). A glucose transporter has previously been characterised in spinach chloroplasts (Schäfer et al., 1977). It catalyzes a facilitated diffusion with highest affinity for D-glucose (K_m , 20 mM). It also catalyzed similar rates of D-xylose, D-mannose, and L-arabinose transport and was inhibited by phloretin, a known inhibitor of glucose transport in erythrocytes.

The existence of maltose transporters in the chloroplast envelopes of spinach provides a further potential route for the export of carbon from starch degradation. Maltose is transported into spinach chloroplasts via a mechanism independent of glucose transport, indicating that there are separate maltose and glucose transporters (Herold et al., 1981; Rost et al., 1996). Carbon could also potentially be exported

as hexose phosphates. Rates of uptake of hexose phosphates by isolated chloroplasts are usually relatively low (Flügge and Heldt, 1991). However, isolated pea chloroplasts appeared to export hexose phosphates in the dark (Stitt and ap Rees, 1980) and glucose-6-phosphate-dependent starch synthesis was substantially induced in spinach and potato chloroplasts isolated from leaves that had been fed glucose for several days through the petiole (Quick et al., 1995). It remains an open question whether maltose or hexose phosphate transporters play a role in exporting the products of transitory starch degradation.

E. Conversion of Starch to Sucrose at Night

The demonstration that carbon can cross the chloroplast envelope either as triose phosphate or as sugars gives rise to the possibility that the products of hydrolytic and phosphorolytic degradation might be exported in different forms and thus have different fates in the cytosol. It is tempting to speculate that hydrolytic degradation gives rise to sugar residues that are exported to support sucrose biosynthesis, whereas phosphorolytic degradation gives rise ultimately to triose phosphate, which is exported from the chloroplast via the TPT to sustain dark respiration (Stitt et al., 1985). Such a division could allow the process of starch degradation to be independently regulated according to the demand for sucrose synthesis and dark respiration. Further, export of free sugar from the chloroplast at night would minimise the requirement for ATP in the stroma to support starch degradation and would be consistent with the apparent absence of hexokinase from chloroplasts. Much further experimental work on the nature and regulation of starch degradation in the chloroplast is clearly required to allow the validity of this hypothesis to be tested. For example, as discussed above, nothing is known about regulatory properties of starch-hydrolysing enzymes that would allow their activities to be modulated to meet a demand for sucrose synthesis. The hypothesis would be invalidated if there is a significant rate of export of hexose phosphate from the chloroplast at night, or if chloroplasts contain significant activity of maltose phosphorylase (Section V.A.4, V.B), since this would allow the products of phosphorolytic degradation to be used in sucrose synthesis.

VI. Conclusions

Transitory starch is of central importance to many plants, serving as a store of carbohydrate for periods when photosynthesis is not possible. There is a reasonable understanding of the enzymes involved in the synthesis of transitory starch, although it remains unclear exactly how the various isoforms involved combine to give rise to the complex and poorly-understood structure of the starch granule. The pathway of starch degradation has yet to be firmly established. Many enzymes with potential roles in starch degradation have been identified in leaves, but in most cases there is no convincing evidence that they are involved in transitory starch degradation, or even localised in the chloroplast. Without a full understanding of the enzymic machinery that mediates starch degradation the regulation of the process can only be guessed at. It is to be hoped that substantial progress can be made on these open questions through the careful analysis of transgenic plants altered in the activities of one or more of the enzymes involved in transitory starch metabolism.

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References

- Abel GJW, Springer F, Willmitzer L and Koßmann J (1996) Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). *Plant J* 10: 981–991
- ap Rees T (1995) Where do plants make ADPglc? In: Pontis HG, Salerno GL and Echeverria EJ (eds) Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology. Am Soc Plant Physiologists Symp 14, pp 143–155. Am Soc Plant Physiologists, Baltimore
- ap Rees T and Hill SA (1994) Metabolic control analysis of plant metabolism. *Plant Cell Env* 17: 587–599
- Baba T, Nishihara M, Mizuno K, Kawasaki T, Shimada H, Kobayashi E, Ohnishi S, Tanaka K and Arai Y (1993) Identification, cDNA cloning, and gene expression of soluble starch synthase in rice (*Oryza sativa* L.) immature seeds. *Plant Physiol* 103: 565–573
- Badenhuizen NP (1969) The Biogenesis of Starch Granules in Higher Plants. Appleton-Century Crofts, New York
- Ball S, Guan H-P, James M, Myers A, Keeling P, Mouille G, Buléon A, Colonna P and Preiss J (1996) From glycogen to amylopectin: A model for the biogenesis of the starch granule. *Cell* 86: 349–352
- Ballicora MA, Laughlin MJ, Fu Y, Okita, TW, Barry GF and Preiss J (1995) Adenosine 5'-diphosphate-glucose from potato tuber. Significance of the N terminus of the small subunit for catalytic properties and heat stability. *Plant Physiol* 109: 245–251
- Barnes SA, Knight JS and Gray JC (1994) Alteration of the amount of the chloroplast phosphate translocator in transgenic tobacco affects the distribution of assimilate between starch and sugar. *Plant Physiol* 106: 1123–129
- Beck E (1985) The degradation of transitory starch granules in chloroplasts. In: Heath RL and Preiss J (eds) Regulation of Carbon Partitioning in Photosynthetic Tissue, pp 27–44. Waverley Press, Baltimore
- Beck E and Ziegler P (1989) Biosynthesis and degradation of starch in higher plants. *Ann Rev Plant Physiol Plant Mol Biol* 40: 95–117
- Beers EP, Duke SH and Henson CA (1990) Partial characterization and subcellular localisation of three α -glucosidase isoforms in pea (*Pisum sativum* L.) seedlings. *Plant Physiol* 94: 738–744
- Bhattacharyya MK, Smith AM, Ellis THN, Hedley C and Martin C (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch branching enzyme. *Cell* 60: 115–121
- Borland AM and Farrar JF (1988) Compartmentation and fluxes of carbon in leaf blades and leaf sheaths of *Poa annua* L. and *Poa x jemtlandica* (Almq.) Richt. *Plant Cell Env* 11: 535–546
- Boyer CD and Preiss J (1978a) Multiple forms of (1–4)- α -D-glucan, (1-4)-m α -D-glucan-6-glycosyl transferase from developing maize kernels. *Carbohydr Res* 61: 312–334
- Boyer CD and Preiss J (1978b) Multiple forms of starch branching enzyme of maize: Evidence for independent genetic control. *Biochem Biophys Res Commun* 80: 169–175
- Briggs DE (1967) A modified assay for α -amylase in germinating barley. *J Inst Brewing* 73: 361–370
- Brisson N, Giroux H, Zollinger M, Camirand A and Simard C (1989) Maturation and subcellular Compartmentation of potato starch phosphorylase. *Plant Cell* 1: 559–566
- Bulipin PV and ap Rees T (1978) Starch breakdown in the spadix of *Arum maculatum*. *Phytochemistry* 17: 391–396
- Burton RA, Bewley JD, Smith AM, Bhattacharyya MK, Tatge H, Ring S, Bull V, Hamilton WDO and Martin C (1995) Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. *Plant J* 7: 3–15
- Buttrose MS (1960) Submicroscopic development and structure of starch granules in cereal endosperms. *J Ultrastruct Res* 4: 231–257
- Buttrose MS (1962) The influence of environment on the shell structure of starch granules. *J Cell Biol* 14: 159–167
- Buttrose MS (1963) Electron microscopy of acid degraded starch granules. *Starch-Stärke* 15: 85–92
- Caspar T, Huber SC and Somerville CR (1986) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase. *Plant Physiol* 79: 11–17
- Caspar T, Lin T-P, Monroe J, Benbow L, Preiss J and Somerville

- CR (1991) Mutants of *Arabidopsis* with altered regulation of starch degradation. *Plant Physiol* 95: 1181–1188
- Chang CW (1979) Starch and its component ratio in developing cotton leaves. *Plant Physiol* 63: 973–977
- Chatterton NJ and Silvius JE (1980) Photosynthate partitioning into leaf starch as affected by daily photosynthetic period duration in six species. *Physiol Plant* 49: 141–144
- Copeland L and Preiss J (1981) Purification of spinach leaf ADPglucose pyrophosphorylase. *Plant Physiol* 68:996–1001
- Corbesier L, Lejeune P and Bernier G (1998) The role of carbohydrates in the induction of flowering in *Arabidopsis thaliana*—comparison between the wild type and a starchless mutant. *Planta* 206: 131–137
- Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL and Smith AM (1998) Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. *Plant Cell* 10: 413–246
- Dang PL and Boyer CD (1988) Maize leaf and kernel starch synthases and starch-branched enzymes. *Phytochemistry* 27: 1255–1259
- Dang PL and Boyer CD (1989) Comparison of soluble starch synthases and starch branching enzymes from leaves and kernels of normal and *amylose-extender* maize. *Biochem Genet* 27: 521–532
- Denyer K and Smith AM (1992) The purification and characterisation of two forms of soluble starch synthase from pea embryos. *Planta* 186: 609–617
- Denyer K, Sidebottom C, Hylton CM and Smith AM (1993) Soluble isoforms of starch synthase and starch branching enzyme also occur within starch granules in developing pea embryos. *Plant J* 4: 191–198
- Denyer K, Barber LM, Burton R, Hedley CL, Hylton CM, Johnson S, Jones DA, Marshall J, Smith AM, Tatge H, Tomlinson K and Wang TL (1995a) The isolation and characterisation of novel low-amylose mutants of *Pisum sativum*. *Plant Cell Env* 18: 1019–1026
- Denyer K, Hylton CM, Jenner CF and Smith AM (1995b) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. *Planta* 196: 256–265
- Denyer K, Clarke B, Hylton C, Tatge H and Smith AM (1996a) The elongation of amylose and amylopectin chains in isolated starch granules. *Plant J* 10: 1135–1143
- Denyer K, Dunlap F, Thørbjørnsen T, Keeling P and Smith AM (1996b) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extraplastidial. *Plant Physiol* 112: 779–785
- Denyer K, Barber LM, Edwards EA, Smith AM and Wang TL (1997) Two isoforms of the GBSSI class of granule-bound starch synthase are differentially expressed in the pea plant (*Pisum sativum* L.). *Plant Cell Env* 20: 1566–1572
- Doehlert DC and Knutson CA (1991) Two classes of debranching enzymes from developing maize kernels. *J Plant Physiol* 138: 566–572
- Doehlert DC, Kuo TM, Juvik JA, Beers EP and Duke SH (1993) Characteristics of carbohydrate metabolism in sweet corn (*sugary-1*) endosperms. *J Amer Soc Hort Sci* 188: 661–666
- Doi A, Doi K and Nikuni Z (1965) ADP-D-glucose- α -1:4-glucan α -4-glucosyltransferase in spinach chloroplasts. Partial purification and some properties of the enzyme. *Biochim Biophys Acta* 113: 312–320
- Dry I, Smith AM, Edwards EA, Bhattacharyya M, Dunn P and Martin C (1992) Characterisation of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs. *Plant J* 2: 193–202
- Duwenig E, Steup M, Willmitzer L and Kossmann J (1998) Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. *Plant J* 12: 323–333
- Edwards A, Marshall J, Sidebottom C, Visser RGF, Smith AM and Martin C (1995) Biochemical and molecular characterisation of a novel starch synthase from potato tubers. *Plant J* 8: 283–294
- Edwards A, Marshall J, Denyer K, Sidebottom C, Visser RGF, Martin C and Smith AM (1996) Evidence that a 77-kilodalton protein from the starch of pea embryos is an isoform of starch synthase that is both soluble and granule bound. *Plant Physiol* 112: 89–97
- Farrar JF and Williams ML (1991) The effects of increased atmospheric carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. *Plant Cell Env* 14: 819–830
- Fisher DK, Gao M, Kim K-N, Boyer CD and Guiltinan MJ (1996) Allelic analysis of the maize *amylose-extender* locus suggests that independent genes encode starch-branched enzymes IIa and IIb. *Plant Physiol* 110: 611–619
- Fliege R, Flügge U-I, Werdan K and Heldt HW (1978) Specific transport of inorganic phosphate, 3-phosphoglycerate and triose phosphate across the inner membrane of the envelope in spinach chloroplasts. *Biochim. Biophys Acta* 502: 232–247
- Flügge U-I and Heldt HW (1991) Metabolite translocators of the chloroplast envelope. *Ann Rev Plant Physiol Plant Mol Biol* 42: 129–144
- Fondy BR and Geiger DR (1982) Diurnal pattern of translocation and carbohydrate metabolism in source leaves of *Beta vulgaris* L. *Plant Physiol* 70: 671–676
- Fondy BR, Geiger DR and Servaites JC (1989) Photosynthesis, carbohydrate metabolism and export in *Beta vulgaris* L. and *Phaseolus vulgaris* L. during square and sinusoidal light regimes. *Plant Physiol* 89: 396–402
- Franceschi VR and Giaquinta RT (1983) The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. *Planta* 157: 422–431
- French D (1984) Organisation of starch granules. In: Whistler RL, BeMiller JN and Paschall FF (eds) *Starch: Chemistry and Technology*, pp 183–247. Academic Press, Orlando
- Fryzman RB and Cardini CE (1964) Soluble enzymes related to starch synthesis. *Biochem Biophys Res Commun* 17: 407–411
- Fryzman RB and Cardini CE (1967) Studies on the biosynthesis of starch. *J Biol Chem* 242: 312–317
- Fukui T, Nakano K, Tagaya M and Nakayama H (1987) Phosphorylase isozymes of higher plants. In: Korpela T and Christen P (eds) *Biochemistry of Vitamin B6*, pp 267–276. Birkhäuser Verlag, Basel
- Galliard T (1987) Starch: Properties and potential. John Wiley and Sons, Chichester
- Gao M, Fisher DK, Kim K-N, Shannon JC and Guiltinan MJ (1996) Evolutionary conservation and expression patterns of maize starch branching enzyme I and IIb genes suggests specialization. *Plant Mol Biol* 30: 1223–1232

- Gao M, Fisher DK, Kim K-N, Shannon JC and Guitinan MJ (1997) Independent genetic control of maize starch-branched enzymes IIa and IIb. *Plant Physiol* 114: 69–78
- Gao M, Wanat J, Stinard PS, James MG and Myers AM (1998) Characterization of *dull1*, a maize gene coding for a novel starch synthase. *Plant Cell* 10: 399–412
- Geiger DR., Shieh W-J and Yu X-M (1995) Photosynthetic carbon metabolism and translocation in wild-type and starch-deficient mutant *Nicotiana sylvestris* L. *Plant Physiol* 107: 507–514
- Gerhardt R, Stitt M and Heldt HW (1987) Subcellular metabolite levels in spinach leaves. *Plant Physiol* 83: 399–407
- Ghiena C, Schulz M and Schabl H (1993) Starch degradation and distribution of the starch degrading enzymes in *Vicia faba* leaves. *Plant Physiol* 101: 73–79
- Gordon AJ, Ryle GJA, Powell CE and Mitchell D (1980) Export, mobilisation and respiration of assimilates in uniculum barley during light and darkness. *J Exp Bot* 31: 461–473
- Guan HP and Preiss J (1993) Differentiation of the properties of the branching isozymes from maize (*Zea mays*). *Plant Physiol* 102: 1269–1273
- Guan H, Kuirki T, Sivak M and Preiss J (1995) Maize branching enzyme catalyzes synthesis of glycogen-like polysaccharide in *glgB*-deficient *Escherichia coli*. *Proc Natl Acad Sci USA* 92: 964–967
- Hanson KR and McHale NA (1988) A starchless mutant of *Nicotiana sylvestris* containing a modified plastid phosphoglucomutase. *Plant Physiol* 88: 838–844
- Harn C, Knight M, Ramakrishnan A, Guan HP, Keeling PL and Wasserman BP (1998) Isolation and characterization of the Ss2 starch synthase cDNA clones from maize endosperm. *Plant Molec Biol* 37: 629–637
- Harrison CJ, Hedley CL and Wang TL (1997) Evidence that the *rug3* locus of pea (*Pisum sativum* L.) encodes plastidial phosphoglucomutase confirms that the imported substrate for starch synthesis in pea amyloplasts is glucose 6-phosphate. *Plant J* 13: 753–762
- Häusler RE, Schlieben NH, Schulz B and Flügge UI (1998) Compensation of decreased triose phosphate/phosphate translocator activity by accelerated starch turnover and glucose transport in transgenic tobacco. *Planta* 204: 366–376
- Hawker JS, Ozburn JL, Ozaki H, Greenberg E and Preiss J (1974) Interaction of spinach leaf ADPglucose starch synthase and branching enzyme in the synthesis of branched α -glucan. *Arch Biochem Biophys* 160: 530–551
- Heineke D, Kruse A, Flügge U-I, Frommer WB, Riesmeier JW, Willmitzer L and Heldt HW (1994) Effect of antisense repression of the chloroplast translocator on photosynthesis metabolism in transgenic potato plants. *Planta* 193: 174–180
- Heldt HW, Chon CJ, Maronde D, Herold A, Stankovic ZS, Walker DA, Kraminer A, Kirk MR and Heber U (1977) Role of orthophosphate and other factors in regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol* 59: 1146–1155
- Herold A, Leegood RC, McNeil PH and Robinson SP (1981) Accumulation of maltose during photosynthesis in protoplasts isolated from spinach leaves treated with mannose. *Plant Physiol* 67: 85–88
- Hewitt JD, Casey LL and Zobel RW (1985) Effect of day length and night temperature on starch accumulation and degradation in soybean. *Ann Bot* 56: 513–522
- Hizukuri S (1986) Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydr Res* 147: 342–347
- Hovenkamp-Hermelink JHM, de Vries JN, Adamse P, Jacobsen E, Witholt B and Feenstra WJ (1988) Rapid estimation of the amylose/amylopectin ratio of small amounts of tuber and leaf tissue of the potato. *Potato Res* 31: 241–246
- Huber SC and Hanson KR (1992) Carbon partitioning and growth of a starchless mutant of *Nicotiana sylvestris*. *Plant Physiol* 99: 1449–1454
- Hylton CM, Denyer K, Keeling P, Chang M-Y and Smith AM (1996) The effect of waxy mutations on the granule-bound starch synthases of barley and maize endosperms. *Planta* 198: 230–237
- Imberty A, Buléon A, Tran V Pérez S (1991) Recent advances in knowledge of starch structure. *Starch-Stärke* 43: 375–384
- Ishizaki Y, Taniguchi H and Nakamura M (1978) A debranching enzyme of the isoamylase-type from potato (*Solanum tuberosum* L.). *Agr Biol Chem* 42: 2433–2435
- Ishizaki Y, Taniguchi H, Maruyama Y and Nakamura M (1983) Debranching enzymes of potato tubers (*Solanum tuberosum* L.) I. Purification and some properties of potato isoamylase. *Agr Biol Chem* 47: 771–779
- Jablonski LM and Geiger DR (1987) Responses of sugar beet plant morphology and carbon distribution to shortened days. *Plant Physiol Biochem* 25: 787–796
- Jacobsen E, Hovenkamp-Hermelink JHM, Krigsheld HT, Nijdam H, Pijnacker LP, Witholt B and Feenstra WJ (1989) Phenotypic and genotypic characterisation of an amylose-free starch mutant of potato. *Euphytica* 44: 43–48
- Jacobsen JV, Hanson AD and Chandler PC (1986) Water stress enhances expression of α -amylase gene in barley leaves. *Plant Physiol* 80: 350–359
- James MG, Robertson DS and Myers AM (1995) Characterization of the maize gene *sugary 1*: A determinant of starch composition in kernels. *Plant Cell* 7: 417–429
- Jenkins PJ, Cameron RE and Donald AM (1993) A universal feature in the structure of starch granules from different botanical sources. *Starch-Stärke* 45: 417–420
- Kacser H and Burns JA (1973) The control of flux. *Symp Soc Exp Biol* 27: 65–104
- Kacser H, Burns JA and Fell DA (1995) The control of flux. *Biochem Soc Trans* 23: 341–366
- Kakefuda G and Preiss J (1997) Partial purification and characterization of a diurnally fluctuating novel endoamylase from *Arabidopsis thaliana* leaves. *Plant Physiol Biochem* 35: 907–913
- Kakefuda G, Duke SH and Hostak MS (1986) Chloroplast and extrachloroplastic starch-degrading enzymes in *Pisum sativum*. *Planta* 168: 175–182
- Kalt-Torres W and Huber SC (1987) Diurnal changes in maize leaf photosynthesis. *Plant Physiol* 83: 294–298
- Kleczkowski LA, Villand P, Preiss J and Olsen O-A (1993) Kinetic mechanism and regulation of ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) leaves. *J Biol Chem* 268: 6228–6233
- Knight ME, Harn C, Lilley CE, Guan HP, Singletary GW, Mu-Foster C, Wasserman BP and Keeling PL (1998) Molecular cloning of starch synthase I from maize (W64) endosperm and expression in *Escherichia coli*. *Plant J* 14: 613–622
- Krishnan HB, Reeves CD and Okita TW (1986) ADPglucose

- pyrophosphorylase is encoded by different mRNA transcripts in leaf and endosperm of cereals. *Plant Physiol.* 81: 642–645
- Kruckeberg AL., Neuhaus HE, Feil R, Gottlieb LD and Stitt M (1989) Decreased-activity mutants of phosphoglucose isomerase in the cytosol and chloroplast of *Clarkia xantiana*. *Biochem J* 261: 457–467
- Kruger NJ and ap Rees T (1983a) Maltose metabolism by pea chloroplasts. *Planta* 158: 179–184
- Kruger NJ and ap Rees T (1983b) Properties of α -glucan phosphorylase from pea chloroplasts. *Phytochemistry* 22: 1891–1898
- Kruger NJ, Bulpin PV and ap Rees T (1983) The extent of starch degradation in the light in pea leaves. *Planta* 157: 271–273
- Kühn C, Quick WP, Schulz A, Riesmeier JW, Sonnewald U and Frommer WB (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Env* 19: 1115–1123
- LaCognata U, Willmitzer L and Müller-Röber B (1995) Molecular cloning and characterization of novel isoforms of potato ADP-glucose pyrophosphorylase. *Mol Gen Genet* 246: 538–548
- Leidreiter K, Heineke D, Heldt HW, Müller-Röber B, Sonnewald U and Willmitzer L (1995) Leaf-specific antisense inhibition of starch biosynthesis in transgenic potato plants leads to an increase in photoassimilate export from source leaves during the light period. *Plant Cell Physiol* 36: 615–624
- Li B, Servaites JC and Geiger DR (1992a) Characterization and subcellular localization of debranching enzyme and endo-amylase from leaves of sugar beet. *Plant Physiol* 98: 1277–1284
- Li B, Geiger DR and Shieh W-J (1992b) Evidence for circadian regulation of starch and sucrose synthesis in sugar beet leaves. *Plant Physiol* 99: 1393–1399
- Lin T-P and Preiss J (1988) Characterisation of D-enzyme (4-alpha glucanotransferase) in *Arabidopsis* leaf. *Plant Physiol* 86: 260–265
- Lin T-P, Spilatro SR and Preiss J (1988a) Subcellular localization and characterization of amylases in *Arabidopsis* leaf. *Plant Physiol* 86: 251–259
- Lin T-P, Caspar T, Somerville C and Preiss J (1988b) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADPglucose pyrophosphorylase activity. *Plant Physiol* 86: 1131–1135
- Lin T-P, Caspar T, Somerville C and Preiss J (1988c) A starch deficient mutant of *Arabidopsis thaliana* with low ADPglucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. *Plant Physiol* 88: 1175–1181
- Lorbeth R, Ritte G, Willmitzer L and Kossman J (1998) Inhibition of a starch-granule bound protein leads to modified starch and repression of cold sweetening. *Nature Biotech* 6: 473–477
- Lorenzen JH and Ewing EE (1992) Starch accumulation in leaves of potato (*Solanum tuberosum* L.) during the first 18 days of photoperiod treatment. *Ann Bot* 69: 481–485
- Ludewig F, Sonnewald U, Kauder F, Heineke D, Geiger M, Stitt M, Müller-Röber B, Gillissen B, Kuhn C and Frommer WB (1998) The role of transient starch in acclimation to elevated atmospheric CO₂. *FEBS Lett* 429: 147–151
- Ludwig I, Ziegler P and Beck E (1984) Purification and properties of spinach leaf debranching enzyme. *Plant Physiol* 74: 856–861
- Manners, DJ and Rowe KL (1969) Studies on carbohydrate-metabolising enzymes. *Carbohydr. Res.* 9: 107–121
- Marshall J, Sidebottom C, Debet M, Martin C, Smith AM and Edwards A (1996) Identification of the major starch synthase in the soluble fraction of potato tubers. *Plant Cell* 8: 1121–1135
- Martin C and Smith AM (1995) Starch biosynthesis. *Plant Cell* 7: 971–985
- Matheson NK (1996) The chemical structure of amylose and amylopectin fractions of starch from tobacco leaves during development and diurnally-nocturnally. *Carbohydr Res* 282: 247–262
- Matheson NK and Wheatley JM (1962) Starch changes in developing and senescing tobacco leaves. *Aust J Biol Sci* 15: 445–458
- Matheson NK and Wheatley JM (1963) Diurnal-nocturnal changes in the starch of tobacco leaves. *Aust J Biol Sci* 16: 70–76
- Mizuno K, Kimura K, Arai Y, Kawasaki T and Shimada H (1992) Starch branching enzyme from immature rice seeds. *J Biochem* 112: 643–651
- Mizuno K, Kawasaki T, Shimada H, Satoh H, Kobayashi E, Okumura S, Arai Y and Baba T (1993) Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. *J Biol Chem* 268: 19084–19091
- Monroe JD, Salminen MD and Preiss J (1991) Nucleotide sequence of a cDNA clone encoding a beta-amylase from *Arabidopsis thaliana*. *Plant Physiol* 97: 1599–1601
- Morell MK, Bloom M and Preiss J (1988) Affinity labelling of the allosteric activator site(s) of spinach leaf ADP-glucose pyrophosphorylase. *J Biol Chem* 263: 633–637
- Morell MK, Blennow A, Kosar-Hashemi B and Samuel MS (1997) Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. *Plant Physiol* 113: 201–208
- Mori H, Tanizawa K and Fukui T (1991) Potato tuber type H phosphorylase isozyme: molecular cloning, nucleotide sequence and expression of a full-length complementary DNA in *Escherichia coli*. *J Biol Chem* 266: 18446–18453
- Mouille G, Maddelein M-L, Libessart N, Tagala P, Decq A, Delrue B and Ball S (1996) Pre-amylopectin processing: A mandatory step for starch biosynthesis in plants. *Plant Cell* 8: 1353–1366
- Mullen JA and Koller HR (1988a) Trends in carbohydrate depletion, respiratory carbon loss, and assimilate export from soybean leaves at night. *Plant Physiol* 86: 517–521
- Mullen JA and Koller HR (1988b) Daytime and nighttime carbon balance and assimilate export in soybean leaves at different photon flux densities. *Plant Physiol* 86: 880–884
- Müller-Röber B, Kossman J, Hannah LC, Willmitzer L and Sonnewald U (1990) Only one of two different ADPglucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. *Mol Gen Genet* 224: 136–146
- Müller-Röber B, La Cognata U, Sonnewald U and Willmitzer U (1994) A truncated version of an ADP-glucose pyrophosphorylase promoter from potato specifies guard cell-selective expression in transgenic plants. *Plant Cell* 6: 601–612
- Nakamura Y (1996) Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. *Plant Sci* 121: 1–18
- Nakamura Y, Umemoto T, Ogata N, Kuboki Y, Yano M and Sasaki T (1996) Starch debranching enzyme (R-enzyme or pullulanase) from developing rice endosperm: Purification,

- cDNA and chromosomal localization of the gene. *Planta* 199: 209–218
- Nakano K, Mori H and Fukui T (1989) Molecular cloning of cDNA encoding potato amyloplast α -glucan phosphorylase and the structure of its transit peptide. *J Biochem* 106: 691–695
- Nakata PA and Okita TW (1995) Differential regulation of ADP-glucose pyrophosphorylase in the sink and source tissues of potato. *Plant Physiol* 108: 361–368
- Neuhaus HE and Stitt M (1990) Control analysis of photosynthate partitioning—impact of reduced activity of ADPglucose pyrophosphorylase or plastid phosphoglucomutase on the fluxes to starch and sucrose in *Arabidopsis thaliana* (L.) Heynh. *Planta* 182: 445–454
- Neuhaus HE, Kruckeberg AL, Feil R and Stitt M (1989) Reduced activity mutants of phosphoglucose isomerase in the cytosol and chloroplast of *Clarkia xantiana*. II Study of the mechanisms which regulate photosynthate partitioning. *Planta* 178: 110–122
- Okita TW, Greenberg E, Kuhn DN and Preiss J (1979) Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaves. *Plant Physiol* 64: 187–192
- Olive MR, Ellis RJ and Schuch WW (1989) Isolation and nucleotide sequences of cDNA clones encoding ADPglucose pyrophosphorylase polypeptides from wheat leaf and endosperm. *Plant Mol Biol* 12: 525–538
- Ozbun JL, Hawker JS and Preiss J (1971) Multiple forms of α -1:4 glucan synthetase from spinach leaves. *Biochem Biophys Res Commun* 43: 631–636
- Ozbun JL, Hawker JS and Preiss J (1972) Soluble adenosine diphosphate glucose- α 1:4-glucan α 1:4-glucosyl transferases from spinach leaves. *Biochem J* 126: 953–963
- Pan D and Nelson OE (1984) A debranching enzyme deficiency in endosperms of the *sugary-1* mutants of maize. *Plant Physiol* 74: 324–328
- Paul MJ and Stitt M (1993) Effects of nitrogen and phosphorous deficiencies on levels of carbohydrates, respiratory enzymes and metabolites in seedlings of tobacco and their response to exogenous sucrose. *Plant Cell Env* 16: 1047–1057
- Pongratz P and Beck E (1978) Diurnal oscillations of amylolytic activity in spinach chloroplasts. *Plant Physiol* 62: 687–689
- Preiss J (1982) Regulation of the biosynthesis and degradation of starch. *Ann Rev Plant Physiol* 33: 431–448
- Preiss J and Sivak M (1996) Starch synthesis in sinks and sources. In: Zamski E and Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops*, pp 63–94. Marcel Dekker, New York
- Prioul J-L, Jeannette E, Reyss A, Grégoire N, Giroux M, Hannah LC and Causse M (1994) Expression of ADP-glucose pyrophosphorylase in maize (*Zea mays* L.) grain and source leaf during grain filling. *Plant Physiol* 104: 179–187
- Quick WP, Scheibe R and Neuhaus HE (1995) Induction of hexose-phosphate translocator activity in spinach chloroplasts. *Plant Physiol* 109: 113–121
- Radwan MA and Stocking CR (1957) The isolation and characterization of sunflower leaf starch. *Amer. J Bot.* 44: 682–686
- Riesmeier JW, Flügge U-I, Schulz B, Heineke D, Heldt HW, Willmitzer L and Frommer WB (1993) Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants. *Proc Natl Acad Sci USA* 90: 6160–6164
- Robinson SP and Walker DA (1979) The control of 3-phosphoglycerate reduction in isolated chloroplasts by the concentrations of ATP, ADP and 3-phosphoglycerate. *Biochim Biophys Acta* 545: 528–536
- Rost S, Frank C and Beck E (1996) The chloroplast envelope is permeable for maltose but not for maltodextrins. *Biochim Biophys Acta* 1291: 221–227
- Rufy TW, Huber SC and Volk RJ (1988) Alterations in leaf carbohydrate metabolism in response to nitrogen stress. *Plant Physiol* 88: 725–730
- Sanwal GG, Greenberg E, Hardie J, Cameron EC and Preiss J (1968) Regulation of starch biosynthesis in plant leaves: Activation and inhibition of ADPglucose pyrophosphorylase. *Plant Physiol* 43: 417–427
- Schäfer G, Heber U and Heldt HW (1977) Glucose transport into spinach chloroplasts. *Plant Physiol* 60: 286–289
- Schnarrenberger C (1990) Characterization and compartmentation in green leaves of hexokinases with different specificities for glucose, fructose and mannose and for nucleoside triphosphates. *Planta* 181: 249–255
- Scholes JD and Farrar JF (1987) Development of symptoms of brown rust of barley in relation to the distribution of fungal mycelium, starch accumulation and localized changes in the concentration of chlorophyll. *New Phytol.* 107: 103–117
- Schulze W, Stitt M, Schulze E-D, Neuhaus HE and Fichtner K (1991) A quantification of the significance of assimilatory starch for growth of *Arabidopsis thaliana* L. Heynh. *Plant Physiol* 95: 890–895
- Scott P and Kruger NJ (1994) Fructose-2,6-bisphosphate levels in mature leaves of tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*). *Planta* 193: 16–20
- Scott P and Kruger NJ (1995) Influence of elevated fructose-2,6-bisphosphate levels on starch mobilisation in transgenic tobacco leaves in the dark. *Plant Physiol* 108: 1569–1577
- Scott P, Lange AJ, Pilkis SJ and Kruger NJ (1995) Carbon metabolism in leaves of transgenic tobacco (*Nicotiana tabacum* L.) containing elevated fructose-2,6-bisphosphate levels. *Plant J* 7: 461–469
- Servaites JC, Geiger DR, Tucci MA and Fondy BR (1989) Leaf carbon metabolism and metabolite levels during a period of sinusoidal light. *Plant Physiol* 89: 403–408
- Shannon JC, Pien F-M, Cao H and Liu K-C (1998) Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperms. *Plant Physiol* 117, 1235–1252
- Sharkey TD and Vanderveer PJ (1989) Stromal phosphate concentration is low during feedback limited photosynthesis. *Plant Physiol* 91: 679–684
- Sharkey TD, Berry JA and Raschke K (1985) Starch and sucrose synthesis in *Phaseolus vulgaris* as affected by light, CO_2 , and abscisic acid. *Plant Physiol* 77: 617–620
- Sharkey TD, Savitch LV, Vanderveer PJ and Micallef BJ (1992) Carbon partitioning in a *Flaveria linearis* mutant with reduced cytosolic fructose bisphosphatase. *Plant Physiol* 100: 210–215
- Shimomura S, Nagai M and Fukui T (1982) Comparative glucan specificities of two types of spinach leaf phosphorylase. *J Biochem* 91: 703–717
- Singletary GW, Banisadr R and Keeling P (1997) Influence of gene dosage on carbohydrate synthesis and enzyme activities in endosperm of starch-deficient mutants of maize. *Plant Physiol* 113: 293–204

- Smith AM (1988) Major differences in isoforms of starch-branched enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.). *Planta* 175: 270–279
- Smith AM and Martin C (1993) Starch biosynthesis and the potential for its manipulation. In: Grierson D (ed) *Biosynthesis and manipulation of plant products*, pp 1–54. Blackie Academic Press, Glasgow
- Smith AM, Neuhaus HE and Stitt M (1990) The impact of decreased activity of starch-branched enzyme on photosynthetic starch synthesis in leaves of wrinkled-seeded peas. *Planta* 181: 310–315
- Smith AM, Denyer K and Martin C (1997) The synthesis of the starch granule. *Ann Rev Plant Physiol Plant Mol Biol* 48: 65–87
- Smith-White BJ and Preiss J (1992) Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. *J Mol Evol* 34: 449–464
- Sonnewald U, Basner A, Greve B and Steup M (1995) A second L-type isozyme of potato glucan phosphorylase: cloning, antisense inhibition, and expression analysis. *Plant Mol Biol*, 27: 567–576
- Steup M (1988) Starch degradation. In: Preiss J (ed) *The Biochemistry of Plants*, Vol 14, pp 255–296. Academic Press, London, UK
- Steup M (1990) Starch degrading enzymes. In: Lea PJ (ed) *Methods in Plant Biochemistry*, Vol 3, pp 103–128. Academic Press, London
- Steup M and Latzko E (1979) Intracellular localisation of phosphorylases in spinach and pea leaves. *Planta*, 145: 69–75
- Steup M and Schächtele C (1981) Mode of glucan degradation by purified phosphorylase forms from spinach leaves. *Planta* 153: 351–361
- Steup M, Robenek H and Melkonian M (1983) In vitro degradation of starch granules isolated from spinach chloroplasts. *Planta* 158: 428–436
- Stitt M (1984) Degradation of starch in chloroplasts: A buffer of sucrose metabolism. In: Lewis DH (ed) *Storage Carbohydrates in Vascular Plants*, pp 205–229. Cambridge University Press, Cambridge
- Stitt M (1990) Fructose-2,6-bisphosphate in plants. *Ann Rev Plant Physiol Mol Biol* 41: 153–185
- Stitt M and ap Rees T (1980) Carbohydrate breakdown by chloroplasts of *Pisum sativum*. *Biochim. Biophys Acta* 627: 131–143
- Stitt M and Heldt HW (1981) Physiological rates of starch breakdown in isolated chloroplasts. *Plant Physiol* 68: 755–761
- Stitt M and Steup M (1985) Starch and sucrose degradation. In: Douce R and Day DA (eds) *Encyclopaedia of Plant Physiol*, Vol. 18, pp 347–390. Springer-Verlag, Berlin
- Stitt M and Quick WP (1989) Photosynthetic carbon partitioning: Its regulation and possibilities for manipulation. *Physiol Plant.* 77: 633–641
- Stitt M, Bulpin PV and ap Rees T (1978) Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. *Biochim. Biophys Acta* 544: 200–214
- Stitt M, Kurzel B and Heldt HW (1984) Control of photosynthetic sucrose synthesis by fructose-2,6-bisphosphate. II. Partitioning between sucrose and starch. *Plant Physiol* 75: 554–560
- Stitt M, Wirtz W, Gerhardt R, Heldt HW, Spencer C, Walker DA and Foyer C (1985) A comparative study of metabolite levels in plant leaf material in the dark. *Planta* 166: 354–364
- Sun Z, Duke SH and Henson CA (1995) The role of pea chloroplast α -glucosidase in transitory starch degradation. *Plant Physiol* 108: 211–217
- Tacke M, Yang Y and Steup M (1991) Multiplicity of soluble glucan-synthase activity in spinach leaves: Enzyme pattern and intracellular location. *Planta* 185: 220–226
- Taira T, Vermatsu M, Nakano Y and Morikawa T (1991) Molecular comparison and identification of the starch synthase bound to starch granules between endosperm and leafblades in rice plants. *Biochem Genet* 29: 301–311
- Takahashi T, Yanase M, Otkada S and Smith SM (1993) Disproportionating enzyme 4-alpha glucanotransferase (EC 2.4.1.25) of potato: purification, molecular cloning, and potential role in starch metabolism. *J Biol Chem* 268: 1391–1396
- Takahashi T, Yanase M, Takata H, Okada S and Smith SM (1996) Potato D-enzyme catalyzes the cyclization of amylose to produce cycloamylose, a novel cyclic glucan. *J Biol Chem* 271: 2902–2908
- Takahashi T, Critchley J, Okada S and Smith SM (1998) Normal starch content and composition in tubers of antisense potato plants lacking D-enzyme (4-a-glucanotransferase). *Planta* 205: 445–451
- Takeda Y, Guan H-P and Preiss J (1993) Branching of amylose by the branching isoenzymes of maize endosperm. *Carbohydr Res* 240: 253–263
- Técső LI, Wang D, Smith AM, Leegood RC and Maule AJ (1992) Red clover mottle virus infection affects sink-source relationships and starch accumulation in pea plants. *J Exp Bot* 43: 1409–1412
- Técső LI, Maule AJ, Smith AM and Leegood RC (1994) Complex localized changes in CO_2 assimilation and starch content associated with the susceptible interaction between cucumber mosaic virus and a cucurbit host. *Plant J* 5: 837–847
- Thorbjørnsen T, Villand P, Denyer K, Olsen O-A and Smith AM (1996) Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J* 10: 243–250
- Tomlinson KL (1996) Starch synthesis in leaves of pea (*Pisum sativum* L.) Ph.D Thesis, University of East Anglia
- Tomlinson KL, Lloyd JR and Smith AM (1997) Importance of isoforms of starch-branched enzyme in determining the structure of starch in pea leaves. *Plant J* 11: 31–43
- Tomlinson KL, Craig J and Smith AM (1998) Major differences in isoform composition of starch synthase between leaves and embryos of pea (*Pisum sativum* L.). *Planta* 204: 109–119
- Trethewey RN and ap Rees T (1994a) A mutant of *Arabidopsis thaliana* lacking the ability to transport glucose across the chloroplast envelope. *Biochem J* 301: 449–454
- Trethewey RN and ap Rees T (1994b) The role of the hexose transporter in the chloroplasts of *Arabidopsis thaliana* L. *Planta* 195: 168–174
- Usuda H, Kalt-Torres W, Kerr PS and Huber SC (1987) Diurnal changes in maize leaf photosynthesis. *Plant Physiol* 83: 289–293
- Vally KJM and Sharma R (1995) Light-induced chloroplast α -amylase in pearl millet (*Pennisetum americanum*). *Plant Physiol* 107: 401–405
- von Schaewen A, Stitt M, Schmidt R, Sonnewald U and Willmitzer L (1990) Expression of a yeast-derived invertase in the cell

- wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J* 9: 3033–3044
- Waigh TA (1997) The structure and side-chain liquid-crystalline polymeric properties of starch. PhD Thesis, University of Cambridge
- Walker DA and Sivak MN (1986) Photosynthesis and phosphate: A cellular affair? *Trends Biol Sci* 11: 176–179
- Wang Q, Monroe J and Sjolund D (1995) Identification and characterization of a phloem-specific beta-amylase. *Plant Physiol* 109: 743–750
- Weber H, Heim U, Borisjuk L and Wobus U (1995) Cell-type specific, coordinate expression of two ADP-glucose pyrophosphorylase genes in relation to starch biosynthesis during seed development of *Vicia faba* L. *Planta* 195: 352–361
- Weier E (1936) The structure of the chloroplast of *Pellionia pulchra*. *Cytologia* 7: 504–509
- Weiner H, Stitt M and Heldt HW (1987) Subcellular compartmentation of pyrophosphate and alkaline pyrophosphatase in leaves. *Biochim Biophys Acta* 893: 13–21
- Williams ML, Farrar JF and Pollock CJ (1989) Cell specialization within the parenchymatous bundle sheath of barley. *Plant Cell Env* 12: 909–918
- Wirtz W, Stitt M and Heldt HW (1980) Enzymic determination of metabolites in the subcellular compartments of spinach protoplasts. *Plant Physiol* 66: 187–193
- Witt W and Sauter JJ (1995) In vitro degradation of starch grains by phosphorylases and amylases from poplar wood. *J Plant Physiol* 146: 35–40
- Witt W and Sauter JJ (1996) Purification and characterization of alpha-amylase from poplar leaves. *Phytochemistry* 41: 365–372
- Yamanouchi H and Nakamura Y (1992) Organ specificity of isoforms of starch-branching enzyme (Q enzyme) in rice. *Plant Cell Physiol* 33: 985–991
- Zeeman S, Umemoto T, Lue WL, Au-Yeung P, Martin C, Smith AM and Chen J (1998a) A mutant of *Arabidopsis thaliana* lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell* 10: 1699–1711
- Zeeman S, Northrop F, Smith AM and ap Rees T (1998b) A starch-accumulating mutant of *Arabidopsis thaliana* deficient in a starch-hydrolysing enzyme. *Plant J* 15: 357–365
- Ziegler P and Beck E (1986) Exoamylase activity in vacuoles isolated from pea and wheat leaf protoplasts. *Plant Physiol* 82: 1119–1121
- Zhu ZP, Hylton CM, Roessner U and Smith AM (1998) Characterisation of starch-debranching enzymes in pea embryos. *Plant Physiol* 118: 581–590
- Zrenner R, Krause KP, Apel P and Sonnewald U (1996) Reduction of the cytosolic fructose-1,6-bisphosphatase in transgenic potato plants limits photosynthetic sucrose biosynthesis with no impact on tuber growth and plant yield. *Plant J* 9: 671–681

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Chapter 10

Control of Photosynthesis, Allocation and Partitioning by Sugar Regulated Gene Expression

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Summary

A variety of genes, whose products are involved in diverse metabolic pathways and cellular functions, are either induced or repressed depending on the availability of soluble sugars in higher plant cells. This metabolic regulation of gene expression can be viewed at two levels. Firstly, at the cellular level, changes in gene expression result in a modification of anabolic or catabolic processes in response to nutrient availability. This mechanism is important in the control of primary carbon metabolism and nutrient homeostasis within the cell. For example, in mesophyll cells when soluble sugar levels are high there is feedback inhibition on photosynthetic gene expression which can actually override the well documented induction effect of light. At the other extreme, catabolic processes are induced at the level of gene expression when soluble sugar levels are depleted in whole leaves or mesophyll protoplasts. Secondly, when viewed at the whole plant level it becomes apparent that sugar regulation of gene expression plays an important role in source-sink interactions. Productivity in source tissues needs to match demand from sink tissues. Sugars play an integral role in achieving this as they act as signals both for repression of genes coding for photosynthetic enzymes and for induction of genes coding for sink-specific enzymes, such as sucrose synthase, extracellular invertase and granule bound starch synthase.

Since changes in the intracellular levels of carbohydrates result in changes in expression of a variety of genes, then any factor, either local or remote, that affects carbohydrate levels will affect gene expression. Such factors include environmental conditions such as light, temperature, availability of macronutrients and developmental

stage of the plant. In this chapter we will discuss the variety of factors that bring about changes in carbohydrate status in photosynthetic cells and the effect these changes have on gene expression. We will then draw upon the extensive literature in metabolic regulation of gene expression in prokaryotes and lower eukaryotes in order to highlight possible mechanisms that may be involved in the sugar regulated gene expression in plant cells.

I. Introduction

Plant tissues can be divided into those that export (source) and those that import (sink) carbon and nitrogen (Turgeon, 1989). Mature green leaves, seed storage tissue during early post-germinative growth and sprouting tubers are examples of source tissues. Sink tissue can be sub-divided into storage sinks, such as developing seeds and tubers which accumulate various proportions of carbohydrate, proteins and lipids; and metabolic sinks such as meristems and roots which accumulate little or no storage material, but rely on net import of metabolites for growth and development. However, this classification into sink and source organs is not static and depends on genetically fixed developmental processes such as the transition of developing seeds and young leaves from sink to source organs during maturation. It also depends on changes in environmental conditions. The ability of a plant to respond to environmental changes is necessary in order to maximize growth, biomass and reproductive capacity when essential resources such as light, CO_2 , water and nitrogen are abundant and to compensate when there is a deficit in one or more of these resources. Coordinated development and interaction of source and sink organs is critical in order to achieve this. One of the key processes that needs to be coordinated between source and sink tissue is the photosynthetic production of carbohydrates with their mobilization, allocation and utilization. This acclimation (maintenance of the supply and demand for photosynthetic assimilates in appropriate balance) in response to fluctuating exogenous factors is achieved by short-term

modulation of enzyme activities and other proteins along with more long-term changes in expression of genes whose products are involved in the production, mobilization, allocation and utilization of photo-assimilates. These genes are controlled by a variety of factors including developmental, hormonal and environmental signals as well as by carbohydrates, either directly or indirectly, since the products of these genes ultimately modify carbohydrate levels.

II. Carbohydrate Regulation of Gene Expression in Source and Sink Tissues

Photosynthetic gene expression is subject to feedback control by metabolites that signal carbohydrate status of photosynthetic cells. To appreciate this control at the whole plant level it is essential first to consider the factors that regulate carbohydrate status in photosynthetic tissues. Carbohydrate status will be a function of the net production of carbohydrate and the partitioning of assimilated carbon to sink tissues. Therefore, any factor which results in a change in either of these parameters will change the carbohydrate status and so effect a change in photosynthetic gene expression. For example, light intensity, temperature and CO_2 availability will directly affect the rate of photosynthesis and so the production of photoassimilates. Availability of essential nutrients such as nitrate or phosphate, will affect the demand for carbon-skeletons for biosynthesis in both source and sink tissues (Paul and Stitt, 1993).

A. Carbohydrate Control of Gene Expression in Source Tissues

A number of model systems have been used to demonstrate that elevated levels of soluble sugars result in repression of photosynthetic gene expression via a feed-back control mechanism. In several cases this control has been demonstrated to occur at the level of transcription. The first example of this type of control came from work using a maize mesophyll protoplast transient expression system in which it was demonstrated that transcription from seven maize

Abbreviations: ADP – adenosine diphosphate; AGP-S – ADP-glucose pyrophosphorylase; AMP – adenosine monophosphate; AMPK – AMP-activated protein kinase; ATP – adenosine triphosphate; CAB – chlorophyl *a/b* binding protein; cAMP – cyclic adenosine monophosphate; CaMV35S-cauliflower mosaic virus 35S promoter; Cra – catabolite repressor/activator; CRP – cAMP receptor protein; FBP – fructose 1,6-bisphosphate; F1P – fructose 1-phosphate; HXK – hexokinase; PC – plastocyanin; PEP – phosphoenolpyruvate; PTS – phosphotransferase system; RBCS – ribulose bisphosphate carboxylase small subunit; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; SNF1 – sucrose non-fermenting protein kinase; VSP – vegetative storage protein

photosynthetic gene promoters was repressed by sugars or acetate whereas other promoters (e.g. CaMV35S) were not significantly affected (Sheen, 1990). In *Arabidopsis*, the light induction of the ribulose bisphosphate carboxylase small subunit gene (*RBCS*) in dark-adapted seedlings is inhibited by growth on 2% sucrose or glucose (Cheng et al., 1992). In rapeseed cell culture 2% sucrose inhibits the light dependent accumulation of the chlorophyll *a/b* binding protein (CAB) (Harter et al., 1993). Moreover sucrose is also able to repress the early, light independent activation of a plastocyanin gene during early *Arabidopsis* seedling development (Dijkwel et al., 1996). Sugar repression of photosynthetic genes has also been demonstrated in a variety of other experimental systems including transgenic tobacco expressing invertase in the apoplast resulting in elevated levels of intracellular sugars, a *Chenopodium* cell culture, and in spinach plants that had been cold-girdled to decrease export of photoassimilates from leaves (von Schaewen et al., 1990; Krapp et al., 1993; Krapp and Stitt, 1995). This work has led to the conclusion that repression of photosynthetic genes by high-carbohydrate-associated metabolic factors represents a basic genetic mechanism (Krapp et al., 1993; Krapp and Stitt, 1995). It should be noted that genes encoding other enzymes including the plastid isoforms of aldolase, triose-phosphate isomerase and phosphoglucomutase, that have a dual role in photosynthesis and respiratory metabolism, are not subject to carbohydrate repression (Krapp and Stitt, 1994) and activities of glycolytic enzymes remain unaltered or increase (Stitt et al., 1991; Krapp and Stitt, 1994).

B. Influence of Carbohydrate Control in Sink Tissues on Gene Expression in Source Tissues

Carbon partitioning to sink tissues depends on the plants developmental program which largely dictates the temporal and spatial distribution of storage and metabolic sinks. Superimposed on this are fluctuations in partitioning in response to changing environmental conditions since many forms of environmental stress can restrict availability of essential resources. In general, genes involved in allocation and accumulation of storage reserves show increased expression when carbohydrate levels are high. Expression of these genes decreases when carbohydrate levels decrease.

One of the key mechanisms regulating carbon partitioning to sink tissue appears to be the metabolic regulation of two families of genes encoding enzymes responsible for the initial metabolism of sucrose (Geiger et al., 1996; Koch et al., 1996). Sucrose is the major form of carbohydrate that is transported in many plants. The genes encoding the two enzymes known to be responsible for sucrose cleavage in the phloem and importing tissues of plant cells, namely sucrose synthase and invertase, are themselves regulated by carbohydrate status (Koch et al., 1992; Xu et al., 1996). Both of these enzymes are encoded by a gene family in maize and in both families one class of genes is up-regulated by elevated sugar levels (*SUCROSE SYNTHASE1* and *INVERTASE2*), whereas a second class of genes in the same family is repressed by sugars and upregulated when sugar levels fall (*SHRUNKEN1* and *INVERTASE1*). Interestingly, the two classes of genes from each of these families are also differentially expressed during development. Those genes that are induced by sugars are expressed in sugar-importing tissues and those that are repressed by sugars are induced during reproductive development. Carbohydrate regulation of sucrose synthase and invertase genes from various other species including potato (Salanoubat and Belliard, 1989), rice (Karrer and Rodriguez, 1992), bean (Heim et al., 1993), *Chenopodium rubrum* (Godt et al., 1995; Roitsch et al., 1995) and *Arabidopsis* (Martin et al., 1993) have also been reported. The metabolic regulation of sucrose synthase and invertase genes therefore provides a possible mechanism for the regulation of import of carbon into sink tissues. This regulation in itself will effect changes in the carbohydrate status in photosynthetic tissues and in so doing ultimately give rise to changes in photosynthetic gene expression.

Expression of extracellular invertase following wounding or bacterial infection is correlated with repression of photosynthetic gene expression (Sturm and Chripeels, 1990). It has been postulated that the resulting increased hydrolysis of apoplastic sucrose to glucose and fructose may play an important role in coordinating the plants defense response by inducing certain genes encoding for proteins such as proteinase inhibitor II and chalcone synthase, and repressing others such as those involved in photosynthesis (Jang and Sheen, 1994).

The majority of sink genes that respond to sugar related signals are also regulated by a number of

other mechanisms including developmental, stress or environmental signals. This leads to a complex regulatory network in which specific genes are responding to a variety of signals. Further details of metabolic regulation of gene expression in higher plants can be found in recent reviews (Graham, 1996; Koch, 1996; Smeekens and Rook, 1997) and more specifically carbohydrate control of photosynthetic gene expression has also been reviewed (Sheen, 1994; Stitt et al., 1995).

III. Influence of Other Metabolites on Sugar Regulated Genes

Photosynthetic acclimation is suggested to be caused by accumulation of carbohydrates in the source leaves resulting in feedback inhibition of photosynthesis (Azcon-Bieto, 1983; Blechschmidt-Schneider et al., 1989). Therefore, any developmental or environmental factor which results in increased levels of carbohydrate in source tissues could cause a repression of photosynthesis.

Photosynthetic acclimation has been observed in some instances when plants are grown at elevated CO_2 (Stitt et al., 1991; Bowes, 1994; Van Oosten and Besford, 1996). Acclimation of tomato plants to elevated levels of CO_2 in which an initial increase in photosynthesis is followed by a long term decrease has recently been linked to sugar repression of photosynthesis (Van Oosten and Besford, 1994). Exposure of tomato plants to either elevated CO_2 or sucrose resulted in decreased levels of nuclear encoded mRNA for photosynthetic enzymes, whereas repression of chloroplast genes was less pronounced (Van Oosten et al., 1994; Van Oosten and Besford, 1996). The similar effects of elevated CO_2 and sugars has led to the proposal that the initial increase in photosynthesis after exposure to elevated CO_2 results in accumulation of sugars (if sink strength is insufficient) and this, in turn, results in repression of nuclear encoded photosynthetic genes (Van Oosten and Besford, 1994, 1996).

Deficiency of nitrogen or phosphorous results in accumulation of carbohydrates in mature leaves and roots within hours after withdrawal of these nutrients (Thorsteinsson and Tillberg, 1990; Henry and Raper, 1991). This implies that nitrogen or phosphorous deficiency limits utilization of carbohydrate more than it limits photosynthesis. In nitrogen-deficient shoots and roots of tobacco seedlings, levels of

hexose phosphates and 3-phosphoglyceric acid are elevated and upon feeding of exogenous sucrose, protein, Rubisco and chlorophyll content is decreased in shoots as nitrogen is recycled (Paul and Stitt, 1993).

Transcripts encoding enzymes involved in nitrate assimilation and starch synthesis increase in response to elevated levels of soluble sugars in photosynthetic tissues. Sucrose can substitute for light in eliciting an increase in steady state levels of nitrate reductase mRNA in dark-adapted green *Arabidopsis* plants (Cheng et al., 1992). In the same experiment, sucrose was shown to override the light induction of the *RBCS* gene. Both light and exogenous sucrose repress accumulation of asparagine synthetase and increase nitrate reductase mRNA levels in *Arabidopsis* (Lam et al., 1994). The partial elimination of sucrose repression of asparagine synthetase expression by amino acids (Lam et al., 1994) suggests that nitrogen:carbon ratio rather than carbon alone is responsible for the metabolic regulation of at least this carbohydrate regulated gene. Control of gene expression by metabolites may therefore regulate major fluxes of carbon and nitrogen in plants.

Allosteric regulation of the starch biosynthetic enzyme ADP-glucose pyrophosphorylase by phosphate and 3-phosphoglyceric acid is a well established mechanism for regulation of starch biosynthesis. An additional level of control appears to act at the level of gene expression. Genes encoding ADP-glucose pyrophosphorylase (*AGP-S*) (Müller-Röber et al., 1990) and granule bound starch synthase (Visser et al., 1991) have been shown to be induced by high levels of sugars in sink tissues. A similar mechanism appears to operate in source tissues. Feeding glucose to autotrophic cell cultures of *Chenopodium* or cold-girdling of spinach leaves results in a rapid increase in the *AGP-S* transcript (Krapp et al., 1993; Krapp and Stitt, 1995). However, other factors must also be involved in the large stimulation of starch accumulation after adding glucose or inhibiting export since ADP-glucose pyrophosphorylase activity does not increase significantly (Krapp and Stitt, 1994). The decrease in activity of the plastidic (but not the cytosolic) isoform of starch phosphorylase when glucose is added to *Chenopodium* cells suggests that carbohydrates may also regulate the breakdown of starch (Krapp and Stitt, 1994).

Transgenic tobacco plants with very low nitrate reductase activity have been used to provide evidence that nitrate acts as a signal to induce organic acid

metabolism and repress starch metabolism in tobacco (Scheible et al., 1997). Addition of nitrate to these plants results in an increase in transcripts for phosphoenolpyruvate carboxylase, cytosolic pyruvate kinase, citrate synthase, and NADP-isocitrate dehydrogenase even though the nitrate is not being reduced and entering metabolism proper. The same treatment results in a decrease of the ADP-glucose pyrophosphorylase transcript and enzyme activity, and starch decreases in the leaves and roots. This work has led to the proposal that nitrate acts as a signal to initiate coordinated changes in carbon and nitrogen metabolism.

IV. Hormones and Sugar Regulation

Animal systems provide the best examples of how sugars and hormones can interact to regulate a given process. One such example is the glucose-induced insulin secretion from pancreatic β -cells (for review, see Holz and Habener, 1992). To trigger the secretion of insulin, glucose has to be phosphorylated by glucokinase and processed by glycolysis and the Krebs cycle. The consequent increase in ATP relative to ADP is thought to mediate a glucose induced depolarization of the β -cell membrane possibly by inhibiting the activity of metabolically regulated potassium channels resulting in an influx of calcium into the cell. The increase in intracellular calcium activates the exocytosis of vesicular insulin. This regulation of insulin secretion by glucose availability is modified by other processes including the inhibitory and stimulatory action of a large number of hormones and neurotransmitters thus allowing a high plasticity of the response reaction (Holz and Habener, 1992).

Do similar interactions between sugars and hormones exist in plants as have been demonstrated in animal systems? Plant hormones have been shown to have an effect on carbohydrate metabolism in both source and sink tissue. For example the activities of cytosolic sucrose phosphate synthase and fructose-1,6-bisphosphatase, which determine to a great extent the amount of sucrose in a leaf, are affected by exogenous application of hormones. Gibberellin application to soybean leaves increases sucrose phosphate synthase activity while the application of abscisic acid inhibits fructose-1,6-bisphosphatase activity (Brenner and Cheikh, 1995).

A recent report of work carried out on a *Chenopodium rubrum* cell suspension culture has

shown that cytokinin treatment results in induction of a cell wall invertase gene (*CIN1*) and a hexose transporter gene at the mRNA level (Ehness and Roitsch, 1997). The increase of cell wall invertase transcripts corresponds to higher steady state protein level and enzymatic activity of an invertase. Cytokinins did not affect the expression of two putative intracellular invertases (*CIN2* and *CIN3*), sucrose synthase or two other hexose transporters present in the cell suspension culture showing the specificity of this response (Ehness and Roitsch, 1997). The increased transcription of *CIN1* and the hexose transporter results in higher rates of sugar uptake in induced compared to non-induced cells. *CIN1* mRNA is also induced by cytokinin in tissues of *C. rubrum* plants thus indicating a physiological role for this hormone in the control of carbohydrate partitioning in plants (Ehness and Roitsch, 1997). Since cell wall invertase is also induced by glucose and 6-deoxyglucose in the same *C. rubrum* cell suspension culture (Roitsch et al., 1995) it would appear that sugars and hormones are involved in the induction of this enzyme at the level of gene expression. In contrast to the induction effect of cytokinin, physiological concentrations of ethylene were found to reduce the enzyme activity of cell wall invertase by 25–47% and this was paralleled by repression of the corresponding gene (Linden et al., 1996).

Dual control by sugars and hormones has also been reported for rice α -amylase and the soybean vegetative storage protein (VSP) gene. The rice α -amylase gene is repressed by sugars and abscisic acid and can be induced by gibberellin (Karrer and Rodriguez, 1992), whereas the activation of VSP gene expression by sugars and methyljasmonate is inhibited by auxins (DeWald et al., 1994).

The possibility that sugar sensing and ethylene signal transduction pathways are integrated was recently highlighted by a report from Sheen's laboratory on the characterization of *Arabidopsis gin* (glucose insensitive) mutants (Zhou et al., 1998). *Arabidopsis gin* mutants were isolated due to their ability to germinate and grow on 333 mM glucose. It was revealed that *Arabidopsis* mutants that produce significantly increased levels of ethylene (*eto*: ethylene overproduction) or mutants which show an ethylene over-production phenotype due to a defect in the ethylene signal transduction pathway (*ctr*: constitutive triple response) behave like *gin* mutants on 333 mM glucose. Furthermore AVG (L- α -(2-

aminoethoxyvinyl)-glycine), an inhibitor of ethylene biosynthesis, reverts the *gin* phenotype to wild type in the *eto* mutants and in at least one of the *gin* mutants (*gin*1.1). However, *CAB1* gene expression, which is insensitive to glucose in the *gin*1.1 mutant, is sensitive to glucose in the ethylene mutants as it is in wild type. This work therefore suggests that ethylene may be playing an important role in the response of germinating seedlings to high levels of glucose whereas it does not appear to be involved in the carbohydrate repression of *CAB* gene expression.

V. Sugar Sensing

All organisms regulate the flux of carbon through key metabolic pathways in order to balance carbohydrate metabolism with nutrient uptake, cellular osmotic potential, growth and physiology. In microorganisms, sugar sensing enables cellular metabolism to be adjusted in response to changes in carbon source availability in the external environment. Glucose is the preferred carbon source for the majority of microorganisms. The availability of glucose acts as a specific signal to repress genes encoding proteins involved in the utilization of alternative carbon sources. Our understanding of sugar sensing and sugar signal transduction pathways is much more advanced in bacteria and yeast than it is in plants or animals.

A. Distinct Sugar Sensing Mechanisms Exist in Evolutionary Divergent Prokaryotes

Carbon catabolite repression is a universal phenomenon in prokaryotes. Multiple mechanisms of control exist within a single cell and distinct mechanisms exist between evolutionary divergent species. The classic picture of catabolite repression in prokaryotes is that involving a cAMP-dependent mechanism. In *E. coli* this involves a phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS) that serves multiple functions one of which is sugar sensing. This system transports, phosphorylates and detects a variety of sugars. In so doing, it initiates sugar metabolism and regulates a number of physiologically important processes including the phenomena of inducer exclusion, inducer expulsion and catabolite repression (Saier et al., 1995). The PTS consists of several phosphoryl transfer proteins (Enzyme I, Hpr, IIA, IIB, and IIC) that must be

sequentially phosphorylated before sugar substrates can be transported and concomitantly phosphorylated. Enzyme I and Hpr are general (non-sugar-specific), soluble, cytoplasmic components of the system while the IIA, IIB and IIC components are sugar-specific membrane bound bifunctional permeases/kinases. Adenylyl cyclase is believed to be allosterically activated by the phosphorylated form of the glucose loaded IIA protein ($\text{IIA}^{\text{glc-P}}$). When active, it synthesizes cAMP, which binds to the cAMP receptor protein (CRP). The cAMP-CRP complex binds directly to DNA to activate transcription. Catabolite repression results when glucose in the medium causes a dephosphorylation of $\text{IIA}^{\text{glc-P}}$, thereby deactivating adenylyl cyclase. This in turn, causes the cytoplasmic cAMP concentration to diminish which causes the cAMP-CRP complex to dissociate from the DNA, thus resulting in catabolite repression (Saier et al., 1995; Saier, 1996).

In recent years, cAMP-independent catabolite repression mechanisms have been delineated in bacteria (Saier et al., 1995). Recent studies have shown that *E. coli* and *Salmonella typhimurium* also utilize other mechanisms which are completely independent of the cAMP-CRP complex to control transcriptional initiation of genes encoding the key enzymes of the major pathways for carbon metabolism (Ramseier, 1996). These pathways include the Embden-Meyerhof and Entner Duodoroff glycolytic pathways (subject to catabolite activation) and the Krebs cycle, glyoxylate shunt and gluconeogenic pathways (subject to catabolite repression). The pleiotropic transcriptional regulatory protein that controls expression of these central metabolic genes has been termed the 'Catabolite repressor/activator' (Cra). In vitro Cra-binding experiments have shown that micromolar concentrations of fructose 1-phosphate (F1P) or millimolar concentrations of fructose 1,6 bisphosphate (FBP), but not other catabolites or gluconeogenic intermediates, displaced the Cra protein from the promoters of all Cra regulated genes examined (Ramseier, 1996). Cra exerts a negative effect on glycolytic genes and a positive effect on genes encoding Krebs cycle, glyoxylate shunt and gluconeogenic enzymes. Under glucose-rich conditions, the concentrations of FBP and F1P increase and these interact with the Cra protein and act to dissociate it from target genes thus mediating catabolite activation of glycolytic genes and catabolite repression of Krebs cycle, glyoxylate shunt and gluconeogenic genes. In regulating such a variety of

genes, the Cra protein effectively controls the direction of carbon flux through metabolic pathways in enteric bacteria.

In evolutionary distinct species, still other mechanisms have evolved to control carbon catabolite repression. For example, in *Bacillus subtilis* and other Gram-positive bacteria cAMP is absent and instead, intracellular carbon metabolites such as fructose-1,6-bisphosphate (FBP) and gluconate-6-phosphate, the common intermediates of the Embden-Meyerhof and Entner Duodoroff pathways, respectively, promote catabolite repression. The mechanism of repression involves a metabolite activated, ATP-dependent protein kinase that phosphorylates a serine residue in HPr, the small phosphocarrier protein of the PTS. HPr-(ser-P) then binds to various protein targets (transcription factors, carbohydrate permeases and hydrolytic enzymes) to regulate their activities.

It is evident, then, that several different catabolite repression and inducer control mechanisms have evolved in prokaryotes, many which are independent of cAMP. Some of these mechanisms may be operative in eukaryotic microorganisms, higher plants and animals. The variety of cAMP independent mechanisms for catabolite repression, particularly those which involve universal metabolite intermediates as effector molecules, provide models for similar mechanisms in higher plants. If this is the case then the rapid accumulation of sequence data for model plant species such as *Arabidopsis* and rice along with already complete genome sequences for some bacteria (eg *Bacillus subtilis*, *Haemophilus influenzae* and *Mycoplasma genitalium*) should allow identification of putative regulatory components from plants in the near future. The task will then be to prove functional significance of these genes and proteins in plants.

B. Sugar Sensing in the Yeast *Saccharomyces cerevisiae*

As in *E. coli*, carbon catabolite repression in *S. cerevisiae* has been found to involve a number of distinct regulatory mechanisms. Addition of fermentable sugars to *S. cerevisiae* grown on non-fermentable carbon sources causes a variety of short-term and long-term regulatory effects. The regulatory switch is characterized by a rapid transient activation of cAMP synthesis, mobilization of the storage carbohydrate trehalose and induction of genes encoding glycolytic enzymes and ribosomal proteins.

Furthermore, high-affinity sugar transport and key gluconeogenic enzymes are rapidly inactivated and transcription of a variety of genes encoding respiratory, gluconeogenic and other enzymes is repressed (Gancedo, 1992; Trumbly, 1992). Several signaling pathways are involved in mediating the above phenomena of which the classic glucose repression pathway and the Ras-cAMP pathways are the best studied. Sensing of the available sugar by both these signaling pathways requires transport and phosphorylation but no further metabolism. *S. cerevisiae* contains two homologous hexokinases, PI (Hxk1) and PII (Hxk2), that can phosphorylate both glucose and fructose, and a single glucokinase (GK1), only capable of phosphorylating glucose (Lobo and Maitra, 1977). Activation of the Ras-cAMP pathway by glucose or fructose is dependent on sugar phosphorylation by any of these three hexose kinases. In contrast, glucose-dependent transcriptional repression exerted through the main glucose repression pathway is primarily dependent on the hexokinase PII. Deletion of the hexokinase PII gene abolishes glucose repression, whereas deletions of the hexokinase PI or glucokinase genes have no effect. Overexpression of hexokinase PI, but not glucokinase, relieves the requirement for PII in establishing glucose repression suggesting a function of the hexokinases in triggering catabolite repression that is separate from enzyme activity (De Winde et al., 1996, and references therein).

A recent report has demonstrated that the main glucose repression pathway involves two distinct mechanisms: an initial rapid response that can be mediated through any one of the three *S. cerevisiae* hexose kinases; and a second, long-term repression response that specifically requires hexokinase PII for glucose repression and either hexokinase PI or hexokinase PII for fructose repression (De Winde et al., 1996). Both the hexokinase PI and the glucokinase genes are themselves repressed by glucose and fructose. However, the repression of the hexokinase PI gene by fructose is transient which is in agreement with the preference of PI for fructose as substrate and its role in long-term fructose repression. These results demonstrate that sugar sensing and establishment of catabolite repression in *S. cerevisiae* is controlled by an interregulatory network, involving all three yeast hexose kinases and the Ras-cAMP pathway (De Winde et al., 1996).

As discussed below, it would now appear that at least part of the hexokinase sensing mechanism may

have been conserved and adapted for sugar sensing in higher plants.

Characterization of the phenotypes of additional *S. cerevisiae* mutants have resulted in the identification of two signaling pathways which operate downstream of sugar sensing. Although one of these pathways is responsible for repression and the other for de-repression of genes that are subject to carbon catabolite control there is substantial crosstalk between the two. Two protein complexes have been identified in the repression pathway. One of these (REG2/REG1/GLC7 type 1 protein phosphatase) mediates a signal by regulating a protein phosphatase activity but no substrate for this activity has as yet been identified. The second complex (SSN6/TUP1) functions as a general repressor of transcription through modulation of chromosome structure. Interaction of the SSN6/TUP1-complex with DNA is mediated by the MIG1 DNA binding protein, which somehow directs binding of the complex to specific sites in the promoter region of genes that are repressed by glucose.

Derepression of gene expression in the absence of glucose is necessary to allow alternative carbon sources to be utilized. This derepression process involves another regulatory pathway containing at least two protein complexes. The first complex (SNF1/SNF4) mediates a signal through activity of the SNF1 protein kinase of which there are plant and mammalian homologues (see below). The second complex (SWI/SNF) consists of at least ten proteins which modulate chromatin conformation and affect expression of a variety of genes. Targeting of this complex to specific gene promoters is mediated by transcription factors. It is not yet known how the SWI/SNF complex receives information from the SNF1 complex in the derepression pathway. The SNF1 complex also directly affects the repression pathway by inactivating the MIG1 repressor protein. Similarly the GLC7 protein phosphatase complex from the repression pathway inhibits the activity of the SNF1 kinase complex.

Our understanding of the mechanisms outlined above, although detailed relative to our knowledge in plants, is still far from complete. One important gap still remaining to be filled is how the metabolite signals involved are sensed and transduced into the regulatory pathway. As discussed above, hexokinase has been implicated as the sugar sensor triggering the repression pathway but a mechanism of action still has to be defined.

An alternative mechanism involving AMP:ATP ratios for signaling intracellular nutritional status to the de-repression pathway has recently emerged through biochemical studies of the *S. cerevisiae* SNF1 kinase (Wilson et al., 1996). The mammalian AMP-activated protein kinase (AMPK) is a homologue of SNF1 kinase. AMPK is made up of three subunits and all of these have homologues that are part of the SNF1 complex. AMPK is activated by the elevation of the cellular AMP:ATP ratio, which occurs during cellular stress in mammalian cells. The mechanism of activation involves allosteric regulation by AMP, promotion of phosphorylation by an upstream protein kinase (AMPK kinase) and inhibition of dephosphorylation of AMPK. The proposed mode of action in mammalian cells is that AMPK functions as a 'fuel gauge' (Hardie and Carling, 1997). When AMPK detects a low fuel situation it protects the cell by switching off ATP-consuming pathways (eg. fatty acid synthesis and sterol synthesis) and switching on alternative pathways for ATP generation (eg. fatty acid oxidation). SNF1 is rapidly activated by phosphorylation on removal of glucose from *S. cerevisiae*, and can be inactivated by protein phosphatases and reactivated by mammalian AMPK kinase, thus demonstrating that the mammalian AMPK and *S. cerevisiae* SNF1 protein kinase cascades are highly conserved (Wilson et al., 1996). The only apparent difference in these two complexes is that the SNF1 complex is not regulated allosterically by AMP in vitro. However, multiple levels of control by AMP:ATP ratio exist for the mammalian complex and a similar situation may exist for the yeast complex. Based on this work, Wilson et al. (1996) put forward a new model for the role of SNF1 protein kinase in carbon catabolite repression. This model proposes that adenine nucleotides, rather than an interaction of glucose with hexokinase, are the metabolic signals which indicate the lack of glucose in the medium. These signals trigger activation of SNF1 kinase and de-repression of glucose repressed genes.

AMPK/SNF1 homologues also exist in plants but their function has not yet been defined. SNF1 homologues from rye and tobacco can complement the *snf1* mutation in *S. cerevisiae* (Alderson et al., 1991; Muranaka et al., 1994). Unlike the plant homologue, the mammalian AMPK does not complement *snf1* regulatory mutants (Woods et al., 1994). Additional SNF1 homologues have been cloned from barley, wheat and *Arabidopsis* (Halford

etal., 1992; Le Guen et al., 1992; Sano and Youssefian, 1994). A tobacco SNF1 homologue, NPK5, constitutively activates expression of the glucose repressible *SUC2* gene in *S. cerevisiae snf1* mutants (Muranaka et al., 1994). However, expression of NPK5 does not rescue mutants disrupted in the *SNF4* gene, the product of which associates with SNF1 and is necessary for maximal activity of the kinase in yeast. These results indicate that NPK5 can substitute for SNF1 in the SNF1/SNF4 complex in *S. cerevisiae snf1* mutants (Muranaka et al., 1994). Carbohydrate starvation of wheat seedlings results in expression of the SNF1 homologue *WPK4* (Sano and Youssefian, 1994). Light and cytokinins also induce *WPK4* gene expression which suggests that this particular kinase may be involved in more than one signal transduction pathway.

An AMPK specific peptide based kinase assay (Davies et al., 1989) has been used to identify AMPK-like activities in extracts from avocado, potato, carrot, pea, oilseed rape, wheat and cauliflower (MacKintosh et al., 1992). Immunological studies indicate that the cauliflower kinase is the homologue of the rye and tobacco SNF1-like protein kinases (Ball et al., 1995). Like the *S. cerevisiae* SNF1, the cauliflower kinase is not sensitive to AMP. In all other respects, including being able to inactivate mammalian acetyl-CoA carboxylase and HMG-CoA reductase, it is similar to the mammalian AMPK. The physiological substrate for the plant AMPK/SNF1 homologue is not yet known but it has been shown that the cauliflower kinase inactivates bacterially synthesized HMG-CoA reductase from *Arabidopsis* by phosphorylating the protein at the same site that is phosphorylated in the mammalian HMG-CoA reductase (Dale et al., 1995).

Much work still has to be done to establish the *in vivo* role(s) of the plant SNF1 homologues. It is possible that this family of protein kinases plays a role in signal transduction of metabolic status in plant cells. Alternatively, or as well, these kinases could play an important role in the post-translational control of key metabolic enzymes as is the case in mammalian cells. Two such enzymes in plant cells are sucrose phosphate synthase which catalyzes a key step in sucrose biosynthesis in source tissues and nitrate reductase which catalyzes the first step in nitrogen assimilation. In spinach leaves both these enzymes are regulated by phosphorylation by multiple protein kinases (Chapter 8, Foyer et al.; McMichael et al., 1995). One of three nitrate reductase kinases detected in extracts from spinach leaves has identical

properties to other SNF1-like enzymes (Douglas et al., 1997). It is, therefore, likely that plant SNF1 homologues do play an important role in regulating metabolic processes in source tissue through post-translational control of key metabolic enzymes.

Experiments involving antisense and over-expression of plant SNF1 homologues are currently underway in several laboratories and these along with specific kinase assays and monitoring expression of appropriate genes under different metabolic conditions should provide useful information on the role of the plant kinase. In addition, identification of plant homologues to other components in the SNF1 signal transduction pathway, such as SNF4 or a possible upstream kinase, along with their functional analysis would provide additional evidence for a conserved mechanism of carbon catabolite repression between plants and yeast.

C. Sugar Sensing in Plants—the Case for Hexokinase

Studies on the sugar mediated repression of the glyoxylate cycle and photosynthetic genes has led to the suggestion that sugar sensing in plants involves a hexokinase (Graham et al., 1994a,b; Jang and Sheen, 1994). In cucumber cell cultures, the malate synthase and isocitrate lyase genes are repressed in the presence of glucose, fructose or sucrose and de-repressed when intracellular levels of these sugars fall below a critical threshold (Graham et al., 1994b). A cucumber mesophyll protoplast transient expression system was used to demonstrate that these same genes are de-repressed at the transcriptional level upon carbohydrate depletion (Graham et al., 1994a). The glucose analogues 2-deoxyglucose and mannose, which are phosphorylated by hexokinase but not further metabolized by the glycolytic pathway, cause repression even though intracellular amounts of sucrose, glucose and fructose fall below the critical levels normally associated with de-repression (Graham et al., 1994b). In contrast to glucose, fructose or sucrose, 3-O-methylglucose, an analogue of glucose that is taken up by the cells but is not phosphorylated by hexokinase, does not result in repression of malate synthase and isocitrate lyase. These results implicate hexokinase or events associated with the hexokinase reaction in the initial sugar sensing step. However it should be noted that experiments with hexose analogues alone do not rule out the possibility of a stereospecific hexose receptor

operating independently of hexokinase.

Using a *C. rubrum* cell culture, Krapp et al. (1993) demonstrated that 6-deoxyglucose or 3-O-methylglucose (both imported into the cell but not phosphorylated by hexokinase) had no effect on *RBCS* gene expression, whereas glucose at the same concentration caused severe repression. It was concluded from this work that transport of carbohydrates does not provide the signal for regulation of *RBCS* and that phosphorylation of glucose is necessary. Using a maize mesophyll protoplast transient expression system, Jang and Sheen (1994) demonstrated that 2-deoxyglucose causes repression of photosynthetic gene promoters whereas 3-O-methylglucose has no effect on expression. The importance of hexokinase in sugar sensing was further demonstrated by feeding experiments using mannoheptulose, an apparently specific inhibitor of hexokinase, which was shown to block the repression effect of glucose and 2-deoxyglucose (Jang and Sheen, 1994). However, more recent work using a *C. rubrum* cell culture has reported that both D-glucose and 6-deoxyglucose treatments result in a similar decrease in *RBCS* mRNA levels and an increase in the mRNAs for a sink specific cell wall invertase and for phenylalanine ammonia-lyase, a key enzyme of defense response (Ehness et al., 1997). An explanation for these conflicting reports is not apparent.

Cloning of two *Arabidopsis* genes, *AtHXK1* and *AtHXK2* has allowed overexpression and antisense experiments to be carried out in order to establish the *in vivo* involvement of hexokinase in the response of seedlings to elevated levels of glucose (Jang et al., 1997; Jang and Sheen, 1997). Wild type seedling growth is inhibited in the presence of 333 mM glucose whereas antisense seedlings with reduced levels of hexokinase show less inhibition of growth. Photosynthetic genes such as *RBCS* and *CAB* are also more strongly repressed by glucose in wild type than in antisense plants. Overexpression of the hexokinase in transgenic seedlings results in increased sensitivity to 333 mM glucose relative to wild type. The authors' interpretation of these results together with other results using the yeast hexokinase, is that hexokinase is a sugar sensor. Decreasing hexokinase protein decreases the capacity of seedlings to sense glucose hence they grow better on 333 mM glucose and do not exhibit the same degree of glucose repression of photosynthetic gene expression as is observed in wild type. Increased levels of hexokinase

lead to increased sugar sensing capacity and increased sensitivity to 333 mM glucose. Another interpretation is that 333 mM glucose is toxic to seedlings, possibly because it results in a massive flux of hexose sugar into the hexose phosphate pool. This might lead to a sequestration of phosphate which could disturb the balance of cellular metabolism. A third possibility is that high concentrations of hexose phosphates in the cell have deleterious effects. Decreasing hexokinase activity will decrease this flux whereas increasing hexokinase activity will increase the flux. The crucial experiment which supports the view that hexokinase is acting as a sugar sensor involved overexpressing the *S. cerevisiae HXK2* gene (which is implicated in sugar sensing in *S. cerevisiae*) in *Arabidopsis*. If the yeast hexokinase were to exhibit sugar sensing in *Arabidopsis* then transgenic seedlings should be more sensitive than wild type to 333 mM glucose. In fact, the opposite phenotype was observed in which the transgenic seedlings were less sensitive to 333 mM glucose than wild type even though the total hexose phosphorylation activity was significantly greater than that in wild type seedlings. The interpretation given for this is that the yeast hexokinase competes effectively for the same substrate as the endogenous glucose sensing hexokinases resulting in reduced sensitivity to glucose.

The hexokinase under- and over-expressing plants were also used to investigate the role of hexokinase in the regulation of sugar inducible nitrate reductase. *Arabidopsis* lines over-expressing *AtHXK* show higher steady state nitrate reductase mRNA levels in response to glucose than wild type seedlings, thus suggesting that hexokinase is also important in signaling expression of sugar-inducible genes. However, other effects such as increased flux of hexose sugars into glycolysis cannot be ruled out.

D. Sugar Transport and Signaling

The generation of a signal by metabolism of a sugar is one way in which carbohydrate status is perceived. An alternative or additional mechanism could involve intra- or extracellular receptors. In the simplest case, a DNA binding protein might act as a sensor and transmit the signal directly to the respective gene as in the case of some hormone induction in mammalian cells. Alternatively, the receptor could be part of a sugar transport system (possibly similar to the PTS system in *E. coli*) in which increased or decreased levels of sugars lead to a chain of signaling events

which ultimately cause a sugar specific response.

The yeast *SNF3* and *RGT2* genes encode putative glucose transporters with high similarities to mammalian, yeast and plant glucose transporters, but differ from them in having a long C-terminal extension. This extension may be located in the cytoplasm and could serve as a signaling domain (Celenza et al., 1988 Marshall-Carlson et al., 1990; Bisson et al., 1993) Mutational analysis has shown that both *SNF3* and *RGT2* may act as sugar sensors and are involved in the induction of glucose induced *HXT* gene regulation in yeast (Ozcan et al., 1996). Furthermore this analysis has shown that *SNF3* and *RGT2* might act as a pair of glucose receptors recognizing either high (*RGT2*) or low (*SNF3*) levels of extracellular glucose. The authors suggest that as in the case of the bacterial PTS system the generation of the sugar signal is coupled to the transport of this sugar and that the components of the sugar signaling pathway are present in the cell even in the absence of the sugar. In this model, the activity of the glucose sensor becomes the limiting factor of the signaling system (Ozcan et al., 1996).

Despite the cloning of a number of sugar transporter genes from plants (Sauer and Tanner, 1993) a sugar transporter/sensor protein equivalent to the yeast *SNF3* or *RGT2* has not yet been identified in plants.

Experiments using non-metabolizable sugar analogues provide evidence for at least two different signal perception mechanisms in plants. As discussed above, source-related genes, e.g. *RBCS* and *CAB*, can be repressed by 2-deoxyglucose, or mannose but not by 3-O-methylglucose (Jang and Sheen, 1994). In contrast, the more sink metabolism-related sucrose synthase, invertase and class I patatin genes are induced by 3-O-methylglucose, 6-deoxyglucose or 2-deoxyglucose (Godt et al., 1995; Roitsch et al., 1995; Ehness et al., 1997; Martin et al., 1997). In addition, the induction of a sugar regulated chimeric patatin class I/uidA (B33GUS) gene in transgenic *Arabidopsis* plants by the glucose analogs 6-deoxyglucose and 3-O-methylglucose shows that at least some sugar signaling pathways in an intact plant system do not require sugar phosphorylation (Martin et al., 1997). The lack of induction of patatin gene expression in transgenic potato tubers with increased carbohydrate levels due to AGPase or sucrose synthase antisense repression is taken as evidence to indicate that the flux rather than the concentration of sugars is sensed in the case of induction of patatin gene expression (Müller-Röber

et al., 1992; Zrenner et al., 1995).

However, the demonstration that the sugar-inducible promoter of the potato proteinase inhibitor II gene is not induced by 6-deoxyglucose in transgenic tobacco (Kim et al., 1991) suggests that at least two mechanisms may operate in parallel to regulate genes that are induced by sugars.

The suggestion that hexose sensing may be associated with the endomembrane system has come from recent work on transgenic tobacco plants expressing yeast-derived invertase targeted to the apoplast and vacuole (Herbers et al., 1996). Targeting of this enzyme to the apoplast or vacuole results in increased levels of monosaccharides in the cells and an associated induction of defense genes and repression of *CAB* genes. Expression of the yeast invertase in the cytosol of tobacco plants results in equally elevated levels of sugars but does not have any effect on defense or photosynthetic genes. Bacterial fructosyltransferase converts sucrose into fructans with a concomitant release of equimolar amounts of glucose. Expression of a bacterial fructosyltransferase gene in the endomembrane system of plants results in a bleached phenotype which suggests accumulation of hexose sugars may be resulting in repression of genes encoding enzymes and proteins in the photosynthetic apparatus (Turk et al., 1997).

E. Sucrose as a Direct Signal

Sucrose is the main transport sugar in most plant species and it is also an important storage compound. Due to the fact that extracellular invertases hydrolyze sucrose to fructose and glucose, it is difficult to prove a direct effect of this molecule in feeding experiments. However, sucrose specific induction of the patatin promoter (Wenzler et al., 1989; Jefferson et al., 1990) has been reported.

VI. Signal Transduction

Specific components of the signal transduction pathways involved in regulation of plant genes by sugars have not yet been identified. As discussed above (Section V.B.) one family of protein kinases that may play a role in the transduction of sugar signals are the plant homologues of the AMPK/SNF1 protein kinase which have been shown to be essential for post-translational control of key

biosynthetic enzymes in mammalian cells and de-repression of genes that are subject to carbon catabolite repression in yeast (Hardie and Carling, 1997).

As in other systems, phosphatases, kinases, calcium and calmodulin appear to be involved in the transduction of sugar signals in plants (Lue and Lee, 1994; Takeda et al., 1994; Ohto et al., 1995).

VII. Sugar Response Elements in Gene Promoters

In contrast to the detailed characterization of various photosynthetic gene promoters in relation to their response to light, there have been few reports characterizing their response to carbohydrates. Sheen (1990) used the maize protoplast transient expression system to demonstrate that sucrose repression of a number of maize photosynthetic genes was mediated by positively acting promoter elements upstream of the TATA box, but no specific consensus sequences were defined. More recently (Urwin and Jenkins, 1997), transient expression experiments with protoplasts from primary leaves of *Phaseolus vulgaris* have been used to identify promoter sequences involved in sucrose repression of the *P. vulgaris RBCS2* gene. A region of this promoter from position -203 to -187 contains sequences resembling elements involved in the sugar stimulation of transcription of several plant and mammalian genes. These include the sucrose response elements (SURE elements) of the patatin gene promoter (Grierson et al., 1994). The SURE elements are similar to elements defined in the sporamin and β -amylase genes of sweet potato both of which are induced by sucrose (Ishiguro and Nakamura, 1992). The -203 to -187 region of the *RBCS2* gene implicated in sugar repression also has remarkable similarity to carbohydrate response elements (ChoRE) found in the promoters of mammalian genes induced by sugars, one of which is the L-type pyruvate kinase gene. Whether the *RBCS2* -203 to -187 promoter region is sufficient to mediate a sucrose response, or whether any functional similarity exists with the elements described above, requires further research.

VIII. Sugar Sensing Mutants

A number of laboratories have designed various

genetic screens in the model plant *Arabidopsis thaliana* in an effort to isolate mutants in the signal transduction mechanisms involved in sugar mediated repression and induction of gene expression. In the majority of cases young seedlings have been screened as these respond to exogenous sugars in various ways and it is also possible to look at large mutagenized populations. One such screen has exploited the fact that intracellular sugar concentrations increase dramatically in *Arabidopsis* seedlings grown on high concentrations of sucrose and growth limiting amounts of nitrate compared to when seedlings are grown on high concentrations of sucrose and sufficient amounts of nitrate. The increase in intracellular sugars results in an increase in anthocyanins and a decrease in chlorophyll. Using high sucrose, low nitrate conditions a number of *carbohydrate insensitive (cai)* mutants have been isolated which do not show the same decrease in chlorophyll and increase in anthocyanins as in the wild type but do show the same increase in intracellular sugar levels (S. Boxall, T. Martin and I. A. Graham, unpublished). As mentioned previously (Section V.C), growth of *Arabidopsis* seedlings on 333 mM glucose results in developmental arrest and this is overcome in plants that have been antisensed for hexokinase I or hexokinase II (Jang et al., 1997). These same conditions have been used to isolate *glucose insensitive (gin)* mutants which are less sensitive to 333 mM glucose in the growth medium (Zhou et al., 1998).

The *Arabidopsis* plastocyanin (*PC*) gene is transiently activated in both the light and dark during early seedling growth and this expression can be repressed by sucrose (Dijkwel et al., 1996). A transgene screening approach using a *PC* gene promoter-luciferase reporter gene construct has been used to isolate *Arabidopsis* mutants that are disrupted in the sugar repression of the plastocyanin *PC* gene during early seedling growth (Dijkwel et al., 1997). *Sucrose uncoupled (sun)* mutants show no or reduced repression of the transgene and, importantly, the endogenous *PC*, *CAB* and *RBCS* mRNA levels are similarly insensitive to sucrose repression. Studies of the affect of 2 mM 2-deoxyglucose on the mature rosette stage *Arabidopsis* have shown that whole plant photosynthesis, plastocyanin gene expression and total extractable Rubisco activity is unaffected in one mutant *sun6* (Van Oosten et al. 1997). This treatment results in reduced photosynthesis, *PC* gene expression and Rubisco activity in wildtype

Arabidopsis.

Many photosynthetic genes that are subject to sucrose repression are also subject to light regulation via phytochrome. The presence of sucrose in the growth medium results in altered *PHYA* responses in the *sun6* mutant compared to wildtype. This suggests that the mechanisms of sugar signaling and light signaling are interrelated (Dijkwel et al., 1997).

Arabidopsis mutants disrupted in the induction of specific genes by sugars have also been isolated. For example, the **β -amylase** gene is induced by sugars in *Arabidopsis* and *low level beta amylase (lba)* mutants have been isolated which do not show this induction response (Mita et al., 1997a,b). In another example the patatin class I promoter, which is induced by sugars in potato, was transferred to *Arabidopsis* where it was found to be similarly regulated. Transgenic *Arabidopsis* harboring a patatin promoter- β -glucuronidase transgene were used to isolate mutants showing a *reduced sucrose response (rsr)* (Martin et al., 1997). The demonstration that the promoter of the potato tuber storage protein is regulated by sugars in the same manner in *Arabidopsis* demonstrates a high degree of conservation between the mechanisms underlying sugar signaling in plants. Characterization of *Arabidopsis* mutants will allow us to identify the key regulatory components involved in sugar signaling. One application of this knowledge will be manipulation of sugar signaling and resource allocation in crop species in order to increase yields of current plant products and to over-ride regulatory mechanisms that may inhibit high yields of genetically engineered novel plant products.

IX. Conclusions

In this chapter we have summarized our current understanding of carbohydrate regulation of gene expression and how this relates at the cellular and whole plant level to control of photosynthetic gene expression. Plants contain several sugar signaling cascades, one of which is important in source tissues, possibly involving hexokinase, and another which is independent of hexokinase possibly located at the plasma membrane in sink tissues. The components of a hexokinase independent sugar signaling pathway could be as in yeast a sugar sensing transporter protein (Oezcan et al., 1997) or simply a sugar sensing protein as the first component of a signaling cascade. The analysis of *Arabidopsis* mutants

disrupted in sugar induced responses should help in understanding how plants sense their sugar status and coordinate sink and source metabolism.

We have given many examples of how metabolites regulate gene expression in other organisms in an attempt to emphasize the variety of mechanism that may operate in plants. It is reasonable to predict that at least some of these mechanism will be conserved across phyla and indeed a precedent for this has already been set in the case of the regulation of heat shock genes (Spina and Schell, 1987). In higher plants various mechanisms appear to have either evolved or been conserved and adapted throughout evolution to meet the physiological requirements of source and sink tissues. Future efforts to manipulate production in source tissues, and ultimately plant yield, will require a thorough understanding of the regulatory mechanisms involved.

References

- Alderson A, Sabelli PA, Dickinson JR, Cole D, Richardson M, Kreis M, Shewry PR and Halford NG (1991) Complementation of *snf1*, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein-kinase cDNA. Proc Nat Acad Sci USA 88: 8602–8605
- Azcon-Bieto J (1983) Inhibition of photosynthesis by carbohydrates in leaves. Plant Physiol 83: 681–686
- Ball KL, Barker J, Halford NG and Hardie DG (1995) Immunological evidence that HMG-CoA reductase kinase-a is the cauliflower homolog of the RKIN1 subfamily of plant protein-kinases. FEBS Lett 377: 189–192
- Bisson LF, Coons DM, Kruckeberg AL and Lewis DA (1993) Yeast sugar transporters. Crit Rev Biochem Mol Biol 28: 259–308
- Blechschmidt-Schneider S, Ferrar P and Osmond CB (1989) Control of photosynthesis by carbohydrate levels in leaves of the C₄ plant *Amaranthus edulis*. Planta 177: 515–525
- Bowes G (1994) Facing the inevitable: Plants and increasing atmospheric CO₂. Annu Rev Plant Physiol Plant Mol Biol 44: 309–332
- Brenner ML and Cheikh N (1995) The role of hormones in photosynthate partitioning and seed filling. In: Davies PJ (ed) Plant Hormones, Second Edition, pp 649–670. Kluwer Academic Publishers, Dordrecht
- Celenza JL, Marshall-Carlson L and Carlson M (1988) The yeast *SNF3*-gene encodes a glucose transporter homologous to the mammalian protein. Proc Natl Acad Sci USA 85: 2130–2134
- Cheng CL, Acedo GN, Cristinsin M and Conkling MA (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc Natl Acad Sci USA 89: 1861–1864
- Dale S, Wilson WA, Edelman AM and Hardie DG (1995) Similar substrate recognition motifs for mammalian AMP-activated protein-kinase, higher-plant HMG-CoA reductase

- kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I. FEBS Lett 361: 191–195
- Davies SP, Carling D and Hardie DG (1989) Tissue distribution of the AMP-activated kinase, and lack of activation by cyclic AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. Eur J Biochem 186: 123–128
- DeWald DB, Sadka A and Mullet JE (1994) Sucrose modulation of soybean *VSP* gene expression is inhibited by auxin. Plant Physiol 104: 439–444
- De Winde JH, Crauwels M, Hohmann S, Thevelein JM and Winderickx J (1996) Differential requirement of the yeast sugar kinases for sugar sensing in establishing the catabolite-repressed state. Eur J Biochem 241: 633–643
- Dijkwel PP, Kock PAM, Bezemer R, Weisbeek PJ and Smeekens SCM (1996) Sucrose represses the developmentally controlled transient activation of the plastocyanin gene in *Arabidopsis thaliana* seedlings. Plant Physiol 110: 455–463
- Dijkwel PP, Huijsman C, Weisbeek PJ, Chua NH and Smeekens SCM (1997) Sucrose control of phytochrome A signaling in *Arabidopsis*. Plant Cell 9: 583–595
- Douglas P, Pigaglio E, Ferrer A, Halford NG and MacKintosh C (1997) Three spinach leaf nitrate reductase-3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca^{2+} ions. Biochem J 325: 101–109
- Ehness R and Roitsch T (1997) Co-ordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by cytokinins. Plant J 11: 539–548
- Ehness R, Ecker M, Godt DE and Roitsch T (1997) Glucose and stress independently regulate source and sink metabolism and defense mechanisms via signal transduction pathways involving protein phosphorylation. Plant Cell 9: 1825–1841
- Gancedo JM (1992) Carbon catabolite repression in yeast. Eur J Biochem 206: 297–313
- Geiger DR, Koch KE and Shieh WJ (1996) Effect of environmental-factors on whole-plant assimilate partitioning and associated gene-expression. J Exp Bot 47: 1229–1238
- Godt DE, Riegel A and Roitsch T (1995) Regulation of sucrose synthase expression in *Chenopodium rubrum*: Characterization of sugar induced expression in photoautotrophic suspension cultures and sink tissue specific expression in plants. J Plant Physiol 146: 231–238
- Graham IA (1996) Carbohydrate control of gene-expression in higher-plants. Research In Microbiology 147: 572–580
- Graham IA, Leaver CJ and Smith SM (1992) Induction of malate synthase gene expression in senescent and detached organs of cucumber. Plant Cell 4: 349–357
- Graham IA, Baker CJ and Leaver CJ (1994a) Analysis of the cucumber malate synthase gene promoter by transient expression and gel retardation assays. Plant J 6: 893–902
- Graham IA, Denby KJ and Leaver CJ (1994b) Carbon catabolite repression regulates glyoxylate cycle gene-expression in cucumber. Plant Cell 6: 761–772
- Grierson C, Du J-S, de Torres Zabala M, Beggs K, Smith C, Holdsworth M and Bevan M (1994) Separate *cis* sequences and *trans* factors direct metabolic and developmental regulation of a potato tuber storage gene. Plant J 5: 815–826
- Halford NG, Vincente-Carballido J, Sabelli PA, Shrewsbury PR, Hannappel U and Kreis M (1992) Molecular analyses of a barley multigene family homologous to the yeast protein kinase gene *SNF1*. Plant J 2: 791–797
- Hardie DG and Carling D (1997) The AMP-activated protein kinase—Fuel gauge of the mammalian cell? Eur J Biochem 246: 259–273
- Harter K, Talke-Messerer C, Barz W and Schäfer E (1993) Light- and sucrose-dependent gene expression in photomixotrophic cell suspension cultures and protoplasts of rape (*Brassica napus* L.). Plant J 4: 507–516
- Heim U, Weber H, Bäumlein H and Wobus U (1993) A sucrose-synthase gene of *Vicia faba* L.: Expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta 191: 394–401
- Henry LT and Raper D (1991) Soluble carbohydrate allocation to roots, photosynthetic rate of leaves, and nitrogen assimilation as affected by nitrogen stress and irradiance. Botanical Gazette 152: 23–33
- Herbers K, Meuwly P, Frommer WB, Metraux JP and Sonnewald U (1996) Systemic acquired-resistance mediated by the ectopic expression of invertase—possible hexose sensing in the secretory pathway. Plant Cell 8: 793–803
- Hickey DA, Benkel KI, Fong Y and Benkel BF (1994) A *Drosophila* gene promoter is subject to glucose repression in yeast-cells. Proc Nat Acad Sci USA 91: 11109–11112
- Holz GG and Habener JF (1992) Signal transduction crosstalk in the endocrine system: Pancreatic β -cells and the glucose competence concept. Trends Biochem Sci 17: 388–393
- Ishiguro S and Nakamura K (1992) The nuclear factor SP8BF binds to the 5' upstream regions of three different genes coding for major proteins of sweet potato tuberous roots. Plant Mol Biol 18: 97–101
- Jang JC and Sheen J (1994) Sugar sensing in higher-plants. Plant Cell 6: 1665–1679
- Jang JC and Sheen J (1997) Sugar sensing in higher plants. Trends Plant Sci 2: 208–214
- Jang JC, Leon P, Zhou L and Sheen J (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell 9: 5–19
- Jefferson R, Goldsbrough A and Bevan M (1990) Transcriptional regulation of a *Patatin-1* gene in potato. Plant Mol Biol 14: 995–1006
- Karrer EE and Rodriguez RL (1992) Metabolic regulation of rice α -amylase and sucrose synthase genes in *planta*. Plant J 2: 517–523
- Kim S-R, Costa MA and An G (1991) Sugar response element enhances wound response of potato proteinase inhibitor II in transgenic tobacco. Plant Mol Biol 17: 973–983
- Koch KE (1996) Carbohydrate-modulated gene-expression in plants. Ann Rev Plant Physiol Plant Mol Biol 47: 509–540
- Koch KE, Nolte KD, Duke ER, McCarty DR and Avigne WT (1992) Sugar levels modulate differential expression of maize *Sucrose Synthase* genes. Plant Cell 4: 59–69
- Koch KE, Wu Y and Xu J (1996) Sugar and metabolic-regulation of genes for sucrose metabolism—potential influence of maize sucrose synthase and soluble invertase responses on carbon partitioning and sugar sensing. J Exp Bot 47: 1179–1185
- Krapp A and Stitt M (1994) Influence of high-carbohydrate content on the activity of plastidic and cytosolic isoenzyme pairs in photosynthetic tissues. Plant Cell Environ 17: 861–866
- Krapp A and Stitt M (1995) An evaluation of direct and indirect mechanisms for the ‘sink-regulation’ of photosynthesis in spinach: Changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-

- girdling source leaves. *Planta* 195: 313–323
- Krapp A, Hofmann B, Schafer C and Stitt M (1993) Regulation of the expression of *RbcS* and other photosynthetic genes by carbohydrates—a mechanism for the sink regulation of photosynthesis. *Plant J* 3: 817–828
- Lam H-M, Peng SS-Y and Coruzzi G (1994) Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. *Plant Physiol* 106: 1347–1357
- Le Guen L, Thomas M, Bianchi M, Halford NG and Kreis M (1992) Structure and expression of a gene from *Arabidopsis thaliana* encoding a protein related to SNF1 protein kinase. *Gene* 120: 249–254
- Linden JC, Ehness R and Roitsch T (1996) Ethylene regulation of apoplastic invertase expression in autotrophic cells of *Chenopodium rubrum*. *Plant Growth Regulation* 19: 219–222
- Lobo Z and Maitra PK (1977) Genetics of yeast hexokinase. *Genetics* 86: 727–744
- Lue M-Y and Lee H (1994) Protein phosphatase inhibitors enhance the expression of an α amylase gene, $\alpha Amy3$, in cultured rice cells. *Biochem Biophys Res Commun* 205: 807–816
- MacKintosh RW, Davies SP, Clarke PR, Weekes J, Gillespie JG, Gibb BJ and Hardie DG (1992) Evidence for a protein kinase cascade in higher plants: 3-hydroxy-3-methylglutaryl-CoA reductase kinase. *Eur J Biochem* 209: 923–931
- Marshall-Carlson L, Celenza JL, Laurent BC and Carlson M (1990) Mutational analysis of the SNF3 glucose transporter of *Saccharomyces cerevisiae*. *Mol Cell Biol* 10: 1105–115
- Martin T, Frommer WB, Salanoubat M and Willmitzer L (1993) Expression of an, *Arabidopsis* sucrose synthase gene indicates a role in metabolism of sucrose both during phloem loading and in sink organs. *Plant J* 4: 367–377
- Martin T, Hellmann H, Schmidt R, Willmitzer L and Frommer WB (1997) Identification of mutants in metabolically regulated gene expression. *Plant J* 11: 53–62
- McMichael RW, Bachmann M and Huber SC (1995) Spinach leaf sucrose-phosphate synthase and nitrate reductase are phosphorylated by multiple protein kinases in vitro. *Plant Physiol* 108: 1077–1082
- Mita S, Hirano H and Nakamura K (1997a) Negative regulation in the expression of a sugar-inducible gene in *Arabidopsis thaliana*—A recessive mutation causing enhanced expression of a gene for beta-amylase. *Plant Physiol* 114: 575–582
- Mita S, Murano N, Akaike M and Nakamura K (1997b) Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for beta-amylase and on the accumulation of anthocyanin that are inducible by sugars. *Plant J* 11: 841–851
- Müller-Röber BT, Koßmann J, Hannah LC, Willmitzer L and Sonnewald U (1990) One of the two ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. *Mol Gen Genet* 224: 136–146
- Müller-Röber BT, Sonnewald U and Willmitzer L (1992) Inhibition of the ADP-Glucose Pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. *EMBO J* 11: 1229–1238
- Muranaka T, Banno H and Machida Y (1994) Characterization of tobacco protein-kinase NPK5, a homolog of *Saccharomyces cerevisiae* SNF1 that constitutively activates expression of the glucose-repressible *SUC2* gene for a secreted invertase of *Saccharomyces cerevisiae*. *Mol Cell Biol* 14: 2958–2965
- Ohto M, Hayashi K, Isobe M and Nakamura K (1995) Involvement of Ca^{2+} signaling in the sugar-inducible expression of genes coding for sporanim and β -amylase of sweet potato. *Plant J* 7: 297–307
- Ozcan S, Dover J, Rosenwald AG, Wolfl S and Johnston M (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proc Natl Acad Sci USA* 93: 12428–12432
- Paul MJ and Stitt M (1993) Effects of nitrogen and phosphorus deficiencies on levels of carbohydrates, respiratory enzymes and metabolites in seedlings of tobacco and their response to exogenous sucrose. *Plant, Cell and Environ* 16: 1047–1057
- Ramseier TM (1996) Cra and the control of carbon flux via metabolic pathways. *Research in Microbiology* 147: 489–493
- Roitsch T, Bittner M and Godt DE (1995) Induction of apoplastic invertase of *Chenopodium rubrum* by D-Glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. *Plant Physiol* 108: 285–294
- Saier MH (1996) Regulatory interactions controlling carbon metabolism—an overview. *Research In Microbiology* 147: 439–447
- Saier MH, Chauvaux S, Deutscher J, Reizer J and Ye JJ (1995) Protein-phosphorylation and regulation of carbon metabolism in gram-negative versus gram-positive bacteria. *Trends Biochem Sci* 20: 267–271
- Salanoubat M and Belliard G (1989) The steady state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis, and sucrose concentration. *Gene* 84: 181–185
- Sano H and Youssefian S (1994) Light and nutritional regulation of transcripts encoding a wheat protein kinase homolog is mediated by cytokinins. *Proc Natl Acad Sci USA* 91: 2582–2586
- Sauer N and Tanner W (1993) Molecular biology of sugar transporters in plants. *Bot Acta* 106: 277–286
- Scheible WR, GonzalezFontes A, Lauerer M, Muller-Röber B, Caboche M and Stitt M (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* 9: 783–798
- Sheen J (1990) Metabolic repression of transcription in higher plants. *Plant Cell* 2: 1027–1038
- Sheen J (1994) Feedback control of gene expression. *Photosynth Res* 39: 427–438
- Smeekens and Rook F (1997) Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiology* 115: 7–13
- Spena A and Schell J (1987) The expression of a heat inducible chimeric gene in transgenic tobacco plants. *Mol Gen Genet* 206: 436–440
- Stitt M, von Schaewen A and Willmitzer L (1991) ‘Sink’-regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell walls involves a decrease in the Calvin cycle enzymes and an increase in glycolytic enzymes. *Planta* 183: 40–50
- Stitt M, Krapp A, Klein D, Roper-Schwarz U and Paul M (1995) Do carbohydrates regulate photosynthesis and allocation by altering gene expression? Carbon Partitioning and Source Sink Interactions in Plant. In: Madore MA and Lucas WJ (eds) *Current Topics in Plant Physiology*, Vol 13, pp 68–77. The American Society of Plant Physiologists, Rockville
- Sturm A and Chripeels M (1990) cDNA cloning of carrot

- extracellular β -fructosidase and its expression in response to wounding and bacterial infection. *Plant Cell* 2: 1107–1119
- Takeda S, Mano S, Ohto M and Nakamura K (1994) Inhibitors of protein phosphatases 1 and 2A block the sugar-inducible gene expression in plants. *Plant Physiol* 106: 567–574
- Thorsteinsson B and Tillberg J (1990) Changes in photosynthesis/respiration ratio and levels of few carbohydrates in leaves of nutrient-depleted barley and pea. *J Plant Physiol* 136: 532–537
- Trumbly RJ (1992) Glucose repression in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* 6: 15–21
- Turgeon R (1989) The sink source transition in leaves. *Ann Rev Plant Physiol Plant Mol Biol* 40: 119–138
- Turk S, DeRoos K, Scotti PA, VanDun K, Weisbeek P and Smeekens SCM (1997) The vacuolar sorting domain of sporamin transports GUS, but not levansucrase, to the plant vacuole. *New Phytologist* 136: 29–38
- Urwin NAR and Jenkins GI (1997) A sucrose repression element in the *Phaseolus vulgaris* rbcS2 gene promoter resembles elements responsible for sugar stimulation of plant and mammalian genes. *Plant Mol Biol* 35: 929–942.
- Van Oosten JJ and Besford RT (1994) Sugar feeding mimics effect of acclimation to high CO₂—rapid down regulation of RuBisCo small subunit transcripts but not of the large subunit transcripts. *J Plant Physiol* 143: 306–312
- Van Oosten JJ and Besford RT (1996) Acclimation of photosynthesis to elevated CO₂ through feedback-regulation of gene-expression—climate of opinion. *Photosynth Res* 48: 353–365
- Van Oosten JJ, Wilkins D and Besford RT (1994) Regulation of the expression of photosynthetic nuclear genes by CO₂ is mimicked by regulation by carbohydrates—a mechanism for the acclimation of photosynthesis to high CO₂? *Plant Cell Environ* 17: 913–923
- Van Oosten JJM, Gerbaud A, Huijser C, Dijkwel PP, Chua NH and Smeekens SCM (1997) An *Arabidopsis* mutant showing reduced feedback inhibition of photosynthesis. *Plant J* 12: 1011–1020
- Visser RGF, Stolte A and Jacobsen E (1991) Expression of a chimeric granule-bound starch synthase-GUS gene in transgenic potato plants. *Plant Mol Biol* 17: 691–699
- von Schaewen A, Stitt M, Schmidt R, Sonnewald U and Willmitzer L (1990) Expression of yeast derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J* 9: 3033–3044
- Wenzler HC, Mignery GA, Fisher LM and Park WD (1989) Analysis of a chimeric class-I *PATATIN-GUS* gene in transgenic potato. *Plant Mol Biol* 12: 41–50
- Wilson WA, Hawley SA and Hardie DG (1996) Glucose repression/derepression in budding yeast—SNF1 protein-kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP-ATP ratio. *Curr Biol* 6: 1426–1434
- Woods A, Munday MR, Scott J, Yang X, Carlson M and Carling D (1994) Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. *J Biol Chem* 269: 19509–19515
- Xu J, Avigne WT, McCarty DR and Koch KE (1996) A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism—evidence from a maize invertase gene family. *Plant Cell* 8: 1209–1220
- Zhou L, Jang J-C, Jones TL and Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. *Proc Nat Acad Sci USA* 95: 10294–10299
- Zrenner R, Salanoubat M, Willmitzer L and Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant J* 7: 97–107

Chapter 11

Intercellular Transport and Phloem Loading of Sucrose, Oligosaccharides and Amino Acids

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Summary

Photoassimilate export from the mesophyll and the processes underlying phloem loading are central to the efficient growth, competitive ability, and reproductive success of a plant. The assimilate flux out of the leaf is regulated on a number of levels. For example, structural aspects including the spatial organization of individual cell types within the leaf and the extent of the symplasmic connections between these cell types control these fluxes at the cellular level. Phloem loading can follow a symplasmic route, or involve an apoplastic step within the vicinity of the companion cell-sieve element (CC-SE) complex. In the latter case, apoplastic transfer is regulated by the capacity of the individual cell types to engage in efflux or uptake (retrieval) of photoassimilates.

Within the autotrophic tissues of the leaf, photoassimilate flow may be regulated through feed-back mechanisms that can modify biochemical pathways, plasmodesmal conductivity, and membrane transport properties. At the membrane transport level, molecular techniques have led to the isolation and characterization of transporters operating at the site of phloem loading. These studies allowed for the molecular manipulation of such transport systems and now offer a powerful method to advance our understanding of the events that underlie both phloem loading and photoassimilate allocation. The recent discovery that plasmodesmata can mediate the cell-to-cell transport of macromolecules suggests that these unique structures may play a role not only in assimilate transport, but also in the integration of cellular processes. A model is proposed where regulatory macromolecules move cell to cell, within the leaf, as well as long-distance, via the phloem, to serve in the coordination and regulation of physiological events taking place in source and sink tissues.

I. Introduction

Assimilates synthesized in the mesophyll cells of mature leaves are the fuel for metabolism and provide the building blocks for growth in all non-photosynthetic plant tissues, including developing leaves, stems, roots, flowers and seeds. Within the leaf, photosynthate is translocated into the conducting tissue of the phloem and then distributed throughout the plant. This process—fundamental for functioning of the specialized, composite higher plant organism—has attracted the attention of plant scientists for decades, sparked passionate discussions on the mechanisms and pathways involved, and is now beginning to be explored by more detailed molecular investigation. Progress in our understanding of the underlying mechanisms associated with assimilate transport and allocation is currently due in large part to the molecular cloning of membrane transporters which has permitted the detailed molecular characterization of their involvement in phloem loading. In addition, new insights into the functioning of plasmodesmata have expanded our understanding of how these intercellular channels, which are unique to plants, may control symplasmic transport and intercellular communication in higher plants. The available evidence suggests that both membrane transport and symplasmic communication and/or flux of assimilates are essential components for photosynthate transfer from the mesophyll into the vascular tissue where subsequent phloem loading takes place.

In this chapter we describe recent progress in our understanding of photoassimilate transport through the use of molecular and cell biological approaches. These findings are discussed in relation to the complex process of assimilate transfer from the site of photosynthesis within the mesophyll to the site of loading into the companion cell-sieve element (CC-SE) complex of the minor vein network in the leaf.

II. Photoassimilate Movement from the Mesophyll to the Phloem

As illustrated in Fig. 1, the basic morphology and anatomy of the leaf are optimized for efficient light harvesting and CO_2 fixation in the mesophyll tissue. Photoassimilates—sugars and amino acids—produced in individual cells have to pass through one, or several, cell layers (Fig. 1B,C) to arrive at a minor vein which functions as the site of assimilate loading into the long-distance transport conduit (Fig. 1D)—the sieve tube system of the phloem (Geiger, 1975). In essence, two pathways are available for the transfer of assimilates: the apoplasm and the symplasm. Assimilates may exit across the mesophyll plasma membrane into the apoplastic space (cell walls and regions exterior to the plasma membrane) from where they can move, by the process of diffusion, into a neighboring minor vein (Fig. 2A,B). Alternatively, as mesophyll cells are interconnected by plasmodesmata (complex cytoplasmic bridges that provide for a spatial continuum of the cytoplasmic domain between cells to form the symplasmic space), assimilates can also diffuse within the symplasm from cell to cell. Following an evaluation of the experimental evidence for the participation of these two routes, we consider the implications of these two pathways in terms of likely regulatory mechanisms.

Abbreviations: BS – bundle sheath; CC-SE – companion cell-sieve element complex; EM – electron microscopy; PCMBS – p-chloromercuri-benzenesulfonic acid; SEL – size exclusion limit; SER – sieve element reticulum; TMV-MP – tobacco mosaic virus movement protein; VP – vascular parenchyma

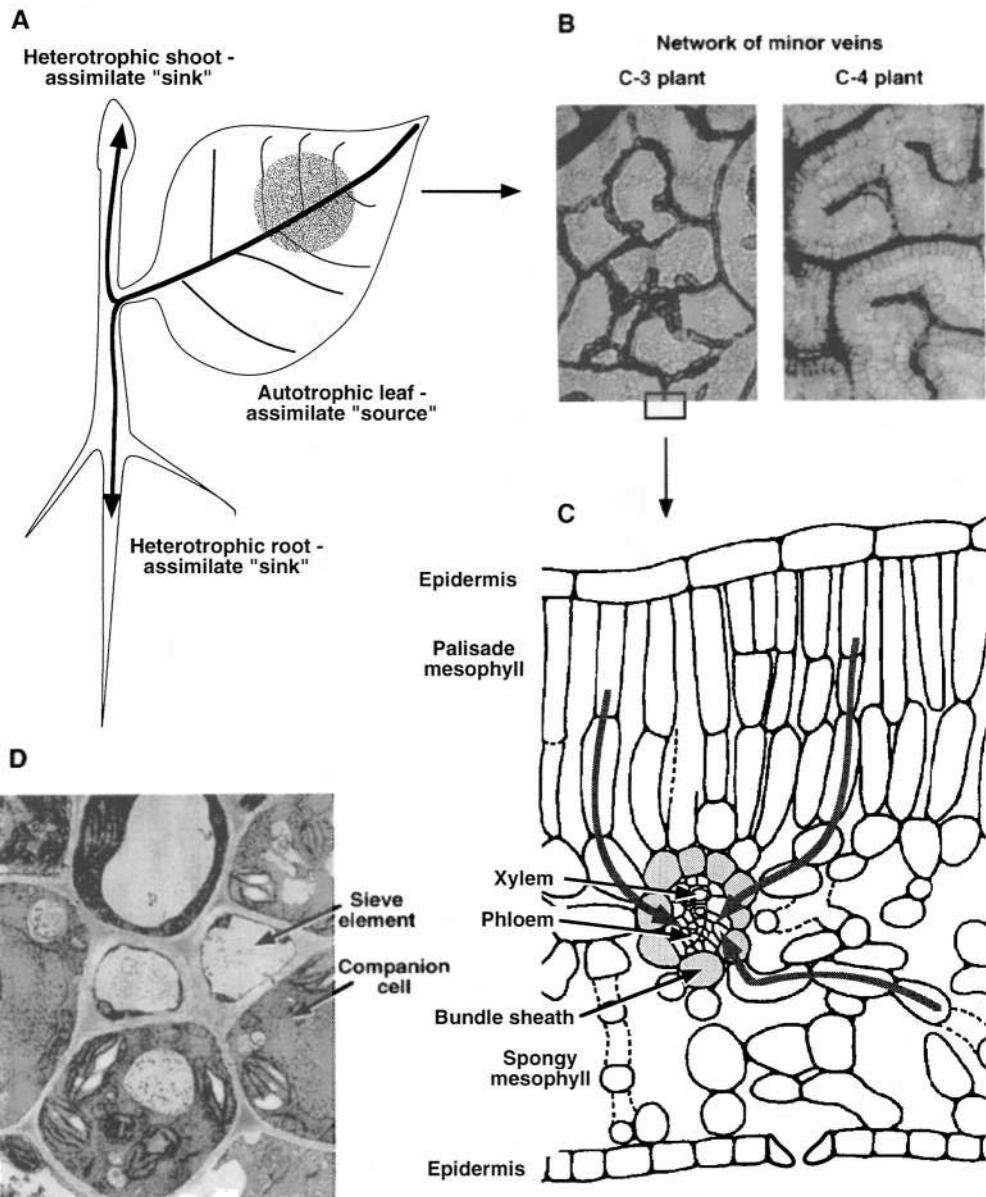


Fig. 1. Assimilate export in higher plants. A, Schematic representation of the plant, illustrating the general relationship between autotrophic leaves and heterotrophic tissues. Photoassimilates produced in the mesophyll are loaded into the minor veins (circle) and the phloem sap then moves into the major vein (dart) of the autotrophic leaf (source) for long-distance translocation (arrows) to the heterotrophic shoot and root (sink) tissues. (Not to scale) B, Illustration of the differences in the minor vein network present in leaves of C₃ (*Coronilla varia*) and C₄ (*Euphorbia maculata*) plants. Note the presence of the predominate bundle sheath cells lining the minor veins in the C₄ leaf. C, Cross sectional drawing of a typical C₃ leaf, illustrating the spatial relationship between the abaxial palisade mesophyll (parenchyma) and adaxial spongy mesophyll cells and the minor vein. The bundle sheath cells (shaded) form a special interface between the mesophyll and the vascular tissues of the xylem and phloem. Shaded arrows depict the pathway followed by photoassimilates from the mesophyll to the site of loading in the phloem. D, Ultrastructural details associated with the companion cell-sieve element complex at the site of loading within a minor vein of a tobacco (*Nicotiana tabacum*) leaf (× 5,200).

A. Photoassimilate Flux through the Apoplastic Pathway

Exchange of solutes across membranes is a general feature of all living cells. Therefore, it is not surprising that a number of studies have demonstrated that leaf discs and mesophyll protoplasts release sugars and amino acids into the surrounding medium—a process which presumably occurs via carrier-mediated mechanisms (Fig. 2) (Secor and Schrader, 1984; Daie, 1989; Laloi et al., 1993; Perez-Alfocea and Larher, 1995). However, in the intact leaf the situation appears to be more complex. It is unfortunate that, because of inherent experimental limitations, only a limited number of attempts have been made to quantify assimilate pools in the apoplasm of source leaves (Ntsika and Delrot, 1986). For this reason, it is not clear how much of the assimilates that are released into the apoplasm, across the mesophyll plasma membrane, move within this compartment for direct transfer to the minor vein. In this context it would be important to ascertain whether there are differential rates of release from mesophyll cells adjacent to the minor veins versus those more distantly located (see Fig. 1C).

The basic question that remains to be resolved is whether the release of sugars and amino acids is controlled by unique efflux mechanisms, or occurs by leakage through carriers normally engaged in transport into the symplasmic compartment (i.e., retrieval of solutes from the apoplasmic compartment). Certainly, uptake into the mesophyll has been demonstrated in numerous systems (Maynard and Lucas, 1982; Wilson and Lucas, 1988; Theodoropoulos and Roubelakis-Angelakis, 1991; Flora and Madore, 1993; Flora and Madore, 1996; Van Bel et al., 1996). Furthermore, both sugar and amino acid carriers have been studied using plasma membrane vesicles isolated from leaves (Bush, 1989, 1993; Gallet et al., 1989; Tubbe and Buckhout, 1992; Buckhout, 1994). Finally, promoters from mono- and disaccharide transporters have been shown to drive expression in mesophyll tissue (Sauer et al., 1994) and the mesophyll plasma membrane has similarly been shown to contain carrier protein for sucrose (Lemoine et al., 1989). These findings are consistent with the concept that carriers regulate the level of sugars within the mesophyll apoplasm. This situation most probably applies also for assimilates such as amino acids, as well as oligosaccharides and

polyols in plant species where these compounds are synthesized.

A careful analysis of the sucrose flux in the *Ricinus* cotyledon established that, in this system, at least 50% of the sucrose mobilized in the endosperm followed an entirely apoplastic pathway through the mesophyll into the minor veins (Orlich and Komor, 1992). However, whereas the importance of an apoplastic route through the mesophyll has been firmly established in the *Ricinus* cotyledon, extrapolating from this experimental system to the situation of a transpiring leaf may be difficult.

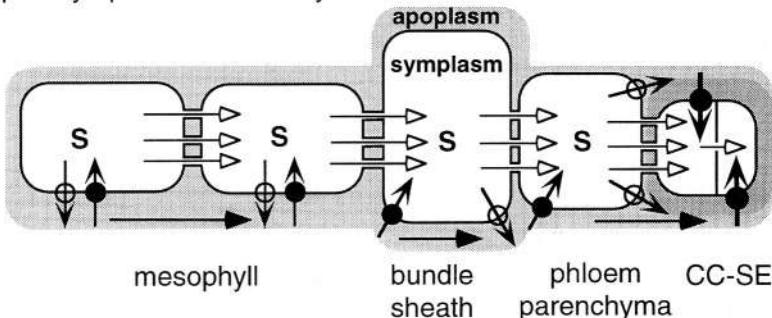
It has often been proposed that the flow of assimilate to the veins would have to be separated from the rapid transpirational flow of water within the leaf (Stitt, 1996). This problem could be avoided if the water coming up the xylem were to enter the vapor phase in the immediate vicinity of the xylem vessels (Boyer, 1985) or be channeled through specific symplasmic routes to evaporation sites next to the stomatal pores. In addition, major transpirational water flow away from the xylem may occur through the walls of the bundle sheath extension cells (see Fig. 6A), followed by lateral transport along the epidermis (Canny, 1993). As minor veins generally lack these extensions, diffusion of solutes through the mesophyll apoplasm may not be disturbed by transpirational water flow (Canny, 1990). Finally, as the apoplastic pathway for assimilate flow could provide a ready source of carbon and nitrogen for invading microbial pathogens, plants would have to react through the development of a variety of defense strategies (Bent, 1996; Dangl et al., 1996; Jackson and Taylor, 1996).

Additional evidence for the presence of an apoplastic sucrose pool within the leaf has been provided by studies on transgenic plants that express and target a yeast acid invertase into the cell wall. In such tobacco (Von Schaewen et al., 1990), tomato (Dickinson et al., 1991) and potato plants (Heineke et al., 1992), starch and sugar levels were found to increase in the leaves, export was reduced and source leaves underwent early senescence. These physiological changes are consistent with cleavage of sucrose, bound for export, in the apoplasm followed by retrieval of monosaccharides predominantly into mesophyll cells. Interestingly, a severe phenotype was not observed in *Arabidopsis* plants expressing an extracellular invertase (Von Schaewen et al., 1990). Finally, the development of secondary plasmodesmata

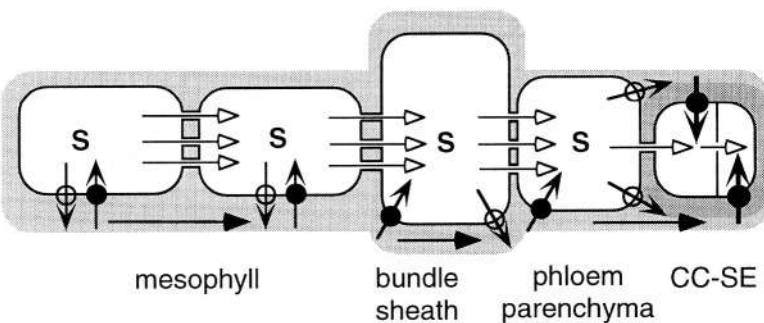
Pathways for Photoassimilate Export

A

Group I - symplasmic continuity

**B**

Group II - near symplasmic isolation of CC-SE complex

**C**

Group II - apoplastic diffusion barrier at the bundle sheath

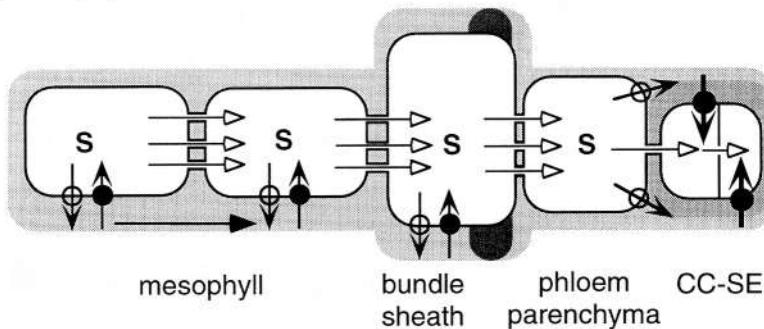


Fig. 2. Potential apoplastic and symplasmic pathways for photoassimilate flux from the mesophyll into the companion cell-sieve element (CC-SE) complex. S denotes a transported photoassimilate; open arrows represent diffusion through the symplasm, via plasmodesmata; closed arrows illustrate diffusion through the apoplasm, via hydrated cell walls. Membrane-bound active and passive sugar transport systems are represented by solid and open circles, respectively. Suberin deposition in the radial cell walls of the bundle sheath is depicted by dark shading in C; these deposits appear to form a barrier to diffusion, within the apoplasm, between the cells of the vascular tissue and the mesophyll.

was found to be inhibited in tobacco leaves which overexpress cell wall invertase (Ding et al., 1993). This latter observation strongly suggests that, at some level, plasmodesmata formation is regulated and coupled to assimilate export.

As the yeast invertase was expressed in these transgenic plants under the control of the 35S CaMV promoter, which is known to drive transcription in mesophyll and phloem tissues, and as the explicit cellular location of the acid invertase within these tissues was not established, it is not clear which apoplastic sucrose pool was intercepted in these transgenic plants that were expressing yeast acid invertase. The range of observed phenotypes may be explained by a varying degree of transgene invertase activity in the minor vein apoplasm, near the presumptive site of loading from the apoplastic compartment into the phloem. Much more would be learned about the relative importance to export of sucrose pools in the apoplasm of the mesophyll and the phloem, by overexpressing the cell wall invertase in the respective tissue via promoters specific for the mesophyll and the phloem (e.g., as was done recently with a viral movement protein in potato: Olesinski et al., 1996).

In some species, transfer of assimilates from the mesophyll to the minor vein via the apoplasm may be blocked by suberin deposition in the cell walls of the bundle sheath (Canny, 1990; Robinson-Beers and Evert, 1991; Botha, 1992; Evert et al., 1996). In this situation, solutes in the mesophyll apoplasm would not be able to pass directly to the site of loading in the minor vein, but rather, they would have to be retrieved by plasma membrane carriers and then pass, symplasmically, across the bundle sheath boundary (Fig. 2C).

It is unfortunate that for the intact photosynthetic leaf it has not been feasible to directly quantify the amount of assimilates that are released into the mesophyll apoplasm. Hence, the importance of the apoplastic route for assimilate transfer across the mesophyll for delivery into the minor vein remains to be resolved. The identification of sucrose and amino acid permeases that may be involved in the controlled transfer of photoassimilates into the apoplasm would certainly open the way for more definitive experiments.

B. Photoassimilate Flux through the Symplasmic Pathway

Mesophyll cells are generally highly interconnected

by plasmodesmata establishing a semi-selective symplasmic pathway which usually extends from the mesophyll through the bundle sheath and into cells of the minor vein. In some plant families, this high degree of symplasmic continuity extends all the way up to the long-distance transport pathway, which comprises files of individual companion cell-sieve element complexes (CC-SE; see Fig. 1D, Fig. 2A). (For a detailed description of plasmodesmal structure, the reader is referred to Lucas et al., 1993a; Overall and Blackman, 1996; Ding, 1997).

Dye injection studies have shown that, in general, plasmodesmata are open for the passage of small molecules (< 1 kDa) and will permit diffusion of such molecules (metabolites) from the mesophyll into the minor vein (Robards and Lucas, 1990). Thus, it is now widely accepted that assimilates may travel from the mesophyll into the minor vein through this symplasmic route. Nevertheless, it has to be borne in mind that the presence of plasmodesmata, as revealed by electron microscopy (EM) studies, does not necessarily indicate that an intercellular pathway is available for the passage of solutes. It is well known that plasmodesmata can be closed by callose deposition (Robards and Lucas, 1990); e.g., after wounding or pathogen attack. Even more important for the regulation of symplasmic photoassimilate transfer is the finding that plasmodesmal conductivity (open or closed state) may be regulated by pressure gradients between neighboring cells (Oparka and Prior, 1992).

The importance of plasmodesmata to the control of assimilate export may be reflected in the coordination of secondary plasmodesmal formation in leaves and the onset of assimilate export. For example, the transition from importing sink tissues to exporting source tissues was found to coincide with the formation of secondary plasmodesmata in the mesophyll of maize (Evert et al., 1996) and with modifications of plasmodesmata in the minor veins of cucurbit (Volk et al., 1996). These observations indicate that formation and/or modification of plasmodesmata can affect the capacity of the symplasmic pathway for the transfer of assimilates. Thus, a dynamic control over plasmodesmal properties could exert a powerful structural level of control over photosynthate export. Indeed, plasmodesmal frequency has been widely used as a measure of symplasmic continuity and taken as an indication of the capacity for symplasmic solute exchange between cells (Van Bel et al., 1988; Beebe and Evert, 1992; Van Bel et al., 1992).

This simplified view of plasmodesmal frequency should be perceived with some caution (Robards and Lucas, 1990; Van Bel and Oparka, 1995). At present there is a paucity of experiments that address the operational characteristics of plasmodesmata. For example, we do not have available experimental values for the conductivity of individual mesophyll plasmodesma in terms of the cell-to-cell diffusion of assimilates. In the absence of this type of information it is presently impossible to develop a quantitative model for the symplasmic route from the mesophyll into the minor veins. In this respect—and this also applies to apoplastic fluxes across the mesophyll—our knowledge has remained more metaphysical than biophysical in nature.

As symplasmic transfer, through plasmodesmata, of small molecules such as photoassimilates is assumed to be driven by diffusion, downhill concentration gradients from the mesophyll to the minor vein should exist for the respective metabolite. For the exchange of C_4 intermediates between the mesophyll and the Kranz cells (specialized bundle sheath cells; see Fig. 3A), such diffusion gradients have been established (Weiner et al., 1988). Unfortunately, the situation is less clear for photoassimilates which are actually exported from the leaves both in C_3 and C_4 plants, due to a lack of information on the relevant assimilate concentrations in the cytoplasm. As the cytoplasmic compartment in the mesophyll is below 10%, and most of the cell volume is occupied by vacuoles, exact data on cytoplasmic sucrose concentrations are difficult to obtain, although feasible for a uniform population of culture cells (Preißer et al., 1992). But it would be difficult, if not impossible, to extrapolate from cell suspension experiments to conditions in the cytoplasm of mesophyll, bundle sheath and phloem parenchyma cells within intact source leaf tissues.

An approach designed to gain empirical insight into the process of symplasmic assimilate transfer used transgenic tobacco plants expressing the viral movement protein of tobacco mosaic virus (TMV-MP). In this experimental system, the TMV-MP has the capacity to increase the size exclusion limit (SEL) of mesophyll plasmodesmata (Wolf et al., 1991; Wolf et al., 1989). If diffusion through plasmodesmata were rate-limiting for sucrose export, this TMV-MP-induced increase in SEL would be expected to result in a stimulation of translocation from the source leaves. However, analysis of carbohydrate levels in the mesophyll and determination of [^{14}C]-export rates revealed that sugar and

starch levels were elevated and export was reduced in these TMV-MP expressing plants (Lucas et al., 1993b; Olesinski et al., 1995). The impact of these results on the concepts of how photosynthate export may be regulated will be discussed later.

These findings on transgenic tobacco plants and other recent insights into plasmodesmal structure and function have challenged the viewpoint that plasmodesmata are simple pores for diffusion of small molecules (Lucas et al., 1993a; Epel, 1994). Microinjection studies have now established that viral movement proteins (Fujiwara et al., 1993; Noueiry et al., 1994; Waigmann et al., 1994) and the plant transcription factor, KNOTTED 1 (Lucas et al., 1995), are able to traffic through plasmodesmata and mediate the cell-to-cell transfer of nucleic acids. These results stimulated the development of novel hypotheses regarding the supracellular nature of plants (Lucas et al., 1993a) and the control of plant development and physiology (Lucas, 1995; Mezitt and Lucas, 1996).

*C. Disruption to Symplasmic Transfer of Photoassimilates in the Maize Mutant *sxd1**

In C_4 plants such as maize, CO_2 is fixed in the mesophyll into C_4 acids (malate) by phosphoenolpyruvate carboxylase (Fig. 1B, Fig. 3A). Malate passes through plasmodesmata into the bundle sheath cells where CO_2 is released for subsequent fixation in the chloroplasts. Interestingly, although the chloroplasts in both bundle sheath and mesophyll cells have the capacity to store starch, the enzymes involved in sucrose synthesis appear to be predominantly located in the mesophyll (Lunn and Furbank, 1997). Thus, plasmodesmata play a central role in facilitating the exchange of intermediates involved in CO_2 fixation and sucrose synthesis. (For more details on various aspects of C_4 photosynthesis the reader is referred to chapters 19 (Leegood), 20 (Dengler and Taylor), 21 (Sage and Pearcy), 22 (Monson and Rawsthorne) and 23 (Cushman et al.))

The importance of plasmodesmata and the symplasmic route in mediating the delivery of sucrose to the site of loading in the phloem gained strong support from a recent study by Russin et al. (1996). These workers reported that sucrose levels in a mutant maize line, termed *sucrose export deficient 1* (*sxd1*), were elevated whereas phloem export was dramatically reduced. An ultrastructural examination of wild-type and *sxd1* leaf tissues revealed that, in this mutant line, the plasmodesmata interconnecting the

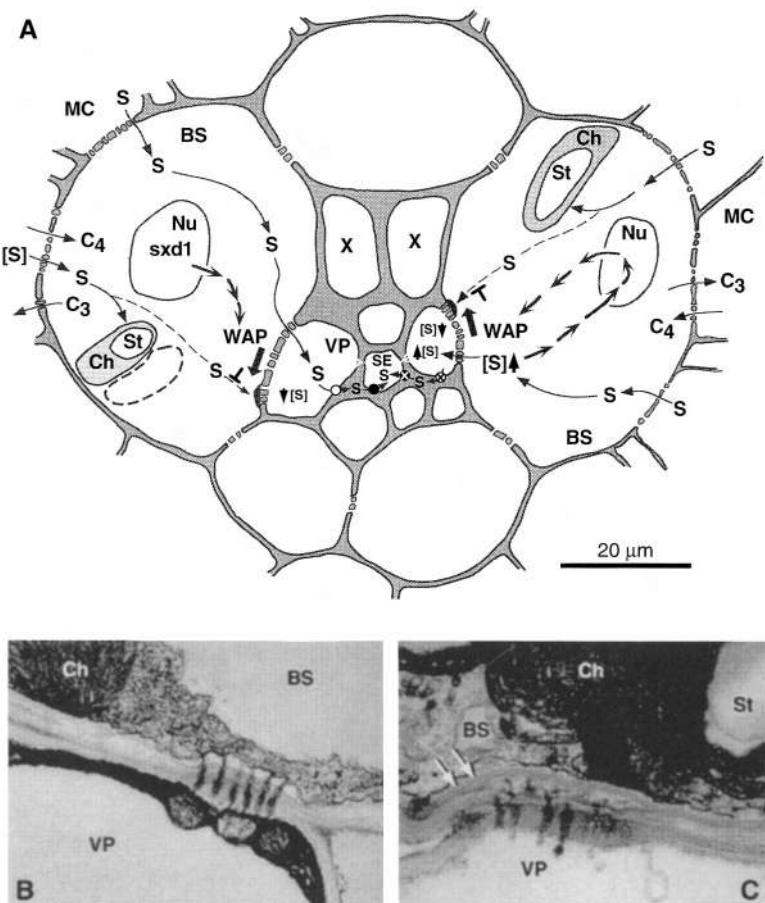


Fig. 3. Essential role of plasmodesmata in photoassimilate transfer for phloem loading demonstrated by the *sxd1* mutation in maize. **A,** Cross sectional drawing of a minor vein within the source leaf of maize (*Zea mays*) illustrating the exchange of metabolites, via plasmodesmata, between the mesophyll and bundle sheath (BS) cells and the symplasmic pathway followed by sucrose from the mesophyll into the vascular parenchyma (VP)/CCs. Two scenarios to account for the phenotype associated with the maize *sxd1* mutant are illustrated. On the left, *sxd1* is shown to cause the activation of a set of genes involved in the sealing of plasmodesmata via wall apposition (WAP). Note that the newly deposited wall material is restricted to the plasmodesmata that connect the BS cells to the VP (or companion) cells, thereby forming a cap, which disrupts the symplasmic continuity between the mesophyll and the CC-SE complex and prevents delivery of sucrose to the phloem loading sites. On the right, *sxd1* is depicted as a mutation in either the passive or active sucrose transport systems thought to be involved in phloem loading into the SEs (illustrated by crossed-circles). In this situation, wall apposition across the BS-VP plasmodesmata is shown to occur as a pleiotropic effect of the increase in sucrose within the BS which would occur when sucrose loading was blocked by the putative dysfunctional sucrose transport system. **($\times 550$)** **B,** Plasmodesmata interconnecting BS and VP cells in wild-type maize source leaves. **($\times 38,000$)** **C,** Sealed plasmodesmata at the BS-VP interface in mature leaves of *sxd1* maize plants (arrows indicate the presence of wall deposits). Symbols are as follows: Ch, chloroplast; C₃, pyruvate; C₄, malate; MC, mesophyll cell; Nu, nucleus; S, sucrose; St, starch; X, xylem vessels. **($\times 38,000$)** (Images in B and C kindly provided by Dr. William Russin, University of Wisconsin)

bundle sheath and vascular parenchyma cells had undergone some form of blockage (Fig. 3B, C). Careful inspection of the transmission electron micrographs of these plasmodesmata revealed that they appeared to be sealed by apposition of additional wall material (Fig. 3C). A similar phenomenon has been reported in studies conducted on guard cells

(Wille and Lucas, 1982), where sealing of plasmodesmata connecting guard cells to the neighboring epidermal cells appears to be essential for the onset of stomatal function. In *sxd1* mutant plants, plasmodesmal closure at the bundle sheath–vascular boundary was restricted to the leaf blade; normal plasmodesmal structures within the leaf base

apparently permitted the delivery of sufficient photosynthate to apical tissues to alleviate the severity of the *sxd1* mutation on plant development.

Two possible scenarios for the manner in which the *sxd1* mutation might elicit the observed phenotype are presented in Fig. 3A. As the sealing of the plasmodesmata occurs later in leaf development (W. Russin, personal communication) and is restricted to plasmodesmata interconnecting bundle sheath–vascular parenchyma (or companion) cells (BS-VP/CC) in the leaf blade (Russin et al., 1996), the *sxd1* mutation may be located within a repressor gene which, in the mutant state, allows ectopic expression of a unique developmental program in the bundle sheath cells. This program then orchestrates the directed apposition of new wall material (WAP in Fig. 3A) across the orifice of plasmodesmata located at the BS-VP/CC boundary. The finding that plasmodesmata interconnecting bundle sheath cells, as well as between bundle sheath and mesophyll cells, were structurally unaffected by the *sxd1* mutation implicates the involvement of positional-dependent information (Fig. 3A; left schematic).

Once the plasmodesmata connecting BS-VP/CC cells have been sealed, symplasmic passage of sucrose into the VP/CC would be prevented, thereby causing the observed increase in soluble sugars in *sxd1* maize plants. In response to elevated sugar levels, chloroplasts in both BS and mesophyll cells would deposit enhanced quantities of starch, as was observed by Russin et al. (1996).

Another interesting feature of the *sxd1* mutant was the finding that during tissue preparation for EM, VP/CC cells were prone to plasmolysis. This would be consistent with closure of the main route for sucrose entry to the site of phloem loading, which would result in a severe depletion of sugar levels within phloem cells. Under these circumstances, the high osmotic potential within these cells would allow plasmolysis to take place in a highly cell-specific manner.

An alternate explanation for the structural and physiological changes induced by *sxd1* is that this mutation alters a critical aspect of sucrose loading into the sieve element. This impediment to sucrose loading could reflect the synthesis of a dysfunctional transporter that is engaged in either the release of sucrose into the apoplasm or its energy-dependent loading into the SE. In this situation, sucrose levels within the VP/CC and BS cells would be expected to quickly increase to a level that may activate a sugar-

induced regulatory cascade. A pleiotropic effect of this cascade could be the activation of the genes involved in sealing the plasmodesmata at the BS-VP/CC boundary (Fig. 3A; right schematic).

Identification of the *SXD1* gene will provide a test for the two scenarios depicted in Fig. 3A. In addition, knowledge of the cellular functions controlled by *SXD1* may well provide a powerful tool for the further analysis of the impact of the symplasmic pathway on physiological and developmental processes in plants.

D. Oligosaccharides and Polyols as Transport Sugars

Most of the experimental data mentioned above were obtained from studies conducted on plants that are either important agricultural plants, such as maize, potato and tomato (Stitt, 1996), or *Ricinus* which lends itself to the study of phloem transport (Komor et al., 1996). All these plants share sucrose as the dominant sugar in the phloem translocation stream. Indeed, a survey of 97 monocot and dicot plant families indicated that sucrose is the exclusive form of sugar translocated in the phloem of some 50 plant families, whereas in a further 33 families other forms of polysaccharides are translocated (Zimmermann and Ziegler, 1975). Raffinose-type oligosaccharides and polyols (e.g., mannitol and sorbitol) represent a major transport compound in 14 and 6 plant families, respectively (Table 1). In every case, sucrose is also present in the translocation stream and at times it is present at a high concentration.

With the presence of various sugars in the leaf, the question arises as to which sugars are synthesized in the mesophyll for transport to the minor veins and which are subsequently loaded into the sieve tube. For example, in *Cucumis melo* leaves sucrose and galactinol are found in both the mesophyll and the minor vein in high concentration, but raffinose and stachyose are mostly restricted to the cells of the minor vein (Haritatos et al., 1996). These findings are consistent with sucrose and galactinol moving from the mesophyll into the phloem where they act as precursors for the synthesis of raffinose and stachyose in the intermediary cells (see Fig. 4) (Beebe and Turgeon, 1991). Similarly, in olive plants, sucrose, galactinol and mannitol are found in the mesophyll, but only stachyose and sucrose are exported (Flora and Madore, 1993). However, in

species, sucrose and mannitol are rapidly labeled

Table 1. Major sugars and polyols translocated in the phloem of plants. The number of plant families is listed where the respective assimilate was found as a dominant (10–30%) or an additional (<10%) component in the phloem sap. Data were compiled from Zimmermann and Ziegler (1975), van Die and Tammes (1975), Fukumorita and Chino (1982) and Weiner et al. (1991). Values in parentheses represent the number of families where the respective assimilate is the exclusive transport form.

Phloem sap constituents	Dominant sugar	Minor constituent	Constituent not detected
Sucrose	83 (50)	14	—
Raffinose-type sugars	14 (0)	40	43
Polyols	6 (0)	33	58

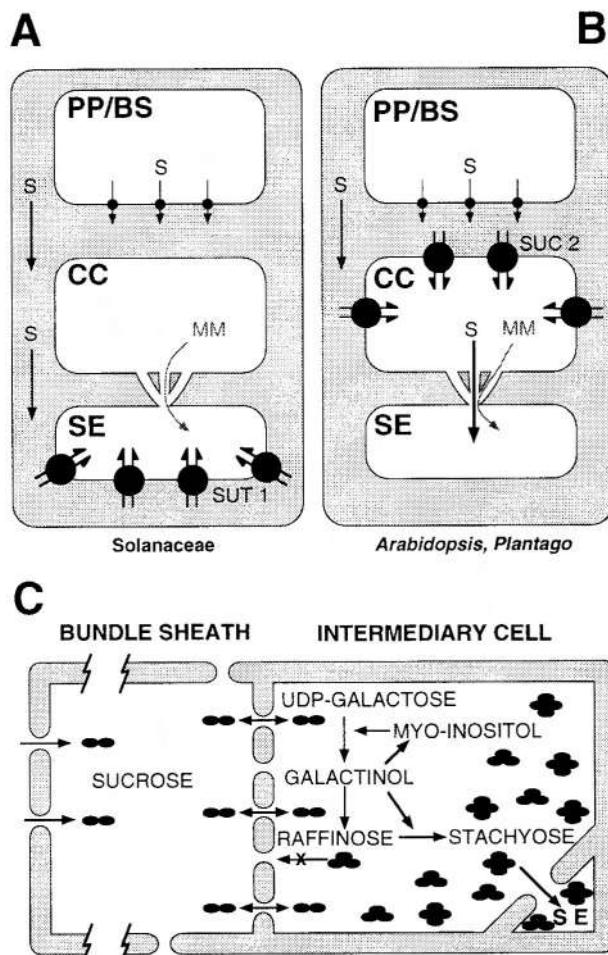


Fig. 4. Models for phloem loading. A, Direct loading of sucrose (S) by the sucrose-H⁺ transporter (SUT1) into the sieve element (SE) after release of sucrose from phloem parenchyma or bundle sheath cells (PP/BS), as suggested by immunolocalization of SUT1 to the SE plasma membrane in Solanaceae species. B, Localization of the sucrose-H⁺ carrier (SUC2) from *Arabidopsis* and *Plantago* in the companion cell (CC) would indicate loading of sucrose via the CC plasma membrane and symplasmic flow of sucrose from the companion cell into the sieve element through branched plasmodesmata. Movement of macromolecules (MM) from the companion cell into the sieve element (SE) occurs in both cases. C, Hypothesis for symplasmic phloem loading via a novel polymer trap mechanism (see text for details). (Modified after Grusak et al., 1996.)

in [$^{14}\text{CO}_2$] pulse-chase experiments and then both forms appear to move from the mesophyll to the minor vein for export from the leaf (Flora and Madore, 1996).

Little information is available on the mechanisms which mediate uptake and/or retrieval of oligosaccharides and polyols across the plasma membrane in plant species which synthesize raffinose-type sugars and polyols. That carriers for polyols exist was shown by uptake studies using leaf discs from parsley (Flora and Madore, 1996) and plasma membrane vesicles from celery petioles (Salmon et al., 1995). Raffinose-induced membrane depolarization also indicate the presence of uptake mechanisms for this type of oligosaccharide in mesophyll cells of *Catharanthus* and *Ocimum* (Van Bel et al., 1996). The challenge ahead will be to develop appropriate molecular cloning techniques to further advance our knowledge of the nature and evolution of these oligosaccharide transport systems.

E. Amino Acid Transfer from the Mesophyll to the Phloem

Depending on plant species and nitrate availability, net amino acid synthesis may proceed to a considerable extent in mesophyll tissues (Andrews, 1986). This allows for direct use of photosynthetic energy in the reduction process and favors shoot over root growth (Burns, 1994; Beck, 1996). As discussed above for sucrose, amino acids may use apoplasmic and/or symplasmic routes for transfer into the minor veins. The respective route taken may depend on the nitrogen supply which can result in large variations in the levels of individual amino acids within the leaf (Shelp, 1987; Schobert and Komor, 1989).

Several classes of amino acid transporter genes have been cloned by yeast complementation studies using cDNAs from *Arabidopsis* (Frommer et al., 1993; Hsu et al., 1993; Kwart et al., 1993; Fischer et al., 1995; Frommer et al., 1995; Rentsch et al., 1996; Chen and Bush, 1997). Using this information and sequence similarities to known amino acid transporters, these plant systems can be classified into two major superfamilies: the plant cationic amino acid transporters (CATs) and the amino acid transporter family 1 (ATF1). The CAT genes are related to the mammalian family of cationic amino acid transporters. To date, only a single member of this family has been identified from plants. The gene encoding this transporter, *AtCAT1*, was cloned by

complementation of a yeast histidine transport mutant (Frommer et al., 1995). Sequence analysis indicated that *AtCAT1* is likely composed of 14 putative membrane spanning domains. Based on indirect evidence, it would appear that *AtCAT1* functions as a high affinity secondary active transport system for basic amino acids; however, the system also appears to recognize a broad spectrum of other amino acids, albeit with a lower affinity (Frommer et al., 1995).

Members of the ATF1 superfamily, which represents the largest and best characterized plant amino acid transporter family, were also cloned by complementation of yeast mutants. Detailed sequence analyses predict that these transporters contain 10 membrane-spanning domains (Rentsch et al., 1998). Several members of the ATF1 family have now been shown to function as amino acid-H⁺ symporters (Boorer et al., 1996; Boorer and Fischer, 1997). Using analysis of substrate specificity, in combination with sequence similarities, four amino acid transporter subfamilies have been identified: namely the proline transporter (ProT), the amino acid permease (AAP), the lysine-histidine transporter (LHT), and the auxin-resistant clones (AUX1). As their name implies, the two members of the ProT subfamily, identified from studies on *Arabidopsis*, exhibit a high transport affinity for proline (Rentsch et al., 1996). In contrast, the members of the AAP subfamily recognize a wide range of different amino acids, including amides and ureides (Fischer et al., 1995; Boorer et al., 1996; Boorer and Fischer, 1997). The AtLHT1 subfamily was identified by homology searches in the database and was subsequently shown to transport lysine and histidine with high affinity (Chen and Bush, 1997). The AtAUX1 subfamily was discovered from studies performed on an auxin-resistant mutant of *Arabidopsis* (Bennett et al., 1996). The current hypothesis is that, because auxins are structurally very similar to tryptophan, during plant evolution an AAP amino acid transporter homolog may have been mutated to yield an auxin transporter (AUX1).

Analyses of CAT and AFT1 expression patterns indicate that individual family members are expressed within specific tissues (Kwart et al., 1993; Fischer et al., 1995; Rentsch et al., 1996). Organ-dependent differences in specificity of amino acid uptake mechanisms were also observed in *Ricinus* (Schobert et al., 1997). However, the expression of a given amino acid transporter can be limited to a particular cell type, or it can overlap with other transporter

genes. Naturally, expression patterns deduced from Northern blot analysis provide only a limited amount of information, and must be followed up by careful in-situ and immunolocalization studies. Studies of this nature will be required to determine which of these transport systems are involved in the transfer of amino acids from the mesophyll to the phloem translocation stream.

III. Phloem Loading

Before reviewing the progress made with respect to identification of the molecular components which drive assimilate accumulation in the CC-SE complex, a definition of the term phloem loading needs to be clearly stated, as it pertains to the present discussion. In the past two decades, the Münch (1930) pressure flow hypothesis has been generally recognized as the only model which can explain all of the experimental observations regarding assimilate flow in the phloem of higher plants. For the sake of clarity, we will use the term phloem loading (Geiger, 1975) to describe the processes and underlying mechanisms: (i) involved in the uptake of assimilates into the CC-SE complex, and (ii) required for the generation and maintenance of the thermodynamic gradient necessary to drive the mass flow of assimilates out of the source leaf.

A. Cellular Organization of the Phloem Long-Distance Transport Pathway

The phloem in the vascular bundles of angiosperms is comprised of parenchyma cells and the companion cell-sieve element (CC-SE) complex, which functions as the long-distance transport pathway (Fig. 1). The CC and SE differentiate after division of a precursor cell, but remain intimately connected by specialized plasmodesmata (Fig. 5B). The SE undergoes a pattern of differentiation resulting in features unique among living plant cells (Sjölund, 1997). The nucleus, vacuole and Golgi apparatus of the differentiating SE become completely degraded and structurally simplified plasmodesmata yield plasma membrane-lined pores in walls that interconnect contiguous SEs (sieve plates and lateral sieve areas), thereby forming the functional sieve tube system. The plasma membrane, a parietally displaced endoplasmic reticulum termed the sieve element reticulum (SER), modified plastids and mitochondria remain within the mature, functional SE (Evert, 1990).

Unlike the situation in animals where a muscle-driven pump generates flow through the vessels which represent a space outside of living cells, long-distance transport in the phloem of higher plants occurs within the highly specialized living SEs. There, as in the animal system, fluid flow is driven by a differential in hydrostatic (turgor) pressure. Turgor within the SEs is generated by the accumulation (loading) of photoassimilates, which results in a lowering of the osmotic potential within the SE. The subsequent reduction in the SE water potential establishes a gradient with respect to the surrounding tissues, which causes water to enter the SE from the nearby xylem conducting elements (Fig. 1). As the CC-SE complex is located deep within the cellular architecture of the vein, this osmotically induced inflow of water does not cause an expansion in SE volume, but rather, it generates a significant increase in turgor pressure. The removal of specific organelles and the anchoring of the remaining organelles to the plasma membrane thus allows efficient turgor-driven mass flow, through the low-resistance sieve plate pores, from sites of assimilate production, termed sources, to sites of assimilate utilization, termed sinks (Fig. 1A).

The differences in SE turgor between the sites of phloem loading and unloading result in mass transport of phloem sap in the direction of this turgor gradient; recorded flow rates range from 0.3 to 3 m/h (Köckenberger et al., 1997). These rates are quite remarkable given the small diameter of the SE (average of 10–20 μm) (Sjölund, 1997). Viewed from another perspective, if we assume an average length of 200 μm for an individual SE, then its entire content would be displaced (in the direction of mass flow) up to 5 times every second!

Considering that the SEs of higher plants are enucleate, their maintenance as living cells over a long period of time appears to rely on the adjacent CCs which are characterized by a dense cytoplasm and an abundance of mitochondria and ribosomes (Fig. 5A, B). The metabolites and proteins needed to sustain sieve tube function are thought to be synthesized in the CC prior to being transported into the sieve tube through specialized plasmodesmata that interconnect these two cell types (Fig. 5A, B). Analysis of sieve tube sap has shown that it contains more than 100 polypeptides (Fisher et al., 1992; Nakamura et al., 1993; Sakuth et al., 1993). Lectins (Bostwick et al., 1992), redox regulatory proteins (Ishiwatari et al., 1995; Szederkenyi et al., 1997), chaperones (Schobert et al., 1995), protein kinases

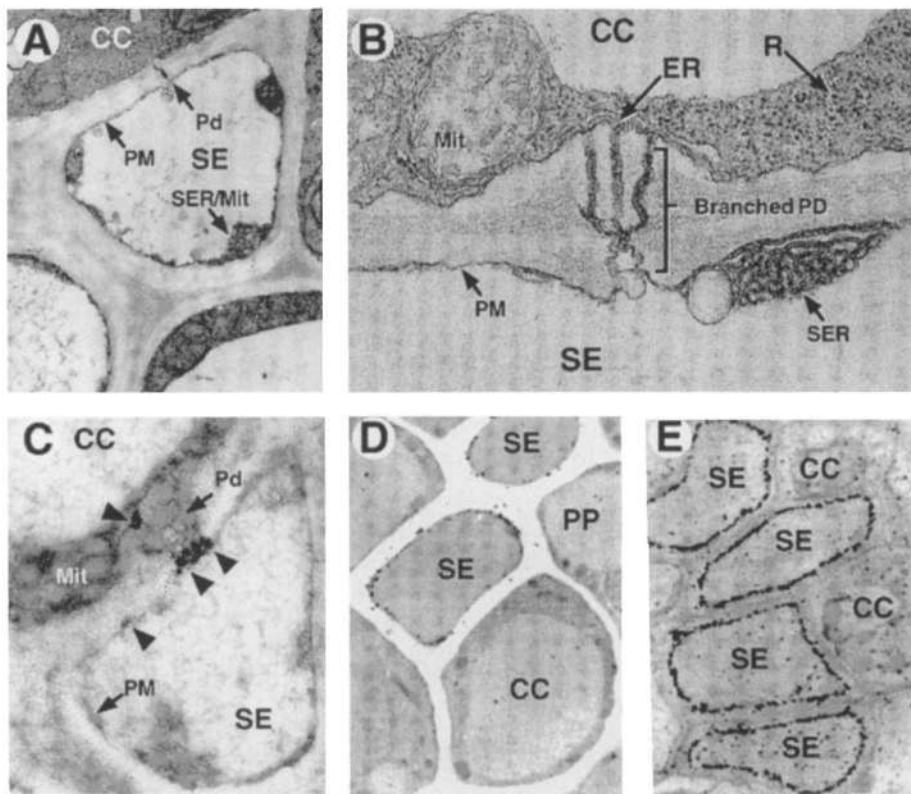


Fig. 5. Role of macromolecular trafficking through CC-SE plasmodesmata in terms of controlling phloem loading and long-distance translocation. **A.** Ultrastructural details of the CC-SE complex. Note the presence of plasmodesmata (Pd) and the plasma membrane (PM) that lines the SE as well as the remaining sieve element reticulum (SER) and mitochondria (Mit) located within the mature, functional SE. ($\times 24,000$). **B.** Branched plasmodesmata interconnecting the CC-SE complex. Note the presence of numerous ribosomes (R) in the CC ($\times 50,000$). (From Esau and Thorsch, 1985). **C.** In-situ detection of SUT1 mRNA within the plasmodesmata and along the SE plasma membrane (darts) in source leaves of potato plants ($\times 35,000$). Immunolocalization of SUT1 to the SE plasma membrane in the minor vein phloem (**D**) from potato leaf ($\times 4,200$), and in petiole phloem (**E**) ($\times 3,200$). (Reproduced from Kühn et al., 1997, with permission). Note the intense labeling in the petiole SE PM.

(Nakamura et al., 1995), protease inhibitors (Murray and Christeller, 1995) and amylase (Wang et al., 1995) have been shown to be constituents of SEs, either by immunolocalization or by their presence in isolated sieve tube sap.

Recent experiments have provided experimental evidence for the hypothesis that phloem proteins have the capacity to mediate their own cell-to-cell transport through plasmodesmata. Squash phloem proteins were isolated from sieve tube sap and then size-fractionated; fractions ranged from 10 to above 100 kDa. After microinjection into squash mesophyll cells these proteins moved from cell to cell and potentiated the movement of fluorescently labeled 20 kDa dextran (Balachandran et al., 1997). Direct microinjection of high-molecular-weight fluoro-

chromes into the SEs of the fascicular stem phloem of *Vicia* confirmed that the plasmodesmata connecting the CC-SE complex are open for the passage of molecules having an SEL greater than 10 kDa but less than 40 kDa (Kempers and Van Bel, 1997). These findings indicate that: (i) significant import of proteins occurs into the sieve tube system, and (ii) unfolding may be required for proteins larger than 20 kDa to pass the plasmodesmata connecting the CC to the SE. For this purpose, agents such as molecular chaperones may well be essential to mediate refolding of these proteins following their transport into the sieve tube (Schobert et al., 1995; Balachandran et al., 1997). Chaperones may also be required for targeting and integration of integral membrane proteins into the sieve tube membrane.

B. Modes of Phloem Loading

The number of plasmodesmata connecting the CC-SE complex to the surrounding cells within the minor vein may vary widely across diverse plant families. These structural differences in the minor vein configuration have been used to classify higher plants into two major groups, with implications on the mechanism of phloem loading. These groups have been defined as follows (Gamalei, 1988):

Group I: The CC-SE complex is highly connected with phloem parenchyma cells, via plasmodesmata, and thus, a symplasmic continuity is maintained between the mesophyll and the CC-SE complex (Fig. 2A). It has been noted that many members in this group transport additional oligosaccharides in the phloem, together with, or in greater amounts than, sucrose (Gamalei, 1991). The inference has been drawn that phloem loading in group I plants is primarily via a symplasmic mechanism.

Group II: A limited number of plasmodesmata connect the CC-SE complex with the surrounding cells of the minor vein (Fig. 2B, C) which results in a constriction of the symplasmic connection between photoassimilate-producing cells and the CC-SE. Here, the inference has been drawn that in group II plants, phloem loading occurs via an apoplastic step between the phloem parenchyma and CC-SE complex.

An elegant hypothesis has been advanced to explain, mechanistically, how symplasmic phloem loading may operate (Turgeon and Gowan, 1990). This process has been referred to as ‘polymer trapping’ and is thought to involve a combination of metabolic and plasmodesmata specializations at the bundle sheath/CC-SE boundaries. For plants such as cucurbits, it has been proposed that plasmodesmata connecting the bundle sheath to the intermediary cell (a specialized form of the companion cell) do not allow the passage of metabolites above 600 Da. However, the plasmodesmata connecting the intermediary cell to the SE are thought to be structurally and/or functionally modified to allow diffusion of larger molecules. Figure 4C illustrates how, in this polymer trap model, metabolic conversion of sucrose and galactinol ($MW < 400$ Da) to raffinose and stachyose ($MW > 600$ Da) would lead to a decrease in the osmotic potential within the CC-SE. In support of this model, it has been shown that synthesis of raffinose and stachyose occurs primarily within the intermediary cells (Beebe and Turgeon,

1991; Haritatos et al., 1996). However, the proposed differences in the SEL of plasmodesmata connecting intermediary cells to the bundle sheath and the SE have yet to gain experimental support.

An important argument for symplasmic loading, in addition to the number of plasmodesmata connecting the CC-SE to the surrounding cells, is based on experiments using p-chloromercuribenzenesulfonic acid (PCMBS), a reported membrane-impermeant sulphydryl-modifying reagent that strongly inhibits sucrose uptake and phloem loading in group II plants by directly blocking the transporter (Delrot, 1987; Bush, 1993). The corollary here is that in group I plants, sucrose uptake and assimilate export should not be, and were not, inhibited by PCMBS (Van Bel et al., 1992, 1993; Flora and Madore, 1996). This has led some workers to conclude that carrier-mediated components are not essential for phloem loading in group I plants. However, given the paucity of our knowledge regarding sugar and/or oligosaccharide carriers in such plants, we cannot discount that the inability of PCMBS to inhibit assimilate export might well reflect the absence and/or inaccessibility of an essential sulphydryl group on membrane proteins involved in loading or retrieval of these molecules from the apoplasm.

As sugar transporters evolved well before the development of higher plants (Marger and Saier, 1993), it would be surprising, from an evolutionary viewpoint, if in group I plants carriers were completely absent from the plasma membrane of the CC-SE complex, thereby forfeiting the advantages of loading and/or retrieving assimilates from the apoplasm (Fig. 2A). In addition, the observation that many group I plants also transport small to major amounts of sucrose may well indicate the potential operation of two parallel phloem loading systems. Clearly, this is an important area that merits further investigation, as it holds great significance with respect to the molecular and structural evolution of phloem loading mechanisms.

C. Molecular Analysis of Sucrose Carrier(s) Involved in Phloem Loading

Sugar, and in particular, sucrose transport in higher plants has been reviewed quite extensively with respect to the progress made by cloning of cDNAs encoding mono- and disaccharide carriers (Sauer et al., 1994; Tanner and Caspary, 1996; Ward et al., 1997). Therefore, we will focus our attention on

experimental results directly related to phloem loading. Considerable efforts were dedicated to the biochemical isolation of plant sucrose transporter proteins from leaf plasma membrane preparations (Gallet et al., 1989; Lemoine et al., 1989). Indeed, the isolation of a sucrose binding protein from soybean was achieved by Ripp et al. (1988) and its association with CCs was demonstrated by Grimes et al. (1992). However, major advances awaited the application of molecular methods. Important breakthroughs came by using a specifically engineered yeast strain that was defective in all forms of invertase expression. This strain was engineered to metabolize sucrose by introducing a sucrose synthase gene that then allowed complementation cloning of sucrose carriers from spinach and potato (Riesmeier et al., 1993, 1992). Because the genes for the cloned sucrose carriers were highly conserved, the cDNAs for sucrose transporters from spinach and potato (e.g., *SUT1*) could be successfully used to isolate genes encoding homologous sucrose carriers from *Plantago*, *Arabidopsis* and *Ricinus* (Gahrtz et al., 1994; Sauer and Stolz, 1994; Weig and Komor, 1996).

Studies performed on yeast cells expressing plant sucrose carriers demonstrated that sucrose uptake was inhibited by protonophores and thiol-modifying agents (e.g., PCMBS). In addition, the K_m values for these heterologously expressed sugar carriers were in the range of 1 mM. These functional characteristics were in agreement with earlier results obtained for high affinity sucrose uptake systems studied in planta (see Delrot, 1987; Bush, 1993). Furthermore, injection of *SUT1* cRNA and expression in *Xenopus* oocytes allowed voltage-clamp measurements to be used to analyze the ionic currents that accompany sucrose uptake, via the expressed *SUT1* transporter. Findings from such experiments were consistent with the operation of a sucrose-H⁺ cotransport system having a functional stoichiometry close to 1:1 (Boorer et al., 1996), values that were in close agreement with earlier studies conducted on plasma membrane vesicles (Bush, 1989).

That these sucrose carriers may indeed play a role in phloem loading was first indicated by in-situ detection of the appropriate mRNA in the minor veins (Riesmeier et al., 1993). Studies employing fusions of the promoter of the sucrose carrier gene to GUS also demonstrated expression within the phloem of leaf tissue (Truernit and Sauer, 1995). In addition, evidence consistent with the functioning of the sucrose carrier in phloem loading was gained by

studies conducted on transgenic potato plants in which expression of the sucrose carrier was reduced by expression of antisense constructs under the control of the strong CaMV 35S promoter, or the phloem-specific RolC promoter (Kühn et al., 1996; Lemoine et al., 1996). In both transgenic potato lines, plants displayed symptoms which were considered typical for inhibition of phloem loading: namely, (i) accumulation of high amounts of soluble and insoluble carbohydrates in source leaves; (ii) inhibition of photosynthesis; and (iii) reduced root growth along with a decrease in tuber yield. Reciprocal graft experiments demonstrated that inhibition of sucrose transporter gene expression in leaves was sufficient to produce the above-described symptoms. Interestingly, within individual leaves of such antisense potato plants, mesophyll cells did not respond uniformly to the inhibition of sucrose export: whereas high accumulations of starch was noted in some cells, other cells responded by a reduction in the rate of photosynthesis.

Intriguing questions still remain to be resolved concerning the cellular localization of the machinery which allows loading of assimilates from the apoplasm into the SE. For example, are all the components required for active sucrose uptake located in the SE plasma membrane; i.e., sucrose carriers energized by proton cotransport and H⁺-translocating ATPases essential for the establishment and maintenance of the proton motive force? Some insight into this aspect of phloem loading was gained from freeze fracture studies performed on cultured *Streptanthus* cells, where a high density of globular structures (presumably H⁺-ATPases) was found in the SE plasma membrane (Sjölund and Shih, 1983). However, recent immunolocalization studies have indicated that, in *Vicia*, the H⁺-ATPase was more concentrated in the plasma membrane of transfer cells in the minor vein as compared with the SE (Bouche-Pillon et al., 1994). In support of these findings, the c-Myc tagged AHA3 (H⁺-ATPase) from *Arabidopsis* was found to be predominantly associated with the CC plasma membrane (Dewitt and Sussman, 1995). Unfortunately, it is not yet known whether another member of the large AHA gene family is targeted to the SE plasma membrane (11 expressed genes have already been identified in *Arabidopsis* that encode H⁺-ATPases (Dewitt et al., 1996).

Even more complexity is added to the picture when one considers recent immunolocalization studies that have been performed on the sucrose

carrier within the phloem of exporting leaves. Depending on the plant species, sucrose carrier protein has been detected either in the CC, or associated with the plasma membrane of the SE. Using specific antibodies directed against the *Arabidopsis* sucrose carrier 2 (SUC2) in conjunction with immunofluorescence techniques, SUC2 was identified in CCs in both *Plantago* and *Arabidopsis* (Stadler et al., 1995; Stadler and Sauer, 1996, see Fig. 4B). On the other hand, in tobacco, potato and tomato plants, antibodies raised against SUT1 detected the sucrose transporter protein in the plasma membrane of sieve elements in the minor vein of the source leaf and in the petiole phloem (Kühn et al., 1997, see Figs. 4A and 5D,E). Interestingly, the abundance of SUT1 was much higher in the plasma membrane of sieve elements in the petiole as compared to the plasma membrane at the loading sites in the minor vein, suggesting that retrieval into the sieve element along the long-distance transport path is another important function of SUT1. Finally, whether these studies revealed the cellular localization of all sucrose carriers involved in phloem loading in these plants is not yet clear.

In any event, the presence of proteins with high turnover rates in the SE plasma membrane, e.g. SUT1 (Kühn et al., 1997), poses an intriguing problem as to how targeting of membrane proteins may be achieved in these unique enucleate cells of the higher plant phloem. As ribosomes have not been reported from electron microscope-based studies performed on mature (functional) SEs and since Golgi vesicles are also absent from these cells, it would appear that transcription and translation of such proteins may well occur in the CC. Although trafficking of soluble proteins through plasmodesmata into the SE has recently gained experimental support (Balachandran et al., 1997; Ishiwatari et al., 1998), studies have not been performed with hydrophobic integral membrane proteins. Clearly, if these integral membrane proteins are indeed first synthesized in the CC either they must be imported into the SE by some form of specialized chaperone system, or they must be synthesized and inserted into the plasma membrane at the CC orifice of the plasmodesmata. Here again, some form of special mechanism would be required to move the protein(s) along the tortuous pathway into the SE. In this regard, it is interesting to note that earlier studies performed on fluorescently labeled membrane lipids suggested that lipid components may be able to move cell to cell in the ER membrane,

whereas lipids in the plasma membrane remained within the target cell (Grabski et al., 1993). Thus, whether the plasma membrane route through the CC-SE plasmodesmata is tenable will require careful experimentation conducted at the CC-SE level.

Finally, in-situ detection of *SUT1* mRNA, within the orifice of the CC-SE plasmodesmata (Kühn et al., 1997), as well as in association with the sieve element reticulum (see Fig. 5C), raises the possibility that translation may well proceed within the functional sieve tube system. Clearly, it will be extremely important to determine whether this *SUT1* mRNA is indeed translated on special ribosomes located within the sieve tube, or whether these transcripts play another role in the long-distance transport system of the angiosperms.

D. Phloem Loading of Amino Acids

Analysis of phloem sap from a variety of plants has shown that nitrogen is transported almost exclusively in the form of amino acids in sieve tubes (Pate, 1980), with amides such as glutamine and asparagine usually being the most abundant species (Hocking, 1980; Fukumorita and Chino, 1982; Fisher and Macnicol, 1986; Shelp, 1987). Experiments with *Ricinus* seedlings, which allow control of amino acid supply to the site of phloem loading, showed that glutamine is the preferred amino acid taken up into the sieve tubes (Schobert and Komor, 1989; Schobert and Komor, 1992). The importance of source metabolism, in determining the mix of amino acid species used for long-distance transport of nitrogen, has been pointed out in other experimental systems. Interestingly, no major differences were found between the content and pattern of amino acids in the leaves and the phloem sap obtained from spinach, barley and sugar beet (Riens et al., 1991; Winter et al., 1992; Lohaus et al., 1994).

Although the functional properties of an amino acid transporter can be studied using a heterologous expression system (Frommer et al., 1994) such as yeast cells, to understand the physiological role of this system requires explicit information on the conditions present within the tissue of interest. Thus, to address the question of which specific transporters are involved in loading amino acids into the CC-SE complex, data will be needed on the actual chemical species and relative concentrations present within the symplasmic and apoplasmic compartments at the site of phloem loading. The importance of this

information can be demonstrated by the following example. A transporter that has been characterized (using a heterologous system) as a high affinity cationic amino acid transporter, but which also functions as a low affinity glutamate transporter, could well function as the glutamate carrier, *in vivo*, because the apoplasmic glutamate concentration in the minor vein could be much higher than that for lysine. The problem is therefore to identify the actual concentration experienced by the carriers located in the plasma membranes along the pathway from the mesophyll to the CC-SE complex.

Unfortunately, currently employed techniques do not permit analysis of the various amino acid concentrations present within the cytosol, vacuole and apoplast of specific cells within an intact leaf. The application of *in vivo* NMR imaging may well prove useful in the resolution of this problem. NMR correlation-peak-imaging was recently successfully applied to the analysis of amino acid and sugar distribution within the hypocotyl of castor bean seedlings. Using this method, Metzler et al. (1995) were able to establish that valine was present only in parenchyma cells of the cortex, whereas glutamine (and/or glutamate) was detected in these same cells as well as in the cells comprising the vascular bundles; lysine and arginine, on the other hand, were present mainly in the vascular tissue. Here we should stress that the finding that valine showed a different distribution from the other amino acids is surprising, because the broad specificity AAPs are able to transport acidic and neutral amino acids, including valine, with similar kinetics. In any event, if NMR imaging techniques can be further refined to provide subcellular resolution, they would provide a powerful method to study amino acid transport at both the cellular and tissue level (see Metzler et al., 1995).

Until such techniques are developed, workers in this field have little choice but to perform experiments that, unavoidably, provide sample averages from an array of cell types within leaf tissues. Nevertheless, the tools for such analyses have become rather highly developed. For example, by using a combination of non-aqueous fractionation, apoplasmic washing and laser stylectomy techniques, the sucrose and amino acid composition of the vacuolar, cytosolic, apoplasmic and phloem sap could be studied on the same plant (Lohaus et al., 1995). As might well be expected, these studies indicated that the apoplasmic concentrations for sugars and amino acids were low in comparison to those present in the cytosol.

However, it is still a matter of some debate as to whether the apoplasmic wash technique can provide meaningful information on the actual metabolite concentrations that are present at the site(s) of phloem loading. This concern is heightened by the recent finding that, within the minor veins, phloem loading of sucrose appears to occur across a small fraction of the total surface area (Kühn et al., 1997). In any event, the presence of a large concentration gradient between the phloem sap and the apoplasm would be consistent with active transport of amino acids into the CC-SE complex, a process which may well be mediated by an amino **acid-H⁺** symport system (AAPs).

IV. Regulation of Assimilate Export

There are important questions to address relating to how the plant integrates the overall rate of photosynthesis with the fraction of assimilate production to be exported from the leaf. In relation to this question, it is equally important to understand how the plant assigns priority to the multitude of sinks that will utilize these photoassimilates. This, in its broadest sense, describes the process of assimilate partitioning, and its regulation is critical to the growth, development, and reproductive success of the plant. In essence, mechanisms have to exist which allow the plant to regulate photoassimilate production in autotrophic tissues relative to the requirements of competing sinks (heterotrophic tissues/organs) (Fig. 1A). These mechanisms must function in concert with the processes that operate to control phloem loading rates and relative direction (distribution) of flow, and must accommodate predictable and unpredictable restrictions and demands: i.e., store photoassimilate during the day to accommodate growth and metabolism of sinks during the night; integrate transitions from sink-to-source status in a diurnal or developmental context; respond to changing biotic and abiotic environmental conditions, etc. Clearly, these are highly complex processes that require input from source and sink tissues and may involve responses such as the regulation of transcription and translation, as well as reversible protein modification, etc. Communication is clearly key to the integration of such physiological and/or developmental processes, whether it be from cell-to-cell (i.e., over short distances) or between organs, in the context of a whole-plant response, which involves

the transfer of information over long distances via the phloem and the xylem.

Exciting breakthroughs on some of these communication processes are only now coming to light (see also section II.B). Here, we will discuss models of how plants cells, tissues and organs may use various modes of communication to direct assimilate partitioning and adapt to a wide range of environmental challenges.

A. The Paraveinal Mesophyll—a Model for Regulation of Assimilate Export

Leaves of soybean, as well as a number of other legume and non-legume species, have a specialized mesophyll layer called the paraveinal mesophyll (PVM) (Fisher, 1967). This system will be used to illustrate some important concepts relevant to the intermediate pathway for symplasmic transfer of photoassimilates within the leaf, as well as the interrelationship between specific sources and sink organs. The PVM forms a paradermally (parallel with the epidermal tissue) arranged one-cell-thick reticulum of very large cells that span the interveinal region between the minor veins, and is symplasmically connected to the bundle parenchyma cells at the level of the phloem (Franceschi and Giaquinta, 1983a). In addition, the PVM is symplasmically connected to the cells of the palisade layer and the spongy mesophyll. As illustrated in Fig. 6, the position of the PVM and its symplasmic continuity with the adjacent cell layers may implicate a central role in the regulation of assimilate transfer within the soybean leaf.

With respect to delivery of photoassimilates to the phloem, the laterally oriented, large cells of the PVM appear to play a role in enhancing the efficiency of assimilate transfer from the photosynthetic parenchyma to the phloem, mainly by reducing the number of cell walls (plasmodesmata) that have to be traversed in the pathway, and thus reducing the resistance associated with the symplasmic route. For example, in *Vigna radiata*, a single PVM cell may span the region between adjacent minor vein phloem, and have 20 or more palisade cells in symplasmic contact (Franceschi and Giaquinta, 1983b). These cells can dramatically reduce the resistance to flow of photoassimilates to the veins for export. In contrast to other cells within the leaf, these cells undergo cytoplasmic streaming (Franceschi et al., 1984) which would be important for mixing and to overcome the

diffusional limitations to transport in the cytoplasm of such large cells. Thus, the PVM acts as a high-capacity assimilate collection and transfer network.

This proposed function of the PVM in the soybean leaf was fully supported by $^{14}\text{CO}_2$ pulse-chase experiments which clearly demonstrated the flux of [^{14}C]-photoassimilate through these specialized cells. In addition, morphometric analyses revealed that the PVM is strongly and positively correlated to the number of palisade cells per unit volume of tissue in the legume leaf, as well as being inversely related to the number of plasmodesmata that have to be traversed in the photoassimilate transport pathway to the phloem (Lansing and Franceschi, unpub. results). Thus, the pattern and efficiency of assimilate flux from mesophyll cells to the export phloem can be affected by: (i) plasmodesmal frequency, (ii) placement of plasmodesmata, and (iii) cell morphology in relation to the three-dimensional anatomical relationship between various cell layers. Here we should emphasize that the concept of a ‘collecting cell’ network is not new, having been first proposed by Haberlandt (1914). However, the important principles developed by this early German botanist/plant physiologist have often been overlooked in recent studies.

The soybean PVM system also plays an important role in terms of nitrogen assimilate storage, in the form of vacuolar storage proteins (Franceschi et al., 1983; Klauer et al., 1996). Studies on these proteins and nitrogen metabolizing enzymes within the PVM further illustrated that this tissue functions as an important and sophisticated control site for amino acid storage, mobilization and delivery to the phloem long-distance transport system. Experiments investigating the deposition and mobilization of amino acids associated with vacuolar storage proteins indicate the presence of a feed-back mechanism that is linked to the nature, or amount, of photoassimilates that are passing through the PVM. For example, the storage proteins accumulate in large amounts in PVM only when nitrogen is not limiting relative to overall growth rate. Subsequently, during seed production, when inorganic nitrogen assimilation is dramatically reduced, the PVM storage proteins are rapidly metabolized, presumably to free amino acids for transport to the developing seeds via the phloem. However, if the plants are continuously depodded, the PVM proteins continue to accumulate to massive levels, even when Rubisco begins to be degraded in the mesophyll cells.

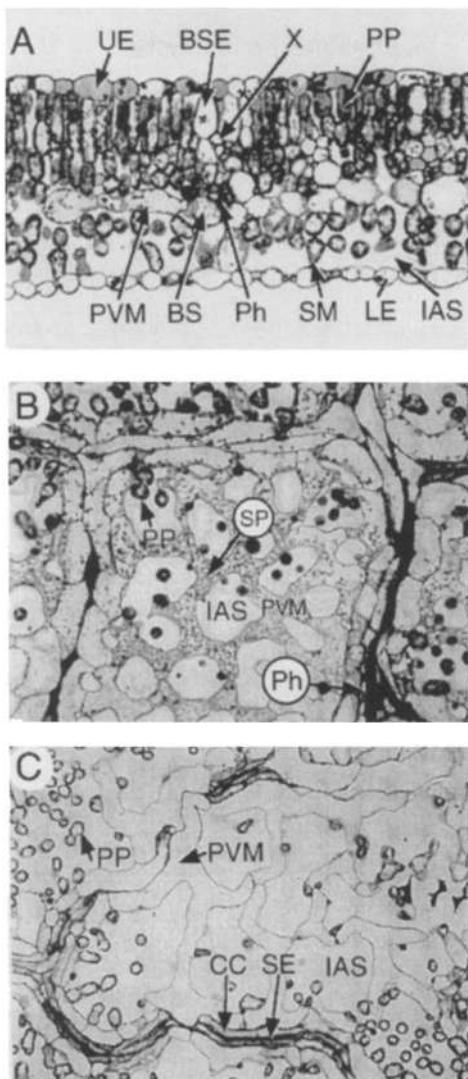


Fig. 6. Light micrographs illustrating the central role played by the paraveinal mesophyll (PVM) in connecting the chlorenchymatous tissues to the minor veins. A, Transverse section through the leaf of *Psophocarpus tetragonolobus* illustrating the typical orientation of the PVM with respect to other leaf tissues; note that the PVM forms a laterally oriented layer of large cells at the interface between the abaxial palisade parenchyma and the spongy mesophyll, with direct connection to the bundle sheath cells of the veins at the region of the phloem. (Dark particles in the PVM cell vacuole represent vegetative storage proteins.) B, Paradermal section through a *P. tetragonolobus* leaf, at the level of the PVM, illustrating the manner in which these large PVM cells form a network that directly interfaces the bundle sheath of the veins. Note that from 5–20 palisade cells can be attached to each PVM cell. C, Paradermal section through a *Vigna radiata* leaf at the level of the PVM. This micrograph demonstrates an extreme example of the highly developed PVM (intermediary transfer tissue). In this legume species, one cell acts as both bundle sheath and PVM cell. To the left side of this micrograph, a single PVM cell is shown spanning the region from a companion cell at one area of a vein to the companion cell at the minor vein region directly across from it. Abbreviations are as follows: BS, bundle sheath cells; BSE, bundle sheath extensions; CC, companion cells; IAS, intercellular air spaces; LE, lower epidermis; Ph, phloem; PP, palisade parenchyma; SE, sieve element; SM, spongy mesophyll; SP, storage protein; UE, upper epidermis; X, xylem. (A-C; $\times 150$)

These results are consistent with the notion that the PVM contains some sort of amino nitrogen ‘sensor’ so that when levels passing through this compartment, in route to the phloem, are higher than

the capacity for phloem loading, excess amino acids and storage proteins synthesized from them are transported across the tonoplast for storage in the vacuole. Interestingly, depodding also leads to

massive buildup of starch in the mesophyll, and this also occurs in the PVM where starch storage rarely takes place (Franceschi and Giaquinta, 1983c). Hence, some form of sugar feedback sensor, with respect to reduced carbon (sugar) build-up, must also reside within these tissues. Collectively, the information obtained on the physiological functions performed by the PVM points to the fact that mechanisms regulating photoassimilate transport out of the leaf exist not only at the site of phloem loading but also in the photoassimilatory cells *and* the cells that comprise the transfer pathway into the phloem. In this regard the symplasmic route from the mesophyll to the site of phloem loading, via plasmodesmata, may represent an efficient route in terms of the exchange of information molecules that could form part of the sophisticated feedback mechanism(s) which appears to operate between the photoassimilatory cells and the phloem transport system.

B. Plasmodesmal Companion Cell-Mesophyll Communication Network

Although the distribution and frequency of plasmodesmata within the minor veins can vary considerably from species to species, in all plants examined thus far, plasmodesmata interconnect the bundle sheath, phloem parenchyma and CCs. As mentioned previously, there is an expanding body of direct and indirect evidence that supports the hypothesis that plasmodesmata have the capacity to engage in, and regulate, the cell-to-cell trafficking of macromolecules (proteins and protein-nucleic acid complexes). This unique property of plasmodesmata would clearly potentiate supracellular control over tissue function (Lucas et al., 1993). It has also become clear that many plant viruses have acquired the capacity to move from cell to cell by a direct interaction with this plasmodesmal macromolecular trafficking system (Lucas and Gilbertson, 1994; Gilbertson and Lucas, 1996; Ghoshroy et al., 1997). In this regard, Lucas and Wolf (1993) advanced the hypothesis that plant viruses gained this capacity by the acquisition of genes used by the plant to mediate supracellular control over plant development and physiology.

Based on these findings and recent models of plasmodesmal trafficking of information molecules, Balachandran et al. (1995) proposed a novel interpretation for the observation that, when expressed in transgenic tobacco plants, the TMV-MP causes

significant changes to carbon metabolism, export and biomass partitioning (Lucas et al., 1993b; Olesinski et al., 1995, 1996). Here it is important to stress that changes in biomass partitioning to the lower stem and root system of these TMV-MP expressing plants required only that the TMV-MP be present in the mesophyll and, further, these effects were shown not to be correlated with MP-induced increase in SEL (Balachandran et al., 1995).

Figure 7 provides a model to account for the TMV-MP results that is based on the concept that plasmodesmata function not only to allow diffusion of metabolites within the symplasm, but also potentiate the selective trafficking of information molecules. Thus, the underlying premise for the model depicted in Fig. 7 is that, under normal physiological conditions, a constant exchange of information molecules occurs between the CC and the mesophyll. These molecules (proteins and/or peptides, protein-mRNA complexes, including short nucleotide sequences, and metabolites) move through plasmodesmata to establish a control network involved in regulating photosynthesis occurring within the mesophyll and photoassimilate (e.g., sucrose and amino acids) loading/export that takes place in the CC-SE complex (see Fig. 7 and Lucas et al., 1996 for full details). Although the sites at which the TMV-MP interacts to induce these changes have yet to be elucidated, the TMV-MP will likely compete with endogenous protein(s) for binding sites that form part of the putative signal transduction cascade. Experimental confirmation of the model presented in Fig. 7 would establish a new conceptual foundation for the study of photosynthesis and carbon partitioning in higher plants.

C. Source-Sink Communication via the Phloem

The above-described local communication network model (Fig. 7) provides a foundation for the study of leaf physiology, but how does the plant coordinate developmental and physiological functions at the whole-plant level? Although it has long been known that plant hormones move within the phloem sap, only recently has it been appreciated that a large number of proteins are also present in this long-distance transport compartment. Based on a thorough study performed on wheat (Fisher et al., 1992), it is clear that a large number of phloem sap proteins move from mature leaves towards the apex, as well as

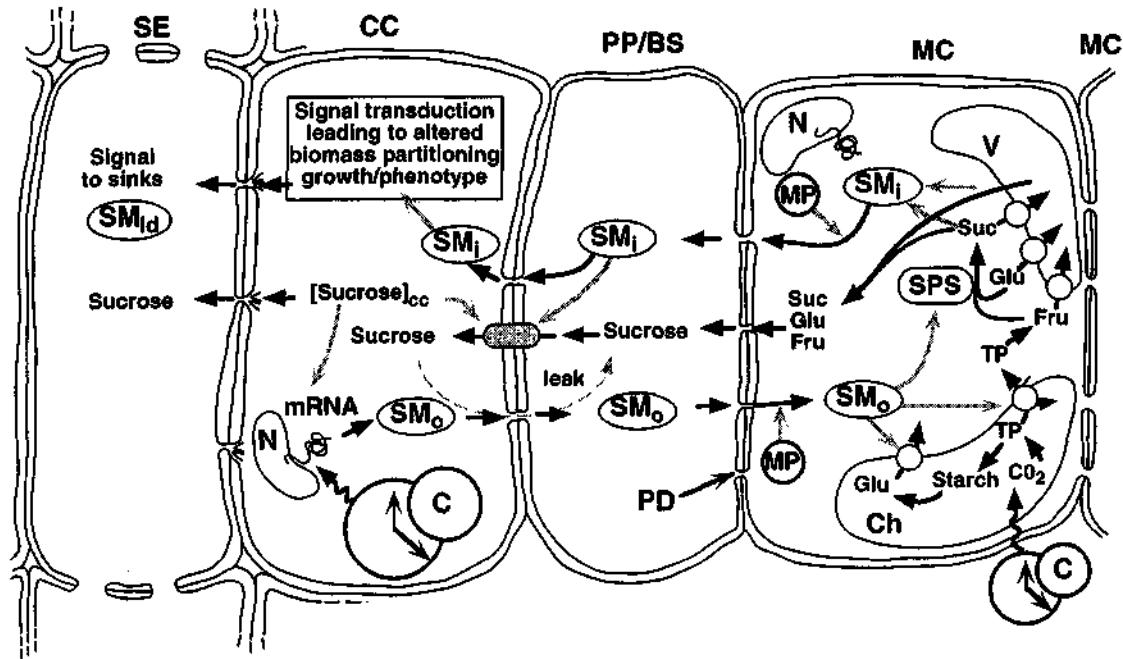


Fig. 7. Model of a communication network proposed to function between companion cells (CC) and the surrounding mesophyll cells (MC) to integrate photosynthesis and sucrose export, via the long-distance transport system of the phloem. Selective trafficking of macromolecules (proteins and/or protein-RNA complexes), via plasmodesmata, forms the basis of the signal molecule (SM) regulatory cascade. Output signals from the CC (SM_o) are trafficked through the intervening phloem parenchyma/bundle sheath (PP/BS) cells, to the MC where they interact at specific sites to control the flow of fixed carbon into sucrose (and amino acids; not shown). Input signals (SM_i) transfer information concerning net photosynthesis from the MC to the CC. Both SM_o and SM_i interact with light-dependent biological control systems (clocks). In addition, the release of long-distance signal molecules (SM_{id}) into the sieve element (SE) is proposed to be under the direct influence of this CC-MC communication network. MP = possible sites of action of the TMV-MP on SM_o and SM_i ; PD = plasmodesmata; N = nucleus; SPS = sucrose phosphate synthase. (Reproduced from Lucas et al., 1996, with permission.)

into strong sink tissues, such as the grain. Furthermore, microinjection studies have demonstrated that phloem sap proteins, collected from squash plants, can undergo cell-to-cell transport through mesophyll plasmodesmata, even when introduced into the target cell at very low levels (Balachandran et al., 1997). The threshold concentration required for plasmodesmal trafficking was estimated to be in the low nM range; i.e., at or below the level required for the action of many plant hormones.

These new findings raise the definite possibility that long-distance signaling, via the phloem, may involve both traditional plant hormones as well as a range of novel macromolecules that have the capability to control either gene expression or enzyme function in distant tissues and organs. Such a long-distance signaling system may well account for the influence of the TMV-MP over resource allocation to the lower stem and roots of TMV-MP expressing

tobacco plants. As illustrated in Fig. 7, the TMV-MP expressed within the mesophyll could act on the putative mesophyll input signal (SM_i) to alter expression and/or plasmodesmal trafficking of a long-distance signaling molecule (SM_{id}) from the CC into the SE.

A rigorous test for this long-distance signaling model will become possible once the proteins and/or nucleic acids within the angiosperm phloem sap become further characterized. Confirmation of this long-distance macromolecular trafficking model would advance our understanding of the manner in which plants operate at a supracellular level (Lucas et al., 1993a). Furthermore, this knowledge would lead to the development of a more complete understanding of the evolutionary processes that gave rise to the enucleate sieve tube system of the angiosperms. Eventually, this information may permit the development of a new generation of plants in which novel controls could be used to manipulate the

delivery of photoassimilates to specific plant organs, such as the grain in the cereals.

V. Conclusions

It has been truly amazing to witness the unfolding of our knowledge regarding the molecular processes engaged in photoassimilate transfer within the leaf from the mesophyll into the phloem. Genes for membrane transport proteins have been cloned which mediate sugar and amino acid uptake from the apoplasm into the symplasm and the role of sucrose carriers in phloem loading has been firmly established. In addition, much information has been gained with respect to the structure and function of plasmodesmata. These unique intercellular channels create a cytoplasmic continuity within the plant body which is the hallmark of the supracellular nature of higher plants. Not only do they allow for the symplasmic flow of photoassimilates but, as exciting recent experiments show, they have the capacity to potentiate the movement of endogenous plant macromolecules, proteins and nucleic acids, from cell to cell. We have advanced the hypothesis that the cell-to-cell movement of macromolecules establishes a control network within the leaf which extends to the growing parts of the plant via the phloem, and allows integration of physiology throughout the plant. This hypothesis provides a framework for the study of the diverse and complex interactions between the various cell types involved in photosynthesis, photoassimilate storage and export which transcends the reductionist approach that mere regulation by metabolites will suffice to explain plant growth and development. The challenges ahead will be to devise experiments to test the hypothesis through the identification and characterization of the postulated supracellular regulatory macromolecules.

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References

- Andrews M (1986) The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant Cell Environ* 9: 511–519
- Balachandran S, Hull RJ, Vaadia Y, Wolf S and Lucas WJ (1995) Tobacco mosaic virus movement protein-induced change in carbon partitioning originates from the mesophyll and is independent of changes in plasmodesmal size exclusion limit. *Plant Cell Environ* 18: 1301–1310
- Balachandran S, Xiang Y, Schobert C, Thompson GA and Lucas WJ (1997) Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell to cell through plasmodesmata. *Proc Natl Acad Sci USA* 94: 14150–14155
- Beck EH (1996) Regulation of shoot-root ratio by cytokinins from roots in *Urtica dioica*: Opinion. *Plant Soil* 185: 3–12
- Beebe DU and Evert RF (1992) Photoassimilate pathway(s) and phloem loading in the leaf of *Moricandia arvensis* (L.) DC. (Brassicaceae). *Int J Plant Sci* 153: 61–77
- Beebe DU and Turgeon R (1991) Galactinol synthase is sequestered in intermediary cells (companion cells) of *Cucurbita* leaf phloem. *Plant Physiol* 96S: 100
- Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schulz B and Feldmann KA (1996) The *Arabidopsis* AUX1 gene: A permease-like regulator of root gravitropism. *Science* 273: 948–950
- Bent AF (1996) Plant disease resistance genes: Function meets structure. *Plant Cell* 8: 1757–1771
- Boorer KJ and Fischer WN (1997) Specificity and stoichiometry of the *Arabidopsis* H⁺/amino acid transporter AAP5. *J Biol Chem* 272: 13040–13046
- Boorer KJ, Loo DDF, Frommer WB and Wright EM (1996) Transport mechanism of the cloned potato H⁺-sucrose cotransporter StSUT1. *J Biol Chem* 271: 25139–25144
- Bostwick DE, Dannenhoffer JM, Skaggs MI, Lister RM, Larkins BA and Thompson GA (1992) Pumpkin phloem lectin genes are specifically expressed in companion cells. *Plant Cell* 4: 1539–1548
- Botha CEJ (1992) Plasmodesmatal distribution, structure and frequency in relation to assimilation in C₃ and C₄ grasses in southern Africa. *Planta* 187: 348–358
- Bouche-Pillon S, Fleurat-Lessard P, Fromont JC, Serrano R and Bonnemain JL (1994) Immunolocalization of the plasma membrane H⁺-ATPase in minor veins of *Vicia faba* in relation to phloem loading. *Plant Physiol* 105: 691–697
- Boyer JS (1985) Water transport. *Annu Rev Plant Physiol* 36: 473–516
- Buckhout TJ (1994) Kinetics analysis of the plasma membrane sucrose-H⁺ symporter from sugar beet (*Beta vulgaris* L.) leaves. *Plant Physiol* 106: 991–998

- Burns IG (1994) A mechanistic theory for the relationship between growth rate and the concentration of nitrate-N or organic-N in young plants derived from nutrient interruption experiments. *Ann Bot* 74: 159–172
- Bush DR (1989) Proton-coupled sucrose transport in plasma-lemma vesicles isolated from sugar beet (*Beta vulgaris* cultivar Great Western) leaves. *Plant Physiol* 89: 1318–1323
- Bush DR (1993) Proton-coupled sugar and amino acid transporters in plants. *Annu Rev Plant Physiol Plant Mol Biol* 44: 513–542
- Canny MJ (1990) Rates of apoplastic diffusion in wheat leaves. *New Phytol* 116: 263–268
- Canny MJ (1993) The transpiration stream in the leaf apoplast–water and solutes. *Phil Trans Royal Soc Lond, Ser B-Biol Sci* 341: 87–100
- Chen L and Bush DR (1997) LHT1, a lysine and histidine specific amino acid transporter in *Arabidopsis*. *Plant Physiol* 115: 1127–1134
- Daie J (1989) Turgor-regulated sugar release from the source leaves of sugar beet (*Beta vulgaris* L.). *Plant Cell Physiol* 30: 1115–1122
- Dangl JL, Dietrich RA and Richberg MH (1996) Death don't have no mercy: Cell death programs in plant-microbe interactions. *Plant Cell* 8: 1793–1807
- Delrot S (1987) Phloem loading: Apoplastic or symplastic? *Plant Physiol Biochem* 25: 667–676
- Dewitt ND and Sussman MR (1995) Immunocytochemical localization of an epitope-tagged plasma membrane proton pump (H^+ -ATPase) in phloem companion cells. *Plant Cell* 7: 2053–2067
- Dewitt ND, Hong B, Sussman MR and Harper JF (1996) Targeting of two *Arabidopsis* H^+ -ATPase isoforms to the plasma membrane. *Plant Physiol* 112: 833–844
- Dickinson CD, Altabella T and Chrispeels MJ (1991) Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiol* 95: 420–425
- Ding B (1997) Cell-to-cell transport of macromolecules through plasmodesmata: A novel signalling pathway in plants. *Trends Cell Biol* 7: 5–9
- Ding B, Haudenschild JS, Willmitzer L and Lucas WJ (1993) Correlation between arrested secondary plasmodesmal development and onset of accelerated leaf senescence in yeast acid invertase transgenic tobacco plants. *Plant J* 4: 179–189
- Epel BL (1994) Plasmodesmata: Composition, structure and trafficking. *Plant Mol Biol* 26: 1343–1356
- Esau K and Thorsch J (1985) Sieve plate pores and plasmodesmata, the communication channels of the symplast: Ultrastructural aspects and developmental relations. *Am J Bot* 72: 1641–1653
- Evert RF (1990) Dicotyledons. In: Behnke HD and Sjölund RD (eds) *Sieve Elements*, pp 103–137. Springer, Berlin Heidelberg New York
- Evert RF, Russin WA and Bosabalidis AM (1996) Anatomical and ultrastructural changes associated with sink-to-source transition in developing maize leaves. *Int J Plant Sci* 157: 247–261
- Fischer WN, Kwart M, Hummel S and Frommer WB (1995) Substrate specificity and expression profile of amino acid transporters (AAPs) in *Arabidopsis*. *J Biol Chem* 270: 16315–16320
- Fischer WN, André B, Rentsch D, Krolkiewicz S, Tegeder M, Breitkreuz K and Frommer WB (1998) Plant amino acid transporters. *Trends Plant Sci* 3: 188–195
- Fisher DB (1967) An unusual layer of cells in the mesophyll of soybean leaf. *Bot Gaz* 138: 215–218
- Fisher DB and Macnicol PIC (1986) Amino acid composition along the transport pathway during grain filling in wheat. *Plant Physiol* 82: 1019–1023
- Fisher DB, Wu Y and Ku MSB (1992) Turnover of soluble proteins in the wheat sieve tube. *Plant Physiol* 100: 1433–1441
- Flora, LL and Madore MA (1993) Stachyose and mannitol transport in olive *Olea-Europaea* L. *Planta* 189: 484–490
- Flora LL and Madore MA (1996) Significance of minor-vein anatomy to carbohydrate transport. *Planta* 198: 171–178
- Franceschi VR and Giaquinta RT (1983a) The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. I. Ultrastructure and histochemistry during vegetative development. *Planta* 157: 411–421
- Franceschi VR and Giaquinta RT (1983b) Specialized cellular arrangements in legume leaves in relation to assimilate transport: Comparison of the paraveinal mesophyll. *Planta* 159: 415–422
- Franceschi VR and Giaquinta RT (1983c) The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. II. Structural, metabolic and compartmental changes during reproductive growth. *Planta* 157: 422–431
- Franceschi VR, Wittenbach VA and Giaquinta RT (1983) The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. III. Immunohistochemical localization of specific glycopeptides in the vacuole after depodding. *Plant Physiol* 72: 586–589
- Franceschi VR, Ku MSB and Wittenbach VA (1984) Isolation of mesophyll and paraveinal mesophyll protoplasts from soybean leaves. *Plant Sci Lett* 36: 181–186
- Frommer WB, Hummel S and Riesmeier JW (1993) Expression cloning in yeast of a cDNA encoding a broad specificity amino acid permease from *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 90: 5944–5948
- Frommer WB, Kwart M, Hirner B, Fischer WN, Hummel S and Ninnemann O (1994) Transporters for nitrogenous compounds in plants. *Plant Mol Biol* 26: 1651–1670
- Frommer WB, Hummel S, Unseld M and Ninnemann O (1995) Seed and vascular expression of a high affinity transporter for cationic amino acids in *Arabidopsis*. *Proc Natl Acad Sci USA* 92: 12036–12040
- Fujiwara T, Giesman-Cookmeyer D, Ding B, Lommel SA and Lucas WJ (1993) Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the red clover necrotic mosaic virus movement protein. *Plant Cell* 5: 1783–1794
- Fukumorita T and Chino M (1982) Sugar, amino acid and inorganic contents in rice phloem sap. *Plant Cell Physiol* 23: 273–283
- Gahrtz M, Stolz J and Sauer N (1994) A phloem-specific sucrose- H^+ symporter from *Plantago major* L. supports the model of apoplastic phloem loading. *Plant J* 6: 697–706
- Gallet O, Lemoine R, Larsson C and Delrot S (1989) The sucrose carrier of the plant plasma membrane: I. Differential affinity labeling. *Biochim Biophys Acta* 978: 56–64
- Gamalei Y (1988) The structural and functional evolution of minor veins of the leaf. *Botanicheskii Zhurnal* 73: 1513–1522
- Gamalei Y (1991) Phloem loading and its development related to plant evolution from trees to herbs. *Trees* 5: 50–64
- Geiger DR (1975) Phloem loading. In: Zimmermann MH and

- Milburn JA (eds) *Transport in Plants I. Phloem Transport*, Encycl Plant Physiol, New Series, Vol. 1, pp 395–431. Springer-Verlag, Berlin Heidelberg New York
- Ghoshroy S, Lartey R, Sheng JS and Citovsky V (1997) Transport of proteins and nucleic acids through plasmodesmata. *Annu Rev Plant Physiol Plant Mol Biol* 48: 25–48
- Gilbertson RL and Lucas WJ (1996) How do plant viruses traffic on the ‘vascular highway’? *Trends Plant Sci* 1: 260–268
- Grabski S, de Feijter AW and Schindler M (1993) Endoplasmic reticulum forms a dynamic continuum for lipid diffusion between contiguous soybean root cells. *Plant Cell* 5: 25–38
- Grimes HD, Overvoorde PJ, Ripp K, Franceschi VR and Hitz WD (1992) A 62-kD sucrose binding protein is expressed and localized in tissues actively engaged in sucrose transport. *Plant Cell* 4: 1561–1574
- Grusak MA, Beebe DU and Turgeon R (1996) Phloem loading. In: Zamsky E and Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships*, pp 209–227. Marcel Dekker Inc. Publishers, New York
- Haberlandt G (1914) *Physiological Anatomy* (English translation). Today and Tomorrow’s Book Agency, New Delhi
- Haritatos E, Keller F and Turgeon R (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L leaves—implications for phloem loading. *Planta* 198: 614–622
- Heineke D, Sonnewald U, Buessis D, Guenter G, Leidreiter K, Wilke I, Raschke K, Willmitzer L and Heldt HW (1992) Apoplastic expression of yeast-derived invertase in potato effects on photosynthesis leaf solute composition water relations and tuber composition. *Plant Physiol* 100: 301–308
- Hocking PJ (1980) The composition of phloem exudate and xylem sap from tree tobacco. *Ann Bot* 45: 633–643
- Hsu LC, Chiou TJ, Chen L and Bush DR (1993) Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. *Proc Natl Acad Sci USA* 90: 7441–7445
- Ishiwatari Y, Fujiwara T, McFarland KC, Nemoto K, Hayashi H, Chino M and Lucas WJ (1997) Rice phloem thioredoxin h has the capacity to mediate its own cell-to-cell transport through plasmodesmata. *Planta* 205: 12–22
- Ishiwatari Y, Honda C, Kawashima I, Nakamura SI, Hirano H, Mori S, Fujiwara T, Hayashi H and Chino M (1995) Thioredoxin h is one of the major proteins in rice phloem sap. *Planta* 195: 456–463
- Jackson AO and Taylor CB (1996) Plant-microbe interactions: Life and death at the interface. *Plant Cell* 8: 1651–1668
- Kempers R and Van Bel AJE (1997) Symplasmic connections between sieve element and companion cell in the stem phloem of *Vicia faba* L. have a molecular exclusion limit of at least 10 kDa. *Planta* 201: 195–201
- Klauer SF, Franceschi VR, Ku MSB and Zhang D (1996) Identification and localization of vegetative storage proteins in legume leaves. *Amer J Bot* 83: 1–10
- Köckenberger W, Pope JM, Xia Y, Jeffrey KR, Komor E and Callaghan PT (1997) A non-invasive measurement of phloem and xylem water flow in castor bean seedlings by nuclear magnetic resonance microimaging. *Planta* 201: 53–63
- Komor E, Orlich G, Weig A and Köckenberger W (1996) Phloem loading—not metaphysical, only complex: Towards a unified model of phloem loading. *J Exp Bot* 47: 1155–1164
- Kühn C, Quick WP, Schulz A, Riesmeier JW, Sonnewald U and Frommer WB (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Environ* 19: 1115–1123
- Kühn C, Franceschi VR, Schulz A, Lemoine R and Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. *Science* 275: 1298–1300
- Kwart M, Hirner B, Hummel S and Frommer WB (1993) Differential expression of two related amino acid transporters with differing substrate specificity in *Arabidopsis thaliana*. *Plant J* 4: 993–1002
- Kwart M, Laubner M, Heineke D, Rentsch D and Frommer WB (1998) An increased C/N ratio in potato tubers due to antisense inhibition of the leaf H^+/amino acid symporter StAAP1. *Plant J* (Submitted)
- Laloi M, Delrot S and M’Batchi B (1993) Characterization of sugar efflux from sugar beet leaf plasma membrane vesicles. *Plant Physiol Biochem* 31: 731–741
- Lemoine R, Delrot S, Gallet O and Larsson C (1989) The sucrose carrier of the plant membrane: II. Immunological characterization. *Biochim Biophys Acta* 978: 65–71
- Lemoine R, Kühn C, Thiele N, Delrot S and Frommer WB (1996) Antisense inhibition of the sucrose transporter in potato: Effects on amount and activity. *Plant Cell Environ* 19: 1124–1131
- Lohaus G, Burba M and Heldt HW (1994) Comparison of the contents of sucrose and amino acids in the leaves, phloem sap and taproots of high and low sugar-producing hybrids of sugar beet (*Beta vulgaris* L.). *J Exp Bot* 45: 1097–1101
- Lohaus G, Winter H, Riens B and Heldt HW (1995) Further studies of the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and the sieve tubes. *Bot Acta* 108: 270–275
- Lucas WJ (1995) Plasmodesmata: Intercellular channels for macromolecular transport in plants. *Curr Opinion Cell Biol* 7: 673–680
- Lucas WJ and Gilbertson RL (1994) Plasmodesmata in relation to viral movement within leaf tissues. *Annu Rev Phytopathol* 32: 387–411
- Lucas WJ and Wolf S (1993) Plasmodesmata: The intercellular organelle of green plants. *Trend Cell Biol* 3: 308–315
- Lucas WJ, Ding B and Van Der Schoot C (1993a) Plasmodesmata and the supracellular nature of plants. *New Phytol* 125: 435–476
- Lucas WJ, Olesinski A, Hull RJ, Haudenschild JS, Deom CM, Beachy RN and Wolf S (1993b) Influence of the tobacco mosaic virus 30-KDa movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants. *Planta* 190: 88–96
- Lucas WJ, Bouche-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B and Hake S (1995) Selective trafficking of KNOTTED 1 homeodomain protein and its mRNA through plasmodesmata. *Science* 270: 1980–1983
- Lucas WJ, Balachandran S, Park J and Wolf S (1996) Plasmodesmal companion cell-mesophyll communication in the control over carbon metabolism and phloem transport: Insights gained from viral movement proteins. *J Exp Bot* 47: 1119–1128
- Lunn JE and Furbank RT (1997) Localisation of sucrose-

- phosphate synthase and starch in leaves of C₄ plants. *Planta* 202:106–111
- Marger MD and Saier MH Jr (1993) A major superfamily of transmembrane facilitators that catalyse uniport symport and antiport. *Trends Biochem Sci* 18: 13–20
- Maynard, JW and Lucas WJ (1982) Sucrose and glucose uptake into *Beta vulgaris* leaf tissues. A case for general (apoplastic) retrieval systems. *Plant Physiol* 82: 432–42
- Metzler A, Izquierdo M, Ziegler A, Köckenberger W, Komor E, Von Kienlin M, Haase A and Decrops M (1995) Plant histochemistry by correlation peak imaging. *Proc Natl Acad Sci USA* 92: 11912–11915
- Mezitt LA and Lucas WJ (1996) Plasmodesmal cell-to-cell transport of proteins and nucleic acids. *Plant Mol Biol* 32: 251–273
- Münch E (1930) Die Stoffbewegungen in der Pflanze. Fischer, Jena
- Murray C and Christeller JT (1995) Purification of a trypsin inhibitor (PFTI) from pumpkin fruit phloem exudate and isolation of putative trypsin and chymotrypsin inhibitor cDNA clones. *Biol Chem Hoppe-Seyler* 376: 281–287
- Nakamura SI, Hayashi H, Mori S and Chino M (1993) Protein phosphorylation in the sieve tubes of rice plants. *Plant Cell Physiol* 34: 927–933
- Nakamura SI, Hayashi H, Mori S and Chino M (1995) Detection and characterization of protein kinases in rice phloem sap. *Plant Cell Physiol* 36: 19–27
- Noueiry AO, Lucas WJ and Gilbertson RL (1994) Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell* 76: 925–932
- Ntsika G and Delrot S (1986) Changes in apoplastic and intracellular leaf sugars induced by the blocking of export in *Vicia faba*. *Physiol Plant* 68: 145–153
- Olesinski AA, Lucas WJ, Galun E and Wolf S (1995) Pleiotropic effects of tobacco-mosaic-virus movement protein on carbon metabolism in transgenic tobacco plants. *Planta* 197: 118–126
- Olesinski AA, Almon E, Navot N, Perl A, Galun E, Lucas WJ and Wolf S (1996) Tissue-specific expression of the tobacco mosaic virus movement protein in transgenic potato plants alters plasmodesmal function and carbohydrate partitioning. *Plant Physiol* 111:541–550
- Oparka KJ and Prior DAM (1992) Direct evidence for pressure-generated closure of plasmodesmata. *Plant J* 2: 741–750
- Orlitz G and Komor E (1992) Phloem loading in *Ricinus communis* L. seedlings: Sucrose pathways via the mesophyll and the apoplasm. *Planta* 187: 460–474
- Overall RL and Blackman LM (1996) A model of the macromolecular structure of plasmodesmata. *Trends Plant Sci* 1: 307–311
- Pate JS (1980) Transport and partitioning of nitrogenous solutes. *Annu Rev Plant Physiol* 31: 313–340
- Perez-Alfocea F and Larher F (1995) Sucrose and proline accumulation and sugar efflux in tomato leaf discs affected by NaCl and polyethylene glycol 6000 iso-osmotic stresses. *Plant Sci* 107: 9–15
- Preißer J, Sprigle H and Komor E (1992) Solute distribution between vacuole and cytosol of sugarcane suspension cells—sucrose is not accumulated in the vacuole. *Planta* 186: 203–211
- Rentsch D, Hirner B, Schmelzer E and Frommer WB (1996) Salt stress-induced proline transporters and salt-stress repressed broad specificity amino acid permease genes identified by suppression of amino acid transport targeting mutant. *Plant Cell* 8: 1437–1446
- Rentsch D, Boorer K and Frommer WB (1998) Molecular biology of sucrose, amino acid and oligopeptide transporters at the plasma membrane of plant cells. *J Membr Biol* 162: 177–190
- Riens B, Lohaus G, Heineke D and Heldt HW (1991) Amino acid and sucrose content determined in the cytosolic, chloroplastic, and vacuolar compartments and in the phloem sap of spinach leaves. *Plant Physiol* 97: 227–233
- Riesmeier JW, Willmitzer L and Frommer WB (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO J* 11: 4705–4713
- Riesmeier JW, Hirner B and Frommer WB (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. *Plant Cell* 5: 1591–1598
- Ripp KG, Viitanen PV, Hitz WD and Franceschi VR (1988) Identification of a membrane protein associated with sucrose transport into cells of developing soybean cotyledons. *Plant Physiol* 88: 1435–1445
- Robards AW and Lucas WJ (1990) Plasmodesmata. *Annu Rev Plant Physiol Plant Mol Biol* 41: 369–420
- Robinson-Beers K and Evert RF (1991) Ultrastructure of and plasmodesmal frequency in mature leaves of sugarcane. *Planta* 184: 291–306
- Russin WA, Evert RF, Vanderveer PJ, Sharkey TD and Briggs SP (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the sucrose export defective maize mutant. *Plant Cell* 8: 645–658
- Sakuth T, Schobert C, Pecsvaradi A, Eichholz A, Komor E and Orlitz G (1993) Specific proteins in the sieve-tube exudate of *Ricinus communis* L. seedlings: Separation characterization and in-vivo labelling. *Planta* 191: 207–213
- Salmon S, Lemoine R, Jamai A, Bouche-Pillon S and Fromont JC (1995) Study of sucrose and mannitol transport in plasma-membrane vesicles from phloem and non-phloem tissues of celery (*Apium graveolens* L.) petioles. *Planta* 197: 76–83
- Sauer N and Stolz J (1994) SUC1 and SUC2: Two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. *Plant J* 6: 67–77
- Sauer N, Baier K, Gahrtz M, Stadler R, Stolz J and Truernit E (1994) Sugar transport across the plasma membranes of higher plants. *Plant Mol Biol* 26: 1671–1679
- Schobert C and Komor E (1989) The differential transport of amino acids into the phloem of *Ricinus communis* L. seedlings as shown by the analysis of sieve-tube sap. *Planta* 177: 342–349
- Schobert C and Komor E (1992) Transport of nitrate and ammonium into the phloem and the xylem of *Ricinus communis* seedlings. *J Plant Physiol* 140: 306–309
- Schobert C, Grossmann P, Gottschalk M, Komor E, Pecsvaradi A and Nieden UZ (1995) Sieve-tube exudate from *Ricinus communis* L. seedlings contains ubiquitin and chaperones. *Planta* 196: 205–210
- Schobert C, Mitsusada N and Aoshima H (1997) Diverse transporters for neutral amino acids in *Ricinus communis* L. seedlings. *Biol Plant* 39: 187–196
- Secor J and Schrader LE (1984) Characterization of amino acid

- efflux from isolated soybean cells. *Plant Physiol* 74: 26–31
- Shelp BJ (1987) The composition of phloem exudate and xylem sap from broccoli *Brassica oleracea* var *italica* supplied with ammonium, nitrate or ammonium nitrate. *J Exp Bot* 38: 1619–1636
- Sjölund RD (1997) The phloem sieve element: A river runs through it. *Plant Cell* 9: 1137–1146
- Sjölund RD and Shin CY (1983) Freeze fracture analysis of phloem structure in plant tissue culture. II. The sieve element plasma membrane. *J Ultrastruct Res* 82: 189–197
- Stadler R and Sauer N (1996) *The Arabidopsis thaliana AtSUC2* gene is specifically expressed in companion cells. *Bot Acta* 109: 299–306
- Stadler R, Brandner J, Schulz A, Gahrtz M and Sauer N (1995) Phloem loading by the PmSUC2 sucrose carrier from *Plantago major* occurs into companion cells. *Plant Cell* 7: 1545–1554
- Stitt M (1996) Plasmodesmata play an essential role in sucrose export from leaves—a step toward an integration of metabolic biochemistry and cell biology. *Plant Cell* 8: 565–571
- Szederkenyi J, Komor E and Schobert C (1997) Cloning of the cDNA for glutaredoxin, an abundant sieve-tube exudate protein from *Ricinus communis* L and characterisation of the glutathione-dependent thiol-reduction system in sieve tubes. *Planta* 202: 349–356
- Tanner W and Caspary T (1996) Membrane transport carriers. *Annu Rev Plant Physiol Plant Mol Biol* 47: 595–626
- Theodoropoulos PA and Roubelakis-Angelakis KA (1991) Glucose transport in *Vitis vinifera* L protoplasts. *J Exp Bot* 42: 477–484
- Truernit E and Sauer N (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H⁺ symporter gene directs expression of beta-glucuronidase to the phloem: Evidence for phloem loading and unloading by SUC2. *Planta* 196: 564–570
- Tubbe A and Buckhout TJ (1992) In vitro analysis of the proton-hexose symporter on the plasma membrane of sugarbeets (*Beta vulgaris* L.). *Plant Physiol* 99: 945–951
- Turgeon R and Gowan E (1990) Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from the apoplast. *Plant Physiol* 94: 1244–1249
- Van Bel AJE and Oparka KJ (1995) On the validity of plasmodesmograms. *Bot Acta* 108: 174–182
- Van Bel AJE, Van Kesteren WJP and Papenhuizen C (1988) Ultrastructural indications for coexistence of symplastic and apoplastic phloem loading in *Commelinaceae benghalensis* leaves. *Planta* 176: 159–172
- Van Bel AJE, Gamalei YV, Ammerlaan A and Bik LPM (1992) Dissimilar phloem loading in leaves with symplasmic or apoplasmic minor-vein configurations. *Planta* 186: 518–525
- Van Bel AJE, Ammerlaan A and Van Dijk AA (1993) A three-step screening procedure to identify the mode of phloem loading in intact leaves: Evidence for symplastic and apoplasmic phloem loading associated with the type of companion cell. *Planta* 192: 31–39
- Van Bel AJE, Hendriks JHM, Boon EJMC, Gamalei YV and Van De Merwe AP (1996) Different ratios of sucrose-raffinose-induced membrane depolarizations in the mesophyll of species with symplasmic (*Catharanthus roseus*, *Ocimum basilicum*) or apoplasmic (*Impatiens walleriana*, *Vicia faba*) minor-vein configurations. *Planta* 199: 185–192
- Van Die J and Tamme PML (1975) Phloem exudation from monocotyledonous axes. In: Zimmermann MH and Milburn JA (eds) *Transport in Plants I. Phloem Transport*, Encycl Plant Physiol, New Series, Vol 1, pp 196–222. Springer-Verlag, Berlin Heidelberg New York
- Volk GM, Turgeon R and Beebe DU (1996) Secondary plasmodesmata formation in the minor-vein phloem of *Cucumis melo* L. and *Cucurbita pepo* L. *Planta* 199: 425–432
- Von Schaewen A, Stitt M, Schmidt R, Sonnewald U and Willmitzer L (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J* 9: 3033–3044
- Waigmann E, Lucas WJ, Citovsky V and Zambryski P (1994) Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. *Proc Natl Acad Sci USA* 91: 1433–1437
- Wang Q, Monroe J and Sjölund RD (1995) Identification and characterization of a phloem-specific beta-amylase. *Plant Physiol* 109: 743–750
- Weig A and Komor E (1996) An active sucrose carrier (Scr 1) that is predominantly expressed in the seedling of *Ricinus communis* L. *J Plant Physiol* 147: 685–690
- Weiner H, Blechschmidt-Schneider S, Mohme H, Eschrich W and Heldt, HW (1991) Phloem transport of amino acids: Comparison of amino acid contents of maize leaves and of the sieve tube exudate. *Plant Physiol Biochem* 29: 19–24
- Weiner H, Burnell JN, Woodrow IE, Heldt HW and Hatch MD (1988) Metabolite diffusion into bundle sheath cells from *C₄* plants—Relation to *C₄* photosynthesis and plasmodesmatal function. *Plant Physiol* 88: 815–822
- Wille AC and Lucas WJ (1984) Ultrastructural and histochemical studies on guard cells. *Planta* 160: 129–142
- Wilson C and Lucas WJ (1988) Wounding and the regulation of apoplasmic retrieval in source leaf tissue of *Spinacia oleracea* L. *J Exp Bot* 39: 529–542
- Winter H, Lohaus G and Heldt HW (1992) Phloem transport of amino acids in relation to their cytosolic levels in barley leaves. *Plant Physiol* 99: 996–1004
- Wolf S, Deom CM, Beachy RN and Lucas WJ (1989) Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. *Science* 246: 377–379
- Wolf S, Deom CM, Beachy R and Lucas WJ (1991) Plasmodesmatal function is probed using transgenic tobacco plants that express a virus movement protein. *Plant Cell* 3: 593–604
- Zimmermann MH and Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmermann MH and Milburn JA (eds) *Transport in Plants I. Phloem Transport*, Encycl Plant Physiol, New Series, Vol 1, pp 480–503. Springer-Verlag, Berlin

Chapter 12

Regulation of Sugar Alcohol Biosynthesis

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Summary

Although we have long known that sugar alcohols can be important primary photosynthetic products involved in storage and translocation, there has been very little information on gene expression or regulation of enzyme activities associated with metabolism of these compounds. Recent studies, however, indicate that sugar alcohol metabolism is probably as tightly regulated as is conventional carbon metabolism in sink and source tissues. Sugar alcohols have also been demonstrated to be associated with the development of tolerance to drought, salt, temperature, and related stresses, and there is quite limited, but increasing evidence of stress-related regulation of genes and enzymes associated with sugar alcohols. Moreover, several studies of plants transformed with a capacity for sugar alcohol biosynthesis now indicate that these plants have enhanced stress tolerance. All this has important implications for crop improvement and developing understanding of stress tolerance mechanisms in plants.

I. Introduction

Why review sugar alcohols in a volume focused on photosynthesis and regulation? Because sugar alcohols frequently occur in many important crop plants, functioning as primary photosynthetic products and playing major roles in translocation and storage. Sugar alcohols have been overlooked or neglected in the past, due in part to analytical problems, and because they infrequently appear in the staples, e.g., wheat, rice, corn, sorghum, and grain legumes. However, their importance and distribution is now much better recognized, especially in horticultural crops. There are a number of reviews, many on distribution (see Loescher and Everard, 1996, for a listing), some extensive (Lewis and Smith, 1967a,b; Bielecki, 1982; Lewis, 1984), and others quite recent (Stoop et al., 1996). Despite the recent reviews, we really know very little about regulation of sugar alcohol metabolism (see Fig. 1), especially compared to the information on metabolism and regulation of other important plant carbohydrates and related enzymes, e.g., starch and ADPG pyrophosphorylase (Preiss and Sivak, 1996), sucrose and sucrose phosphate synthase (Huber and Huber, 1996), sucrose synthase, and the invertases (Koch, 1996), or even the raffinose-related oligosaccharides (Keller and Pharr, 1996).

Metabolism of sugar alcohols, like other photosynthetic products, must be closely controlled, and it has been reasonable to presume that control is modulated by environmental and developmental factors as well as sink/source interactions. That these controls are manifest at the gene, protein, or enzyme/substrate level is a given, but only a few such studies of sugar alcohol metabolism in higher plants have been completed. It has also been presumed, based until recently almost entirely on correlative evidence in higher plants and more direct evidence from work on algae (Cowan et al., 1992), fungi (Brown, 1978), and lichens (Honegger, 1991), that sugar alcohols play roles in tolerance of environmental stresses, especially those related to cold, salinity, and drought. Several quite recent studies now show that engineering a capacity for sugar alcohol biosynthesis can affect the ability of transgenic plants to withstand salt and

drought stress (see below). Since these data confirm roles for sugar alcohols in stress tolerance, it also seems reasonable to presume that stress in turn may profoundly affect partitioning, sink-source transitions, and regulation of these phenomena. Indeed, other quite recent studies show unequivocal and sometimes dramatic effects of stress on gene expression and enzyme activities related to sugar alcohol metabolism. Our focus here is mostly on the straight chain (acylic) polyols. Our goal here is to summarize the recent literature on these compounds and to assess its further implications for regulation.

II. Primary Physiological Roles

A. Photosynthetic Products

Bielecki (1982) has estimated that sugar alcohols account for 30% of global primary production. In algae the sugar alcohol may be the only primary product, but in all sugar alcohol-producing higher plants that have been studied, analyses of $^{14}\text{CO}_2$ assimilation have usually revealed two major soluble products, the sugar alcohol and the invariably present sucrose. In apple leaves, for example, about 70% of the newly fixed carbon was equally distributed (on a molar basis) between sucrose and glucitol (Grant and ap Rees, 1981). In celery 80 to 90% of the label in mature leaves was recovered as mannitol and sucrose, with about equal amounts of label (on a molar basis) in each (Loescher et al., 1992). Similar results were noted in lilac and apricot, mannitol and glucitol synthesizers, respectively (Trip et al., 1963; Bielecki and Redgwell, 1977). In species where the galactosyl-sucrose oligosaccharides are significant products (e.g., privet) sucrose and mannitol collectively accounted for less of the total label but were still synthesized in considerable quantities (Loescher et al., 1992). Alternatively, the sugar alcohol may be a secondary photosynthetic product. Hamamelitol, for example, is relatively unimportant initially in *Hedera* leaves, comprising only a small fraction of the labeled compounds, but it accumulates nonetheless due to slow turnover and little transport (Moore et al., 1997). This labeling pattern is similar to that of several cyclitols, e.g., an extended pulse of $^{14}\text{CO}_2$ was required for label to first appear and a chase of several days was necessary for label to accumulate in L-quebrachitol in *Acer pseudoplatanus* (Schilling et al., 1972).

Abbreviations: A6PR – aldose 6P reductase; G6PDH – glucose 6P dehydrogenase; GAPDH – non-reversible glyceraldehyde 3P dehydrogenase; M6PR – mannose 6P reductase; MTD – mannitol 1-oxidoreductase; SDH – sorbitol (glucitol) dehydrogenase

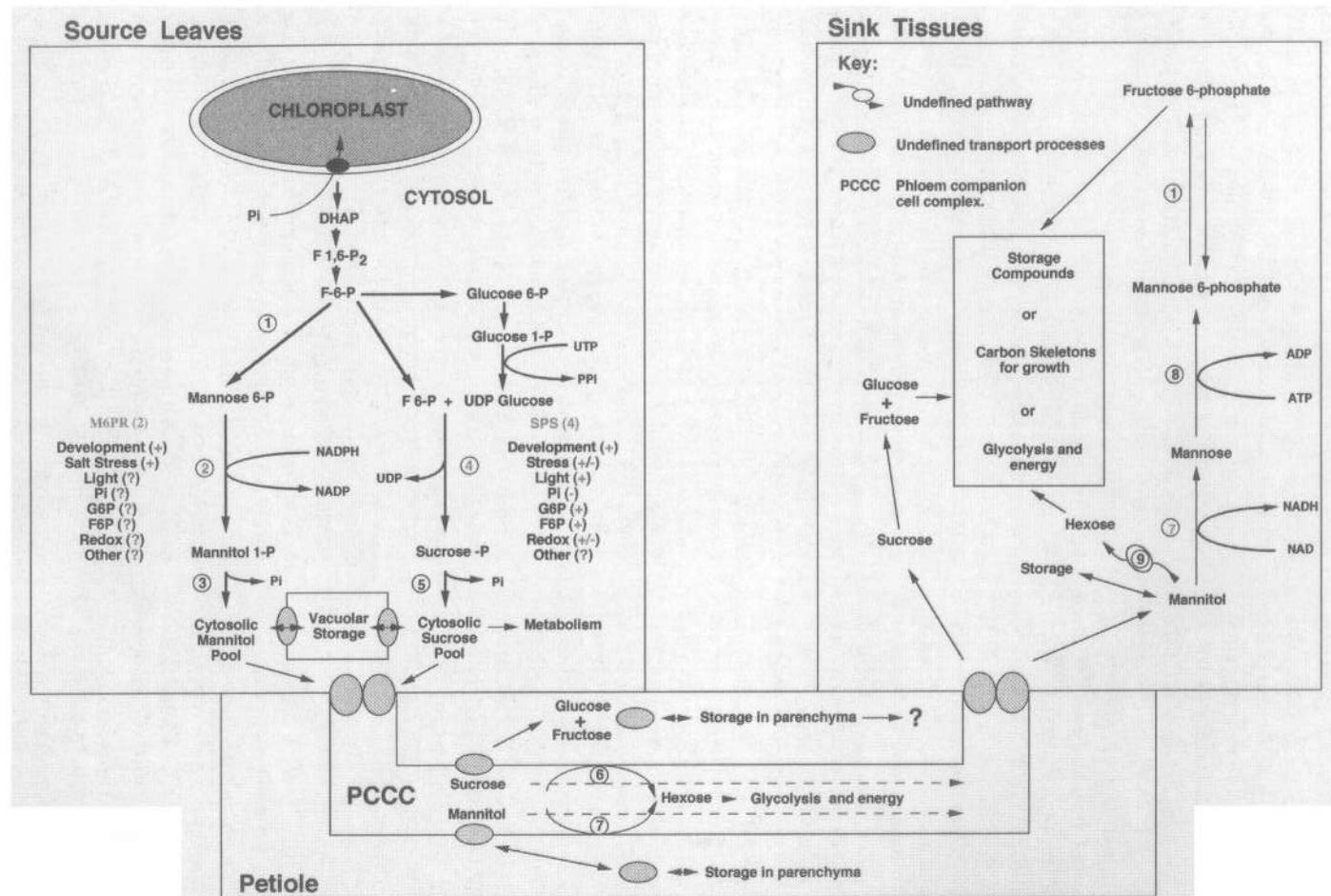


Fig. 1. Schematic diagram showing important features involved in the metabolism and transport of the primary soluble products of photoassimilation (mannitol and sucrose). The information for this diagram comes primarily from work on celery (*Apium graveolens* var. *dulce*), but the synthetic pathway has been described in other higher plant species (see text). The diagram illustrates the parallel (and presumably competitive, in terms of substrates) mannitol and sucrose biosynthetic pathways. Various documented (+/-), and possible (?) modulators of the two key regulatory steps [M6PR (2) and SPS; (4)] in the mannitol and sucrose biosynthetic pathways, respectively, are listed. The diagram also illustrates the spatial separation of the anabolic and catabolic pathways for mannitol, and it is the dynamic balance between sink and source activity, translocation between them and sequestration in long and short term storage pools (involving as yet poorly described transport processes) that is ultimately responsible for regulating mannitol pools in the plant. Important enzymatic steps shown in the diagram are: 1) mannose 6P isomerase (note the roles in both anabolic and catabolic pathways); 2) mannose 6P reductase (M6PR); 3) mannitol-1-P-phosphatase; 4) sucrose-phosphate synthase (SPS); 5) sucrose-phosphate phosphatase; 6) sucrose synthase (SS); 7) mannitol-1-oxidoreductase (MTD); 8) hexokinase. Mannitol catabolism involves MTD (7), but alternative degradative enzymes exist in other species, e.g. mannitol 2-dehydrogenase (9) (see Table 1). See also Color Plate 4.

B. Long Distance Translocation

Transport of sugar alcohols has not been systematically investigated except for a study showing that mannitol, glucitol, and galactitol were transported in 57 of more than 500 species (Zimmermann and Ziegler, 1975). Evidence for translocation of other acyclic sugar alcohols is lacking, although the cyclitol inositol may be present in trace amounts in most phloem exudates. Sucrose, however, seems invariably present as a translocated carbohydrate in sugar alcohol-synthesizing higher plants, even when 60 to 90% of the photosynthate consists of a sugar alcohol.

Evidence for sugar alcohol phloem transport comes primarily from labeling studies (Webb and Burley, 1962; Bielecki, 1969; Davis and Loescher, 1990; Flora and Madore, 1993), although aphid stylet exudates have been analyzed in at least two reports (Moing et al., 1992, 1997). Knowledge of phloem transport mechanisms is otherwise limited. Most information comes from celery, from which vascular tissues can be isolated relatively easily (Daie, 1987a,b), although glucitol transport has been studied in isolated apple phloem (Bielecki, 1969). Export of both glucitol and mannitol is often related to source synthetic capacity and tissue concentrations (Moing et al., 1994), but in some cases it is not. In olive, for example, stachyose accounted for only 5% of the total leaf label while mannitol accounted for 30%, yet stachyose was the predominant label in phloem sap (Flora and Madore, 1993). Other results suggest that the sugar alcohol is synthesized secondarily (Moing et al., 1992), or is first partitioned into a vacuolar storage pool with sucrose apparently being exported at a faster rate from a transitory (cytosolic) storage pool (Davis and Loescher, 1990).

C. Storage

There is considerable evidence for sugar alcohols as storage compounds. Sugar alcohols often represent the majority of the photosynthetic carbon pools in leaves and other vegetative tissues. There can, however, be distinct differences depending on species, tissue, stage of development, and environmental conditions. Storage in leaves especially depends on the stage of growth. Glucitol in apple seedling and mature apple leaves increased rapidly with maturation, e.g., from 0.9% of dry wt in June to 4.8% in late July, decreasing in August and September, but finally increasing in October, suggesting environ-

mental as well as developmental influences (Whetter and Taper, 1963; Chong, 1971).

Also, although glucitol is the major photosynthetic product and translocate, and is the dominant storage carbohydrate during active growth of sweet cherry, only low concentrations occur in dormant roots, which instead store massive amounts of starch (Keller and Loescher, 1989). However, glucitol becomes a significant component of the xylem-translocated compounds exported from roots to developing shoots in early spring (Loescher et al., 1990). In celery, mannitol synthesized in the mesophyll accumulates temporarily in the petiole parenchyma of mature leaves, being remobilized to the younger (sink) tissues during senescence (Keller and Matile, 1989; Davis and Loescher, 1990). In petiole parenchyma mannitol is stored predominantly in the vacuole (81%), with a lesser amount in the cytosol (19%), but cytosolic concentrations may be quite high (Keller and Matile, 1989). On a shorter time scale, sugar alcohols appear to be a temporary carbon store in source leaf mesophyll tissues, accumulating in the light and being translocated in the dark once sucrose pools, which also perform a temporary storage role, have been depleted (Fellman and Loescher, 1987; Davis and Loescher, 1990). Taken together, all these results not only suggest distinct storage roles for these compounds, but also provide evidence of developmental and tissue specific regulation of acyclic sugar alcohol metabolism, storage, and transport.

Sucrose is more readily metabolized than mannitol in both young and mature celery leaves as well as in sink tissues; mannitol is only utilized in nongreen sink tissues or in young (immature and nonphotosynthetic) leaves before they undergo the sink-source transition in celery (Fellman and Loescher, 1987). Similarly, in olive, despite similar uptake rates of ¹⁴C-labeled fructose, glucose, sucrose, and mannitol in disks from mature leaves, mannitol utilization was barely detectable whereas the sugars were catabolized (Gucci et al., 1996). Hamamelitol turnover in *Hedera helix* leaves is extremely slow ($t_{1/2}$, ca. 4 years) (Moore et al., 1997). These results demonstrate that the sugar alcohol is metabolically more sequestered than sucrose, and this may have several important physiological implications in storage, translocation, and stress tolerance.

There is now evidence in celery cell cultures and other sink tissues that sequestration or limited metabolism is due at least in part to repression of the mannitol-catabolizing mannitol dehydrogenase

(MTD) by hexose sugars (Prata et al., 1997). It was already known that glucose-grown cell cultures expressed little MTD activity during active growth, but underwent a marked increase in MTD activity, protein and mRNA upon glucose starvation (Pharr et al., 1995), or when mannitol was the primary carbon source (Stoop and Pharr, 1993). This repression appears to be mediated by hexokinase(s) in a manner comparable to the sugar repression of photosynthetic genes (Stitt et al., 1995; Jang et al., 1997), and these observations are related to the multiple and general effects of tissue carbohydrate status on regulation of gene expression (Koch et al., 1992; Koch, 1996).

D. Roles in Parasitic Plants

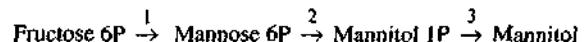
Do parasitic plants require special considerations or interpretations of sugar alcohol metabolism? Parasitic species differ widely in their capacity to import and assimilate carbon and inorganic nitrogen. However, all parasites invariably have, compared to their hosts, lower water potentials which are, not surprisingly, accompanied by higher osmolarities and less susceptibility to drought (Stewart and Press, 1990). The osmotic gradient between host and parasite is sometimes due in part to accumulation of organic materials, i.e., glucose, fructose and cyclitols in the mistletoes (Richter and Popp, 1992) and mannitol in many other parasitic species. It is not always clear whether these are derived from their hosts or synthesized by the parasite, but in *Orobanche* (Harloff and Wegmann, 1993) and perhaps several other genera, e.g., *Thesium* (Fer et al., 1993; Simier et al., 1993, 1994), this gradient appears to be largely the result of a mannitol synthesizing pathway quite similar to that in celery, i.e., with a mannose 6P isomerase, a mannose 6P reductase, and a mannitol 1P phosphatase all present (Harloff and Wegmann, 1993). It is not yet clear whether these synthetic enzymes are all kinetically similar to those in celery (Simier et al., 1994; A. Fer and P. Simier, personal communication). *Orobanche* also contains a low affinity mannitol:NAD 2-oxidoreductase (EC 1.1.1.67) which, by providing a capacity for mannitol degradation to fructose is evidence for a complete mannitol cycle in this taxon (Harloff and Wegmann, 1993). Another feature of parasitic plants distinguishing them from conventional polyol synthesizers is the developmental expression of the biosynthetic enzymes. In non-parasitic species the capacity for sugar alcohol biosynthesis peaks in newly expanded leaves with

little or no activity detectable in young or sink tissues (see Section IV); however, in parasitic plants the highest activities are often found in the youngest tissues (Harloff and Wegmann, 1993; Simier et al., 1994, 1998).

III. Metabolism

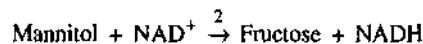
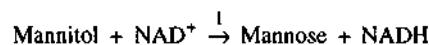
Sugar alcohol metabolism occurs in microbial, animal, and fungal systems as well as higher plants. Higher plant metabolism has recently been reviewed (Loescher and Everard, 1996). Here we focus only on those enzymes involved in mannitol and glucitol metabolism (see Fig. 1 and also Table 1) because these sugar alcohols can be primary photosynthetic products, and both have been studied far more extensively than any other sugar alcohol in higher plants.

Mannitol is synthesized in green celery tissues as a result of three cytosolic enzymes (Rumpho et al., 1983; Loescher et al., 1992): (1) mannose 6P isomerase (E.C.5.3.1.8), (2) NADPH-dependent mannose 6P reductase (E.C.1.1.1.224), and (3) mannitol 1P phosphatase (E.C.3.1.3.22) (Fig. 1).



Glucitol is similarly synthesized via a NADPH-dependent glucose 6P reductase (aldose 6P reductase, E.C. 1.1.1.200) (Hirai, 1981; Negm and Loescher, 1981) and a sorbitol (glucitol) 6P phosphatase (E.C. not assigned) (Grant and ap Rees, 1981). Mannitol may be synthesized in *Orobanche* either by a mannose 6P reductase or perhaps by a low affinity mannitol 1P oxidoreductase (fructose 6P reductase) (E.C. 1.1.1.17) (Harloff and Wegmann, 1993).

The path for mannitol breakdown appears to depend on the species and involves one of two NAD dependent enzymes: (1) mannitol 1-oxidoreductase (E.C. not assigned) (Stoop and Pharr, 1992), or (2) mannitol NAD oxidoreductase (fructose reductase) (E.C. 1.1.1.67) (Harloff and Wegmann, 1993) (Fig. 1).



Glucitol is apparently primarily metabolized by an NAD-dependent sorbitol (glucitol) dehydrogenase (EC 1.1.1.14) (Negm and Loescher, 1979), but other

Table 1. This table lists the known synthetic, degradative and selected ancillary enzymes associated with acyclic sugar alcohol metabolism in higher plants. Also listed are EC numbers (where assigned), the source, and accession numbers if they have been cloned from higher plants. Please note sorbitol is synonymous with glucitol. Further information is given in the footnote.

Name, synonyms and abbreviation	EC Number	Organism	Accession number	Reference if cloned
Synthetic Enzymes				
NADP-dependent sorbitol 6P dehydrogenase; NADPH-dependent aldose 6P reductase; A6PR	1.1.1.200	<i>Eriobotrya</i> <i>Malus</i>	D11080	Hirai, 1981 (p) Negm and Loescher, 1981 (p) Kanayama et al., 1992 (c)
mannose 6P reductase; M6PR	1.1.1.224	<i>Apium graveolens</i> var. dulce	U83687	Loescher et al., 1992 (p) Everard et al., 1997 (c)
mannitol 1P:NAD oxidoreductase	1.1.1.17	<i>Orobanche</i>	*	Harloff and Wegmann, 1993 (p)
NADPH-dependent aldose reductase; galactitol synthesising aldose reductase	unassigned	<i>Euonymus japonica</i>		Negm, 1986 (p)
NADPH-dependent ribose 5P reductase	unassigned	<i>Adonis</i>		Negm and Marlow, 1985 (p)
sedoheptulose reductase; ketose reductase; SedR	unassigned	<i>Primula</i>		Häfliger et al, 1998 (p)
Synthetic and Degradative Enzymes				
NAD-dependent polyol dehydrogenase; ketose reductase; sorbitol dehydrogenase; L-iditol:NAD 5-oxidoreductase/dehydrogenase; SDH	1.1.1.14	<i>Malus</i> <i>Zea mays</i> <i>Glycine max</i> <i>Viscum album</i>	*	Negm and Loescher, 1979 (p) Doehlert, 1987 (p) Kuo et al., 1990 (p) Wanek and Richter, 1993 (p)
Degradative Enzymes				
mannitol dehydrogenase; mannitol: mannose 1-oxidoreductase; MDH; MTD	unassigned	<i>Apium graveolens</i> var. rapaceum and dulce	U24561	Stoop and Pharr, 1992 (p) Williamson et al., 1995 (c)
NADP-dependent sorbitol dehydrogenase	unassigned	<i>Malus</i>		Yamaki, 1984 (p)
sorbitol oxidase	unassigned	<i>Malus</i>		Yamaki, 1980 (p)

mannitol NAD:oxidoreductase; mannitol 2-dehydrogenase; fructose reductase	1.1.1.67	<i>Orobanche</i>	*	Harloff and Wegmann, 1993 (p)
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Associated Enzymes

mannose 6P isomerase; phosphomannose isomerase; phosphohexomutase; PMI	5.3.1.8	<i>Apium graveolens</i> var. dulce	*	Rumpho et al., 1983 (p)
mannitol 1P phosphatase	3.1.3.22	<i>Apium graveolens</i> var. dulce <i>Orobanche</i>		Rumpho et al., 1983 (p) Harloff and Wegmann, 1993 (p)
sorbitol 1P phosphatase	not assigned	<i>Malus</i>		Grant and ap Rees, 1981 (p)
glyceraldehyde 3P:NADP reductase; non-reversible or non-phosphorylating triosephosphate dehydrogenase; GNR	1.2.1.9	<i>Apium graveolens</i> var. dulce	*	Rumpho et al., 1983 (p)

Function undefined

NADPH-aldoxide reductase; AR	1.1.1.21	<i>Glycine max</i> <i>Hordeum vulgare</i> <i>Bromus inermis</i>	P23901	Kuo et al., 1990 (p) Bartels et al., 1991 (p,c) Lee and Chen, 1993a (c)
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* indicates where the corresponding genes have been cloned from sources other than higher plants. By using EC numbers (where available) in conjunction with the web site "Enzymes" (<http://teosinte.agron.missouri.edu/enzyme.html>) access to all clone and sequence information currently available on public data bases can be obtained (via the associated "hot links"). The letters in parentheses following references indicate whether the study covers the protein and enzyme activity (p), or molecular cloning (c).

enzymes may be important in some situations (Yamaki, 1982; Yamaki and Ishikawa, 1986; Moriguchi et al., 1990) (See Table 1).

Important to regulatory questions are recent advances in what we know of these enzymes at the gene and protein level. Sequences have been available for a number of sugar alcohol related enzymes in animal and microbial systems, and now at least three higher plant enzymes have been cloned and sequenced: the aldose 6P reductase from apple (Kanayama et al., 1992), a celery mannitol 1-oxidoreductase (Williamson et al., 1995), and a celery mannose 6P reductase (Everard et al., 1997) (Table 1). M6PR and A6PR are closely related, with their deduced amino acid sequences showing 64% identity and 84% similarity. Considerable homology also exists between these proteins and the mammalian ALR2 class of aldose reductases which are NADPH-specific, monomeric 35kD proteins (see Section VIII.A). The monomeric masses of A6PR and M6PR, either directly measured (Loescher et al., 1992; Kanayama and Yamaki, 1993) or from the deduced amino acid sequences (Kanayama et al., 1992; Everard et al., 1997), are 35 kD. However, gel filtration chromatography indicated the native molecular masses of A6PR and M6PR to be 65 kD (Hirai, 1981) and 58 kD (Loescher et al., 1992), respectively, suggesting that they may be homodimers. Whether this indicates a genuine difference between the plant and animal aldose reductases or is a result of anomalous behavior of the plant enzymes during gel filtration needs to be resolved.

Why aren't the isomerases, mutases, epimerases, and phosphatases important regulatory factors? Two studies (Grant and ap Rees, 1981; Loescher et al., 1992) suggest that the mannose 6-phosphate isomerase and the glucitol 6P and mannitol 1P phosphatases are possible, but unlikely candidates for several reasons. The hexose phosphates, fructose 6P, glucose 6P, and mannose 6P are in equilibrium in celery leaf tissues, extractable activities of these enzymes are all far in excess of flux rates through the biosynthetic pathways, and the pool sizes of the phosphorylated hexitols (the last intermediate in the biosynthetic pathway) are all very small.

IV. Developmental Regulation and Primary Roles

Studies of sink-source transitions can provide

excellent insight into the relative roles that specific enzymes and processes play in plant metabolism (Turgeon, 1989). However, only a few such studies are known in sugar alcohol-synthesizing plants, and these have focused on either glucitol or mannitol metabolism where they usually involved the enzymes aldose 6P reductase (A6PR) and sorbitol (glucitol) dehydrogenase (SDH) or mannose 6P reductase (M6PR) and mannitol 1-oxidoreductase (MTD), respectively. The results have invariably indicated that sugar alcohol metabolism is generally under very tight developmental control. For example, sink-source interconversion studies of apple and celery leaves show that developing leaves undergo transitions from sinks with high rates of sugar alcohol oxidation to sources with reduced or negligible capacity for sugar alcohol degradation and increased capacities for synthesis. These transitions are accompanied by increases in the corresponding synthetic enzyme. A6PR increases in glucitol-synthesizing apple leaves (Loescher et al., 1982; Yamaki and Ishikawa, 1986) and cotyledons (Yamaki, 1980; Kanayama et al., 1995) and developing peach leaves (Merlo and Passera, 1991). Similar changes occur in M6PR and SPS in mannitol- and sucrose-synthesizing celery leaves (Davis et al., 1988). Data from young celery leaves are also consistent with enzyme assays that show little detectable M6PR in young leaves and non-green tissues. Other evidence shows that as leaves undergo the sink-source interconversion there are corresponding decreases in degradative enzymes (Loescher et al., 1982; Fellman and Loescher, 1987; Merlo and Passera, 1991).

Lewis (1984) has reviewed other reports comparing synthesis and degradation that show that the ability to synthesize, accumulate, and degrade sugar alcohols can vary tremendously among different plant tissues during development within a given plant tissue and between otherwise closely related taxa. Sinks, for example, have little or no capacity to synthesize sugar alcohols. Apple root tips, bark and wood of stems, and root tissues can synthesize only a little glucitol from fructose (Grant and ap Rees, 1981). Celery root tips can accumulate and metabolize mannitol, but do not synthesize mannitol from other sugars (Stoop and Pharr, 1992). Celery petioles, however, which contain some green tissues, can synthesize mannitol, although at lower rates than leaves (Keller and Matile, 1989). Similarly, although glucitol is commonly translocated in many Rosaceous species, it is found in only trace amounts in nectar.

These nectaries rapidly convert glucitol to glucose, fructose, and even sucrose, but apparently cannot convert these sugars to the hexitol (Bielecki and Redgwell, 1980).

Unfortunately, very little else has been done to correlate activities of the sugar alcohol enzymes with development of either photosynthetic (source) competence, storage or sink activity. However, more comprehensive studies of gene regulation are now possible, and there has been progress. Genes for several of the key anabolic and catabolic enzymes have recently been cloned. In celery, for example, M6PR gene expression and enzyme activity are now known to be limited to green tissues and to be under tight transcriptional regulation during leaf initiation, expansion and maturation. These data confirm a close relationship between development of photosynthetic capacity, mannitol synthesis, and M6PR activity (Everard et al., 1997). Another study (Prata et al., 1997) of MTD gene expression and enzyme activity suggests that mannitol oxidation is transcriptionally regulated in celery suspension cultures (a model sink tissue). Transcriptional regulation appears to be controlled by endogenous carbohydrate levels by mechanisms like those involved in repression of photosynthetic gene expression via hexokinase (Sheen, 1994). However, there are as yet no data to show how the synthetic and degradative pathways are coordinated.

V. Localization of Synthetic and Degradative Steps

Both indirect (Lewis, 1984) and direct immunolocalization (Everard et al., 1993) evidence now leads to the conclusion that sugar alcohol biosynthesis is predominantly cytosolic, and not associated with vacuoles, chloroplasts, mitochondria, peroxisomes, or membranes, although these same studies suggest that M6PR may also be present in the nuclear compartment. Both A6PR and M6PR genes appear to lack signal peptides, and there is no evidence that the M6PR peptide is post-translationally processed *in vivo* (J. D. Everard, unpublished), further confirming the cytosolic location. As the key step in mannitol biosynthesis, M6PR is also predominantly found in green spongy and palisade parenchyma of leaf mesophyll tissues and bundle sheaths (Everard et al., 1993), but not the vascular parenchyma. This contrasts with galactinol synthase, which immuno-

localization suggests is located in the intermediary cells of the phloem of cucurbit source leaves, where it may facilitate the phloem loading of galactosyl-sucrose oligosaccharides like raffinose (Beebe and Turgeon, 1992).

Consistent with these immunocytochemistry results in both mannitol and sucrosyl oligosaccharide synthesizing species, labeling studies in olive (*Olea*) (Flora and Madore, 1993) suggest spatial separation of stachyose and mannitol biosynthesis, with relatively rapid mannitol synthesis occurring within the photosynthetic mesophyll tissues and slower raffinose synthesis occurring closer to, and probably within, the minor veins. Somewhat similar differences in labeling of mannitol and stachyose were noted in privet (*Ligustrum*, also in the Oleaceae), but no differences between sucrose and mannitol labeling were observed in celery which does not transport or synthesize raffinose oligosaccharides (Loescher et al., 1992).

There is no recent or immunocytochemical evidence suggesting that sugar alcohol biosynthesis is chloroplastic, but targeting a bacterial enzyme for mannitol biosynthesis to that compartment in transgenic plants has some implications for stress tolerance (see Section VII.E). The mannitol degrading enzyme MTD is also cytosolic, and is found in various sink tissues, i.e., meristems of celery root apices, in young expanding leaves, in the vascular cambium, and in the phloem, including the parenchyma and the sieve-element/companion cell complexes (Zamski et al., 1996). The association between MTD and phloem has been suggested as a means by which mannitol may reenter the metabolically active carbohydrate pool and thus provide energy to support phloem function (Zamski et al., 1996). This is analogous to the proposed role of phloem associated sucrose synthase (Geigenberger et al., 1993; Nolte and Koch, 1993) (Fig. 1).

VI. Membrane Transport

Single saturable, or biphasic, active, PCMB-sensitive, turgor-sensitive specific mannitol uptake systems have been described in celery storage parenchyma (Keller, 1991). Keller speculated that the mannitol carrier was at the plasmalemma and involved in active proton-cotransport. Similar results were reported for glucitol transport in apple fruit tissue protoplasts and isolated vacuoles, where

transport was facilitated by a PCMBS, CCCP, and orthovanadate sensitive saturable carrier on the plasmalemma (Yamaki and Asakura, 1988) and a somewhat similar ATPase-coupled carrier on the tonoplast (Yamaki, 1987). Studies of older apple fruit tissue discs indicated that this system was also turgor sensitive (Berüter, 1993). Berüter also concluded from compartmental analyses that the cytosol was part of the apparent free space, although this was dependent on osmotic strength of the bathing medium and the age of the tissue. Sugar alcohol transport in these sink systems appears to have some characteristics common to those reported for sucrose in sink tissues (Patrick, 1993). In isolated celery phloem preparations (Daie, 1986; Daie, 1987a,b) and olive leaf disks (Flora and Madore, 1993), however, mannitol uptake appeared to be insensitive to PCMBS, perhaps because the mannitol carrier does not always have an exposed sulfhydryl group as part of its active site. Alternatively, in species lacking PCMBS sensitivity, anatomical studies suggest that phloem loading may be symplastic (Turgeon and Beebe, 1991; van Bel et al., 1992).

Another option is that phloem loading and uptake of mannitol in source tissues may involve a distinctly different carrier from that in sink tissues. Recent work (Salmon et al., 1995) with plasma-membrane vesicles (PMVs) from phloem strands isolated from mature celery petioles or from mature petioles devoid of vascular bundles showed that the PMVs from phloem strands had a higher ATPase activity suggesting the possibility of different carriers. Further work supported this by showing that PMVs from phloem tissues accumulated mannitol and sucrose in response to an artificial proton-motive force, in agreement with the existence of proton/substrate carriers, but PMVs from petioles devoid of vascular bundles accumulated only mannitol under the same conditions. Similarly, modeling of glucitol and sucrose export from mature leaves suggested that glucitol and sucrose export capacities involved different transporters with different kinetics, i.e., glucitol transport was saturated at normal photosynthetic rates resulting in glucitol accumulating during the day while sucrose content appeared to be maintained at a constant level (Moing et al., 1994). More recently, Moing et al (1997) reported that glucitol concentrations in the leaf were quite high and not significantly different from the phloem concentrations, thus enough for symplastic loading; however, transport of both glucitol and sucrose was

inhibited by PCMBS, an indicator of apoplastic loading (van Bel et al., 1994).

In some bacteria, specific phosphorylase-linked permeases for mannitol, glucitol, or galactitol transport are well characterized. The mtl operon, for example, consists of a number of genes, including a permease and a mannitol 1P dehydrogenase (Weng and Jacobson, 1993; Henstra et al., 1996), but no equivalent system has been reported for sugar alcohol transport in higher plants. On the other hand, Bielecki and Redgwell (1980), studying secretion in *Prunus* nectaries, noted that glucitol transport (perhaps as an integral part of phloem unloading) was associated with conversions to fructose and glucose. Similarly, Yamaki (1982) reported evidence in apple for a tonoplast-associated glucitol oxidase or a cytosolic glucitol dehydrogenase (Yamaki and Asakura, 1988) that may be involved in glucitol transport and conversion, resulting in hexoses accumulating as the predominant carbohydrates in fruit vacuoles.

Another consideration involves inorganic nutrition. Although boron has historically been considered a phloem-immobile element, recently Brown and coworkers (Hu et al., 1997) successfully isolated and characterized boron-sugar alcohol complexes from the phloem sap of celery and the extrafloral nectar of peach. In celery they were able to directly analyze phloem sap by matrix-assisted laser desorption-Fourier transform mass spectrometry, with verification by HPLC and gc-ms (Penn et al., 1997). Their data revealed that B is present in the phloem as the mannitol-B-mannitol complex. Molecular modeling further predicted that this complex is present in the 3,4 3',4' bis-mannitol configuration. In the extrafloral nectar of peach, B was present as a mixture of glucitol-B-glucitol, fructose-B-fructose, or glucitol-B-fructose. These findings provide a mechanistic explanation for the observed phloem B mobility in these species and associated susceptibility to B deficiency (Hanson, 1991), and they may also have further implications for sugar alcohol transport.

VII. Secondary Physiological Roles

Immobility means that plants must respond to environmental changes, and carbohydrate-regulated genes represent an especially valuable mechanism for acclimation (Koch, 1996). If it is a reasonable presumption that environmental stresses affect regulation of sugar alcohol metabolism, then there

should be examples of stress affecting sugar alcohol synthesis, breakdown, storage, transport, partitioning, and sink-source transitions. Such changes leading to accumulation and/or synthesis may be evident at the whole plant level, but others could be quite subtle, e.g., evident only in changes in cellular compartmentation. Although evidence of stress effects on sugar alcohol metabolism is limited compared to that available for some other carbohydrates, the results taken as a whole are clear and convincing.

A. Roles as Compatible Solutes and in Osmoregulation

Stresses that drastically alter cellular water potential, e.g., salinity, drought, and cold stresses, can also specifically cause a number of compounds to accumulate (Schobert, 1977; Pollard and Jones, 1979; Yancey et al., 1982; Wyn Jones, 1984; Popp, 1984; Bonnett and Incoll, 1993). These compounds, which include both acyclic and cyclic sugar alcohols, the imino acid proline, quaternary ammonium compounds (e.g., glycine betaine), and tertiary sulfonium compounds, were originally thought to act primarily as osmotic regulators, by maintaining 'osmotic balance'. But other compounds, both inorganic (K^+ , Cl^- ; Na^+ , SO_4^{2-}) and organic (hexose reducing sugars), can play roles in osmotic adjustment (Munns and Weir, 1981; Sharp et al., 1990), and it is now understood that the first group of compounds may have other protective qualities. This change in view came from work on sugar-tolerant yeasts which accumulate nonreducing sugars (trehalose) and sugar alcohols (including glycerol) in response to osmotic stress. From this latter work the term 'compatible solute' was first introduced (Brown and Simpson, 1972).

Data on compatible solutes comes primarily from studies in which high concentrations did not interfere with *in vitro* enzyme activities (Flowers et al., 1977; Ahmad et al., 1979; Pollard and Jones, 1979; Smirnoff and Stewart, 1985; Manetas et al., 1986; Karsten et al., 1996), and sometimes even protected them from deleterious effects of salt (Ahmad et al., 1979; Manetas et al., 1986) or heat (Paleg et al., 1981; Smirnoff and Stewart, 1985). Compatible solutes also protected the cellular transcriptional apparatus from perturbations by salt *in vitro* (Gibson et al., 1984) and increased thermal tolerance of isolated chloroplast thylakoids (Seemann et al., 1986). However, for compatible solutes to perform *in vivo*

like they do *in vitro*, they would seem to have to accumulate to very high concentrations, e.g., up to 500 mM. Indeed, many of the compounds listed above do achieve these critical levels if asymmetric compartmental distribution is assumed. But, in transgenic plants where the capacity to synthesize these compounds has been engineered, concentrations of the compatible solute may be well below 100 mM (a value achieved only by assuming that all accumulation occurs in the cytosol). Protective effects have nonetheless been demonstrated, and the term 'osmoprotectant' has been coined to accommodate these observations (Bohnert and Jensen, 1996).

Several biophysical roles have been proposed for sugar alcohols and other compatible solutes (e.g., proline) and their interactions with biological structures (Galinski, 1993). In the first, they may substitute for water in protein hydration shells and membrane structures or, at least in the case of proline, protect exposed hydrophobic domains of proteins and thus maintain conformation at water activities that otherwise cause dehydration and denaturation (Schobert, 1977; Crowe et al., 1988). A second alternative suggests uneven distribution of compatible solutes in a protein solution where compatible solutes are excluded from protein hydration spheres; in some way bulk water structure is ordered so that biological structures become preferentially hydrated and conformation is maintained as the bulk water activity is otherwise lowered. There are data supporting both hypotheses, i.e., direct interactions (Webb and Bhorjee, 1968) or exclusion (Timasheff, 1993). In the third and perhaps supplemental protective role, proline and sugar alcohols may scavenge hydroxyl radicals, protecting enzymes from denaturation during stress (Smirnoff and Cumbes, 1989; Jennings and Burke, 1990; Popp and Smirnoff, 1995) (see also Section VII.E).

B. Cold Hardiness

As compatible solutes, sugar alcohols could play roles in cold tolerance and freezing resistance. Cold injury often results from desiccation due to extracellular freezing and consequent withdrawal of water from the protoplast by the growing ice crystals. Work on insects strongly suggests a role in cold hardiness (Storey and Storey, 1991), especially now that there is a glucitol related gene associated with cold acclimation (Niimi et al., 1993). Trends in woody plants may be analogous to insects, but despite

numerous efforts to demonstrate such relationships the mostly correlative evidence is less than convincing. There is little evidence of other than passive increases in sugar alcohols that could be explained by reduced demand and decreased export due to low temperatures, there are no compartmentation studies, there are as yet no cold regulated genes associated with sugar alcohol metabolism in higher plants, and there are no transgenic plants with improved cold tolerance resulting from sugar alcohol accumulation.

For example, Sakai (1960), studying 19 woody species with a range of sugar alcohol-synthesizing capabilities, found no direct correlation between sugar alcohol content and cold hardiness; however, later work showed increases in both sugar alcohols and raffinose in response to cold stress (Sakai, 1966). Some apple tissue culture experiments suggested that using glucitol as a carbon source increased cold tolerance (Pieniazek et al., 1978). In other work with xylem sap extracted from apple shoots, glucitol increased with leaf senescence and low (0 to 4°C) temperatures. As temperatures continued to fall during dormancy, glucitol levels generally increased, with concentrations fluctuating sharply with changes in temperature (Williams and Raese, 1974; Ichiki and Yamaya, 1982). Although xylem sap contained 10 to 12 times as much glucitol in December and January as compared with October, the amount of glucitol in the entire tissue remained fairly constant throughout dormancy except for a slight increase during December and January. Exposing apple shoots to near zero temperatures could increase sap glucitol as much as 40-fold within 24 h (Williams and Raese, 1974). Thus, some increases in sap concentrations may indeed be related to changes in tissue distribution (and perhaps cellular compartmentation) rather than synthesis.

Similar changes in sap glucitol were found in sweet cherry (*Prunus avium*), with the highest levels following exposure to subfreezing temperatures in January. As in apples, overall tissue glucitol content increased little, but there were increases in xylem sap levels. Later in the season, however, these changes were not correlated with decreasing temperatures: although total carbohydrate levels rose, glucitol levels declined, and reducing sugars increased until just before full bloom where they may have been important in early bud development (Sauter and Ambrosius, 1986; Loescher et al., 1990).

Further evidence of relationships between cold

hardiness and sugar alcohols in deciduous perennials is limited, and interpretations are complicated by studies where unidentified 'carbohydrates' are correlated with hardiness (Flinn and Ashworth, 1995). Hirai (1983), however, reported glucitol content and activity of the glucitol synthesizing enzyme (A6PR) to increase in late autumn and decrease in spring in leaves of loquat (*Eriobotrya*, a subtropical evergreen). Similar results were obtained with seedlings in controlled environments where activity was increased by low temperature. In winter, in bark of the evergreen olive, mannitol levels were more than double what they were in the summer (Drossopoulos and Niavis, 1988). Studies of low temperature acclimation in several cacti (*Opuntia*) species showed, depending on the tissue, from $2\times$ to over $9\times$ increases in soluble sugars, including a nearly $3\times$ increase in mannitol in the hardiest species (Goldstein and Nobel, 1994).

As already mentioned, there are no cold regulated genes associated with sugar alcohol metabolism in higher plants. There may, however, be several cold regulated genes similar to genes related to sugar alcohol metabolism. For example, cDNA of an aldose reductase-related gene has been isolated from ABA-treated bromegrass cell suspension cultures during the induction of freezing tolerance (Lee and Chen, 1993b). It appears that both aldose reductase-related mRNA and enzyme activity were elevated during ABA-induced hardening in these cell suspension cultures; however, polyethylene glycol treatments (to mimic dehydration) did not elicit similar effects (Lee and Chen, 1993b).

C. Drought Stress

Very few studies focus specifically on acyclic polyols and drought stress, and interpretations of these and other stresses may be confounded by confusing primary and secondary effects. Osmotic adjustment generally does not occur without a decrease in growth rate (Munns, 1988), and photosynthetic products could accumulate due to reduced sink demand resulting from stress-induced growth inhibition, in the same way that low temperature-induced accumulations could also be explained by reduced demand and decreased export from source leaves (Popp and Smirnoff, 1995). Also, it is not always clear whether osmotic adjustment is due to active synthesis and accumulation of solutes or passive due to leaf dehydration and shrinkage (Hsiao, 1973). Nonetheless, glucitol was the dominant soluble

carbohydrate and its content doubled in drought-stressed *Prunus* (Ranney et al., 1991). In apple, water stress resulted in preferential accumulation of glucitol and glucose instead of sucrose and starch (Wang et al., 1995; Wang et al., 1996). In *Fraxinus* mannitol not only changed significantly in leaves throughout the diurnal cycle, but also played a large role in the seasonal osmotic adjustment which occurred in response to summer drought (Marigo and Peltier, 1996). More recent comparisons of drought stressed and control trees and seedlings showed that these acclimated to quite low predawn leaf water potentials (-4 MPa and lower) without apparent wilting or cessation of photosynthesis. Drought resulted in very high mannitol and malate concentrations, i.e., calculated at 280 and 600 mM, respectively, while sucrose remained relatively low, less than 30 to 50 mM (Guicherd et al., 1997). Similar recent studies in olive showed that both mannitol and glucose collectively accounted for approximately 30% of the leaf's osmotic potential, and studies of salt stress showed that both mannitol and the mannitol to glucose ratio increased rapidly, but it was not clear how these related to drought stress. Cytosolic concentrations were, however, calculated to be adequate for mannitol to be effective as a compatible solute (Gucci et al., 1997).

Despite the limited studies of acyclic polyols, there are numerous examples of drought-induced accumulation of other soluble carbohydrates (for a review see Popp and Smirnoff, 1995). In systems involving cyclic polyols, pinitol increased five-fold in drought stressed *Cajanus* (Keller and Ludlow, 1993), in *Artemisia* accumulations of a cyclitol, probably quebrachitol (Schilling et al., 1972), were clearly drought related (Evans et al., 1992), and in *Pinus pinaster* pinitol doubled when the osmotic potential of the nutrient solution was lowered (Nguyen and Lamont, 1988). In systems involving sucrose there is evidence that water-stress may generally result in higher sucrose and lower starch levels than in non-stressed leaves (see Zrenner and Stitt, 1991, and papers cited therein).

In alternative approaches using transgenic plants, two recent studies have shown that plants with genetically modified capacities for soluble carbohydrate biosynthesis were more drought tolerant. Engineering tobacco plants for biosynthesis of trehalose, a non-reducing disaccharide, greatly increased their capacity to survive drought, but phenotype was altered and growth rate was decreased

30–50% (Holmström et al., 1996). Similar work was done with transgenic tobacco plants that had the capacity to accumulate bacterial fructans (polyfructose). The transgenic plant grew significantly better than controls under polyethylene glycol-mediated drought stress, growth rates were higher, as were fresh and dry weight yields. Weight differences were observed in all organs and were particularly pronounced in roots. However, unlike the trehalose-synthesizing transgenic tobacco plants, under unstressed control conditions the presence of fructans had no significant effect on phenotype, growth rate, and yield (Pilon-Smits et al., 1995).

Work with other presumptive compatible solutes has shown distinct changes in gene expression with drought stress. Proline is one of the most common compatible osmolytes in water-stressed plants (Taylor, 1996), and several studies have shown drought related changes in gene expression, i.e., activation of proline biosynthesis and inactivation of degradation. For example, a nuclear gene for mitochondrial proline dehydrogenase is upregulated by proline but downregulated by dehydration (Kiyosue et al., 1996). There is also induction (osmoregulation) of the proline synthesizing pyrroline-5-carboxylate reductase gene with either seed maturation or salt stress (Verbruggen et al., 1993), and there are salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases (Rentsch et al., 1996).

Although no sugar alcohol related plant genes have been studied in the context of drought stress, expression of a barley gene homologous to aldose and aldehyde reductases has been found that is restricted to the embryo and temporally correlated with acquisition of desiccation tolerance (Bartels et al., 1991; Roncarati et al., 1995). Aldose reductase may also be present in corn seedling tissues which can utilize glucitol as a carbon source (Swedlund and Locy, 1993), and there is evidence in soybean seedlings which appear to have a functioning glucitol pathway of both aldose and ketose reductase activities (Kuo et al., 1990). As already mentioned, however, although Lee and Chen (1993b) were able to increase cold hardiness using polyethylene glycol for osmotic stress treatments, there was no detectable aldose reductase mRNA accumulation in bromegrass suspension cultures.

D. Salt Stress

The correlation between salinity stress and sugar

alcohol accumulation in higher plants is quite strong. Such studies represent not only the majority of the publications on stress and sugar alcohol accumulation, but also where the most progress has been made. The first work was on glucitol metabolism in *Plantago* (Ahmad et al., 1979; Lambers et al., 1981) and then later in numerous other species (Gorham et al., 1981; Briens and Larher, 1982; Everard et al., 1994; Stoop et al., 1994; Popp and Smirnoff, 1995; Tattini et al., 1996) which showed that acyclic polyols increased substantially with salt stress. There are similar studies of the cyclic polyols (Popp and Smirnoff, 1995). All of this is further supported by the literature documenting osmoregulatory roles for polyols in marine algae (Kirst, 1990). However, like much of the algal literature, most of the higher plant literature is correlative, and the mechanisms involved have not been studied. There are exceptions: mechanisms leading to glycerol accumulation in the euryhaline alga *Dunaliella* have been studied extensively (Cowan et al., 1992). If we accept that sugar alcohols function as compatible solutes or osmoprotectants in transgenic plants (Tarczynski et al., 1993; Thomas et al., 1995) as apparently do glycine betaine (Nomura et al., 1995) and proline (Kishor et al., 1995), the conclusion that accumulation is an adaptive response is compelling.

The literature on sugar alcohols and salt stress in higher plants often presents interpretational problems. Other compatible solutes, e.g., glycine betaine (Rhodes and Hanson, 1993), proline (Delauney and Verma, 1993), and the cyclic polyols (Popp, 1995; Popp and Smirnoff, 1995), are synthesized in response to stress and are not significant products under non-stressed conditions. However, in higher plants the acyclic polyols are, as already discussed, often primary photosynthetic products. Accumulation may, therefore, represent a consequence rather than an adaptation to stress, the result of suppressed growth and/or reductions in tissue water content. Differential changes between the sugar alcohols and other photosynthetic products (e.g., sucrose and starch) may also be misleading due to the relatively sequestered nature (in terms of their capacity to be metabolized) of polyols (Trip et al., 1964; Fellman and Loescher, 1987; Gucci et al., 1996). For example, passive accumulation of mannitol in response to salt stress has been reported for olive. Mannitol increased in olive leaves in response to salinity stress, but the magnitude of the increase had little relationship to the intensity of the salt treatment or the salt tolerance

of the cultivar or species. Indeed, in olive increases in mannitol accumulation (concentration) could result from a decrease in tissue water content (Tattini et al., 1996; Gucci et al., 1997a; Gucci et al., 1997b; Tattini pers. comm.) without any change in carbon partitioning or mannitol metabolism, but other data suggest that mannitol actively accumulates with salt stress (Gucci et al., 1998). Since starch is frequently depleted under salt stress (Everard et al., 1994; Tattini et al., 1996), data on tissue pool sizes should be supplemented with measures of carbon flux into these various carbon pools to understand the metabolic processes underlying accumulation.

Studies of celery stressed with sodium chloride (Everard et al., 1994) or excess macronutrients (Stoop and Pharr, 1994a,b) have shown that stress effects on mannitol accumulation were due to changes in both carbon partitioning and utilization. These changes can be linked to regulation at both the enzyme and gene level (Everard et al., 1994; Stoop and Pharr, 1994b; Loescher et al., 1995; Pharr et al., 1995; Williamson et al., 1995; Stoop et al., 1997). For example, in newly fully expanded leaves of celery plants subjected to 300 mM NaCl carbon flux into mannitol was maintained at control rates despite a 70% decrease in overall carbon assimilation. Maintaining mannitol synthesis came at the expense of sucrose, and the ratio of label in mannitol to sucrose increased four-fold. Associated with this shift was a two fold increase in extractable M6PR activities. However, salt-induced increases in M6PR extractable activity were not associated with a concomitant rise in M6PR protein which suggests post-translational enzyme regulation (Everard et al., 1994).

In developmental studies, as photosynthetic capacity increased and as leaves developed and matured, more carbon was partitioned into mannitol with proportionately less into sucrose, and these changes were accompanied by increased activities of enzymes in the mannitol biosynthetic pathway (Davis et al., 1988). With salt stress, however, mannitol biosynthesis was further enhanced in younger leaves, with 40% of the carbon partitioned into mannitol at 300 mM NaCl compared to 10% in controls. This change in carbon fixation was mostly at the expense of sucrose (Loescher et al., 1995). This salt enhanced increase in mannitol partitioning was accompanied by a six-fold increase in extractable M6PR activity (Everard et al., 1994). Recent results (J. D. Everard, unpublished) show that these increased M6PR

activities were associated with concomitant increases in M6PR transcript and protein levels, indicating salt-induced transcriptional control. Such results are certainly consistent with other observations of salt-induced gene expression (Bohnert et al., 1995; Cushman and Bohnert, 1995; Deutch and Winicov, 1995). See chapters in this volume on CAM for other examples.

On a whole plant basis, mannitol pool sizes are dictated by a balance between synthesis and degradation, processes that are spatially and developmentally separated in celery (Figure 1) and also presumably influenced by long distance transport as well as salinity stress. Although nothing is yet known about mannitol transport in salt stressed plants, considerable information is now available on degradative processes and their regulation. Just as M6PR and subsequent mannitol synthesis and storage in source tissues are enhanced by salt stress, salt induced decreases in mannitol degradation and subsequent accumulation in sink tissues are due to a down regulation of mannitol catabolism via the enzyme MTD (Stoop and Pharr, 1994b; Williamson et al., 1995; Stoop et al., 1996).

E. Free Radical Scavengers

Drought and salt stress are closely related. Indeed, imposition of salt stress may generally be related to a two phase response (Munns et al., 1995) with the first involving a large decrease in growth rate caused by the salt outside the roots, i.e., an osmotic response with decreases in stomatal conductance. In the second phase there could be a salt-specific response to toxic effects within the plant. Thus, in the first phase decreased stomatal conductance could increase production of free radicals and the damage associated with photoinhibition. In this context there is evidence in fungi and other organisms that sugar alcohols like glucitol and mannitol and cyclic polyols like myoinositol serve a protective function as free radical scavengers (Smirnoff and Cumbes, 1989; Jennings and Burke, 1990), but most of this evidence has been *in vitro*. There is also evidence that an ability to deal with these active oxygen species may be important in desiccation tolerance (Smirnoff, 1993). However, *in vivo* data are quite limited. With no data available for higher plant systems, the beneficial effects for stress tolerance have been controversial (Munns, 1993). Recently, however, tobacco was transformed with a bacterial mannitol 1P dehydrogenase targeted to the

chloroplast by the addition of an amino-terminal transit peptide (Shen et al., 1997). These transgenic tobacco plants accumulated mannitol from 2.5 to 7 $\mu\text{mol/g fr wt}$. One transgenic line accumulated approx 100 mM mannitol in chloroplasts and was identical to the wild type in phenotype and photosynthetic performance. The presence of mannitol in chloroplasts resulted in an increased resistance to methyl viologen (MV)-induced oxidative stress, documented by the increased retention of chlorophyll in transgenic leaf tissue following MV treatment. It is especially important to note that mannitol did not reduce production of reactive oxygen species, but it did apparently provide additional protection beyond that normally present in nonstressed plants.

Alternatively, in a sequencing study of the mannitol degrading MTD from celery a protein database search has revealed that the ELI3 pathogenesis-related (PR) proteins from parsley and *Arabidopsis* are also very closely related to MTD, and treatment of celery cells with salicylic acid resulted in increased MTD activity and mRNA (Williamson et al., 1995). This has led to the conclusion that increased MTD activity may provide an additional source of carbon and energy for response to pathogen attack (Williamson et al., 1995; Stoop et al., 1996). Since mannitol is a scavenger of active oxygen species, it may also be likely that MTD by decreasing mannitol levels enhances a plant's defensive arsenal which often includes pathogen-induced production of active oxygen species (Sutherland, 1991). These MTD data provide additional support for mannitol as an important component of a plant's response to divergent types of stress.

VIII. Regulation at the Molecular Level

As is clearly evident from the numerous examples above, both developmental and environmental factors affect expression of sugar alcohol related genes and extractable activities of sugar alcohol related enzymes. In other systems and organisms, enzyme activation or inactivation or modulation of enzyme kinetics by oxidation and reduction of enzyme thiols or by protein phosphorylation is common, as is sensitivity to substrates and activators. Unfortunately, no enzyme involved in sugar alcohol metabolism is as well characterized in terms of its regulation as any of those involved in the parallel pathways of sucrose metabolism, i.e., phosphorylation and dephos-

phorylation of SPS (Huber and Huber, 1996) and sucrose synthase (Huber et al., 1996) or the translational mechanisms related to expression of the invertases (Koch, 1996). We are unaware of any data to suggest that any of the sugar alcohol-related enzymes are phosphorylated or dephosphorylated as part of a post-translational regulatory mechanism, nor is there as yet any evidence for a role for fructose 2,6-bisphosphate which is a factor in several other aspects of carbohydrate metabolism (Stitt, 1990). It remains to be determined if these and other mechanisms are involved in regulation of sugar alcohol metabolism in higher plants; however, some data are quite suggestive.

A. Evidence from Other Systems and Organisms

In the alga *Tetraselmis* (*Platymonas*) *subcordiformis*, regulation of mannitol levels during osmotic acclimation appears to depend on enzyme properties rather than on gene activation. The key and apparently irreversible synthetic step is a mannitol 1-phosphate dehydrogenase, which exhibits increased activities in the presence of high NaCl concentrations. In contrast, the degradative pathway starts with a mannitol dehydrogenase (a 2-oxidoreductase) that is sensitive to NaCl. Such results suggest that mannitol levels are directly regulated via alternative pathways, with activities differentially sensitive to NaCl (Richter and Kirst, 1987). Regulation of glycerol content in the algal *Dunaliella* demonstrates similar features: pathways with enzymes with irreversible actions with different substrate affinities and pH optima for the backward and forward reactions (Kirst, 1990; Cowan et al., 1992).

Animal systems may provide some important clues. Sequence analysis of cDNA clones showed M6PR to be closely related to A6PR from apple (Everard et al., 1997). The analysis also showed both M6PR and A6PR to be members of the aldose reductase ALR2 division (EC 1.1.1.21) of the aldo-keto reductase super-family. This family of monomeric NADPH-specific oxidoreductases has been described extensively in mammalian systems at the gene (Bohren et al., 1989; Carper et al., 1989), enzyme (Wermuth, 1985) and protein structure levels (Wilson et al., 1992). In mammals, these aldose reductases play a central role in osmotic adjustment in renal inner medulla cells (Burg, 1995) and are almost certainly associated with the etiology of the disabling

complications associated with diabetes in several tissues (Kador and Kinoshita, 1985). Recent evidence shows mammalian aldose reductases to be transcriptionally regulated in response to osmotic perturbations (Ferraris et al., 1994; Burg, 1995), and in the context of stress tolerance mechanisms and osmoregulation, osmotic (tonicity) responsive elements (promoters) have been identified (Ferraris et al., 1994; Ruepp et al., 1996; Daoudal et al., 1997).

Aldose reductase-like proteins and genes have been identified in higher plants, but for the most part their physiological roles remain undefined. However, two have been shown to be stress induced (Bartels et al., 1991; Lee and Chen, 1993b; Roncarati et al., 1995), and promoter analysis has identified motifs that confer the developmental expression observed in vivo and perhaps the responses to ABA (Roncarati et al., 1995). As already discussed (Section VII.D), transcription of M6PR is induced in young celery leaves under salt stress and MTD is repressed, but a description of the mechanisms involved must await further analysis of the flanking regions of genomic clones.

Despite similarities to the mammalian aldose reductases, M6PR and A6PR differ from their counterparts in their substrate specificities, i.e., requiring aldose phosphates instead of aldoses although the mammalian enzymes may utilize sugar phosphates (Srivastava et al., 1982). Sequence analysis indicates that the residues lining the putative sugar substrate pocket differ considerably, which may explain the substrate differences; however, those amino acids involved in holding NADPH in position are fully conserved in both plant and animal enzymes (Wilson et al., 1992; J. D. Everard, unpublished). The mammalian enzymes are also generally much less selective than M6PR and A6PR, with an array of aromatic and aliphatic carbonyl compounds as other effective substrates. The k_m for glucose (the substrate leading to glucitol accumulation in kidneys and other tissues subjected to hyperglycemia) is 9×10^3 times higher than that for DL-glyceraldehyde, the usual experimental substrate (Morjana et al., 1989).

In animals, large changes in cellular redox states do not occur normally in most tissues and there is also little evidence for reversible enzyme S-thiolation in the regulation of metabolic reactions (Ziegler, 1985). Nonetheless, post-translational redox mediated regulation of the mammalian aldose reductases has been extensively reported as causing changes in their

activation state and their sensitivity to aldose reductase inhibitors (Vander Jagt et al., 1990; Petrash et al., 1992; Bohren and Gabbay, 1993). Phosphorylation is apparently not a factor, and disulfide bridges are not involved (Grimshaw et al., 1989; Borhani et al., 1992). Nonetheless, redox regulation likely involves mixed sulfides which have been implicated in modifying the activation state and inhibitor sensitivity *in vitro* (Bhatnagar et al., 1989; Cappiello et al., 1996) and *in vivo* (Cappiello et al., 1995; Grimshaw and Lai, 1996).

Redox modification is certainly important in plants, especially during short-term adaptation of various photosynthetic enzymatic activities (Scheibe, 1994), and it may also be a factor in sugar alcohol biosynthesis which is often closely linked to photosynthesis. In chloroplasts, in the light, electrons are transferred to thioredoxin via ferredoxin and the reduced thioredoxin then modulates the activities of key enzymes (Buchanan, 1980; Ziegler, 1985; Scheibe, 1991; Scheibe, 1994). This is a common regulatory mechanism in chloroplasts where redox levels vary enough during a diurnal cycle to enable disulfide linkages to form reversibly in the presence of a suitable catalysts (i.e., the thioredoxins). But, both M6PR and A6PR are cytosolic (see Section V), and the majority of redox-regulated plant enzymes are plastidic (Scheibe, 1994). Very few redox-regulated extra-plastidic plant enzymes have been identified (Florence et al., 1993; Anderson et al., 1995). Also, the biochemical requisite for reductive (and/or oxidative) modification reactions is the presence of regulatory sequences carrying cysteine residues which are subject to reversible redox changes (Scheibe, 1991; von Schaewen et al., 1995). For example, differences in cysteine content apparently distinguish between a redox sensitive chloroplastic glucose-6-phosphate dehydrogenase (G6PDH) and an insensitive cytosolic G6PDH in potato (von Schaewen et al., 1995). Although M6PR and A6PR contain cysteines, four and five respectively, the apparently critical cysteine residue (cys-298) that is sensitive to redox regulation in mammalian aldose reductases (Bohren and Gabbay, 1993; Cappiello et al., 1996) is missing from both M6PR (Everard et al., 1997) and A6PR (Kanayama et al., 1992) as well as two plant aldose reductases (Bartels et al., 1991; Lee and Chen, 1993a). On the other hand, many sugar alcohol metabolizing enzymes in plants are sensitive to sulphydryl reagents and oxidation (Ikawa et al.,

1972; Negm and Loescher, 1979; Negm and Loescher, 1981; Negm, 1986; Stoop et al., 1995, 1998). M6PR is particularly sensitive: high levels of DTT are essential to maintain enzyme activity during extraction (Loescher et al., 1992).

There are examples of redox related mechanisms of enzyme regulation that are distinct from those involving sulphydryl residues. Many enzymes are sensitive to NADP/NADPH ratios. For example, in fungi, where mannitol is not only common but often so abundant that it is the common currency of carbon metabolism (Lewis and Smith, 1967a), a number of specific synthetic and catabolic fungal enzymes have been identified, e.g., various mannitol and mannitol 1-phosphate dehydrogenases that are responsible for polyol and sugar interconversions. Some of these enzymes, e.g., mannitol dehydrogenase (mannitol: NADP 2-oxidoreductase, EC 1.1.1.138), are apparently very sensitive to *in vitro* alterations in the NADP/NADPH ratio (Ruffner et al., 1978), leading to the proposal that small changes in the physiological NADP and NADPH concentrations, i.e., small changes in the redox state of the coenzyme, would have a considerable effect on the rate of mannitol oxidation (Morton et al., 1985). Other similar enzymes, however, were not sensitive to changes imposed by growth on some substrates (Singh et al., 1988) which argues against the hypothesis that in fungi these enzymes are involved in meeting or responding to cellular demands for NADPH (Niehaus and Jiang, 1989). However, pH effects and unresolved compartmentation questions may confound these analyses for both forms of the coenzyme as well as the putatively sensitive enzymes (and their isozymes).

B. Sources and Recycling of Reductant

The NADPH necessary for sugar alcohol biosynthesis is apparently supplied by the non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (E.C. 1.2.1.9). This cytosolic enzyme is found in all photosynthetic organisms except cyanobacteria (Mateos and Serrano, 1992; Michels et al., 1994). It has been proposed that this enzyme is involved in a photosynthetic shuttle transferring reducing power as NADPH from the chloroplast to the cytosol (Kelly and Gibbs, 1973b). In support of its role in sugar alcohol biosynthesis GAPDH was found in celery (Rumpho et al., 1983) at levels substantially higher than in several species

that do not synthesize sugar alcohols (Kelly and Gibbs, 1973a). Whether this enzyme contributes to regulation of sugar alcohol biosynthesis awaits further evaluation of its kinetic parameters and substrate sensitivities in sugar alcohol synthesizing species. In non-sugar alcohol synthesizing spinach, however, the *in vivo* concentrations of substrates, cofactors, and products suggest that the GAPDH reaction is distant from thermodynamic equilibrium and is regulated, i.e., inhibited primarily by the product NADPH (Scaglia et al., 1990; Trost and Pupillo, 1993). Demand for NADPH in sugar alcohol biosynthesis via M6PR and subsequent phloem export of the sugar alcohol from the leaves could therefore modulate GAPDH activity and in conjunction with Pi levels also modulate the 3-PGA/G3P shuttle and photosynthesis.

In the context of NADPH export, sugar alcohol biosynthesis may have the same implications for maintaining a high photosynthetic capacity as does activity of the mitochondrial electron transport chain in the light (Krömer, 1995). Photosynthetic rates in many sugar alcohol producing plants are high compared to 'typical' C₃ species. Celery, for example, has been reported to have a very high photosynthetic rate and to differ from typical C₃ species in having a low CO₂ compensation point (Fox et al., 1986). High photosynthetic rates (for a C₃ species) have been confirmed, but anatomical and immunocytochemical studies and reevaluations of gas exchange in celery rule out the usual CO₂ concentrating mechanisms and the unusually low CO₂ compensation point (Everard et al., 1994; W. Loescher, unpublished).

The hypothesis that sugar alcohol synthesis may provide further use for reductant is not without precedent. For example, respiration of glucose through the alternative oxidase in *Plantago coronopus* roots was diverted into glucitol synthesis when these tissues were exposed to NaCl (Lambers et al., 1981). By so doing the plants diverted carbon and utilized excess energy, normally dissipated through the alternative oxidase, to accumulation of a compatible solute (glucitol) associated with enhanced salt tolerance in this and other species (see Section VII.D). Jennings and Burke (1990) have also proposed that the cycling of carbon, and hence reducing power, through sugar alcohol synthesis and degradation may provide a pathway by which fungi 'spill' energy during periods of stress.

IX. Conclusions and Considerations for Future Research

Our understanding of sugar alcohol metabolism, particularly of the enzymes and a few of the genes related to glucitol and mannitol, has only recently begun to progress. As can be seen from this discussion, we are beginning to develop some insights into the biochemical, transcriptional and post-transcriptional control of biosynthesis and degradation of mannitol. Although transgenic glucitol synthesizers are also available (Tao et al., 1995; Sheveleva et al., 1998), little is known about glucitol regulation, and nothing is known about regulation of other sugar alcohols found in higher plants. We know very little about intracellular transport (which relates to compartmentation issues) or the mechanisms related to intercellular or long-distance transport (which relate to phloem and partitioning issues) for any of the sugar alcohols. The systems involved are complex for two major reasons. The need to regulate carbon partitioning in plants has been discussed in detail (Stitt et al., 1987), and all the arguments for the need to regulate carbon flow in starch and sucrose synthesizers are equally relevant to sugar alcohol synthesizers, with the added complications of another synthetic pathway with one or more critical regulatory steps competing for the same pools of substrates and intermediates (Fig. 1). If we accept the increasing evidence that sugar alcohols have a role in stress tolerance in higher plants, this role certainly requires further modulation of metabolism, storage, and transport. In finding answers to these problems, we will not only develop insight into regulation of carbon partitioning and crop productivity, but also into some mechanisms of stress tolerance. As others have argued (Bartels and Nelson, 1994; Stitt and Sonnewald, 1995; Bohnert and Jensen, 1996), use of transgenic plants and the results of even seemingly minor improvements in stress tolerance could have major implications for plant breeding and crop improvement.

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References

- Ahmad I, Larher F and Stewart GR (1979) Sorbitol, a compatible osmotic solute in *Plantago maritima*. *New Phytol* 82: 671–678
- Anderson LE, Li D, Prakash N and Stevens FJ (1995) Identification of potential redox-sensitive cysteines in cytosolic forms of fructosebisphosphatase and glyceraldehyde-3-phosphate dehydrogenase. *Planta* 196: 118–124
- Bartels D and Nelson D (1994) Approaches to improve stress tolerance using molecular genetics. *Plant Cell Environ* 17: 659–667
- Bartels D, Engelhardt K, Roncarati R, Schneider K, Rotter M and Salamini F (1991) An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. *EMBO J* 10: 1037–1043
- Beebe DU and Turgeon R (1992) Localization of galactinol, raffinose, and stachyose synthesis in *Cucurbita pepo* leaves. *Planta* 188: 354–361
- Berüter J (1993) Characterization of the permeability of excised apple tissue for sorbitol. *J Exp Bot* 44: 519–528
- Bhatnagar A, Liu S, Das B and Srivastava SK (1989) Involvement of sulphydryl residues in aldose reductase-inhibitor interaction. *Mol Pharmacol* 36: 825–830
- Bielecki RL (1969) Accumulation and translocation of sorbitol in apple phloem. *Aust J Biol Sci* 22: 611–620
- Bielecki RL (1982) Sugar alcohols. In: Loewus FA and Tanner W (eds) *Plant Carbohydrates I. Intercellular Carbohydrates Encyclopedia Plant Physiol*, N.S., 13A, pp 158–192. Springer-Verlag, New York
- Bielecki RL and Redgwell RJ (1977) Synthesis of sorbitol in apricot leaves. *Aust J Plant Physiol* 4: 1–10
- Bielecki RL and Redgwell RJ (1980) Sorbitol metabolism in nectaries from flowers of Rosaceae. *Aust J Plant Physiol* 7: 15–26
- Bohnert HJ and Jensen RG (1996) Metabolic engineering for increased salt tolerance—the next step. *Aust J Plant Physiol* 23: 661–667
- Bohnert HJ, Nelson DE and Jensen RG (1995) Adaptations to environmental stresses. *Plant Cell* 7: 1099–1111
- Bohren KM, Bullock B, Wermuth B and Gabbay KH (1989) The aldo-keto reductase superfamily. *J Biol Chem* 264: 9547–9551
- Bohren KM and Gabbay KH (1993) Cys²⁹⁸ is responsible for reversible thiol-induced variation in aldose reductase activity. In: Weiner H (ed) *Enzymology and Molecular Biology of Carbonyl Metabolism*, pp 267–277. Plenum Press, New York
- Bonnett CD and Incoll LD (1993) Effects on the stem of winter barley of manipulating the source and sink during grain-filling. 1. Changes in accumulation and loss of mass from internodes. *J Exp Bot* 44: 75–82
- Borhani DW, Harter TM and Petrasch JM (1992) The crystal structure of the aldose reductase NADPH binary complex. *J Biol Chem* 267: 24841–24847
- Briens M and Larher F (1982) Osmoregulation in halophytic higher plants: A comparative study of soluble carbohydrates, polyols, betaines and free proline. *Plant Cell Environ* 5: 287–292
- Brown AD (1978) Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Adv Microbiol Physiol* 17: 181–242
- Brown AD and Simpson JR (1972) Water relations of sugar-tolerant yeasts: The role of intracellular polyols. *J Gen Microb* 72: 589–591
- Buchanan BB (1980) Role of light in the regulation of chloroplast enzymes. *Annu Rev Plant Physiol* 31: 341–374
- Burg MB (1995) Molecular basis of osmotic regulation. *Am J Physiol* 268: F983–F996
- Cappiello M, Vilardo PG, Cecconi I, Leverenz V, Giblin FJ, del Corso A and Mura U (1995) Occurrence of glutathione-modified aldose reductase in oxidatively stressed bovine lens. *Biochem Biophys Res Commun* 207: 775–782
- Cappiello M, Voltarelli M, Cecconi I, Vilardo PG, Dal Monte M, Marini I, del Corso A, Wilson DK, Quiocho FA, Petrasch JM and Mura U (1996) Specifically targeted modification of human aldose reductase by physiological disulfides. *J Biol Chem* 271: 33539–33544
- Carper DA, Wistow G, Nishimura C, Graham C, Watanabe K, Fujii Y, Hayashi H and Hayaishi O (1989) A superfamily of NADPH-dependent reductases in eukaryotes and prokaryotes. *Exp Eye Res* 49: 377–388
- Chong C (1971) Study of the seasonal and daily distribution of sorbitol and related carbohydrates within apple seedlings by analysis of selected tissues and organs. *Can J Bot* 51: 519–525
- Cowan AK, Rose PD and Horne LG (1992) *Dunaliella salina*: A model system for studying the response of plant cells to stress. *J Exp Bot* 43: 1535–1547
- Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ and Anchordoguy TJ (1988) Interactions of sugars with membranes. *Biochim Biophys Acta* 947: 367–384
- Cushman JC and Bohnert HJ (1995) Transcriptional activation of CAM genes during development and environmental stress. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, pp 135–158. Springer Verlag, Heidelberg
- Daie J (1986) Kinetics of sugar transport in isolated vascular bundles and phloem tissue of celery. *J Amer Soc Hort Sci* 111: 216–220
- Daie J (1987a) Interaction of cell turgor and hormones on sucrose uptake in isolated phloem of celery. *Planta* 171: 1033–1037
- Daie J (1987b) Sucrose uptake in isolated phloem of celery is a single saturable transport system. *Planta* 171: 474–482
- Daoudal S, Tournaire C, Halere A, Veysiére G and Jean C (1997b) Isolation of the mouse aldose reductase promoter and identification of tonicity-responsive element. *J Biol Chem* 272: 2615–2619
- Davis JM and Loescher WH (1990) [¹⁴C]-Assimilate translocation in the light and dark in celery (*Apium graveolens*) leaves of different ages. *Physiol Plant* 79: 656–662
- Davis JM, Fellman JK and Loescher WH (1988) Biosynthesis of sucrose and mannitol as a function of leaf age in celery (*Apium graveolens* L.). *Plant Physiol* 86: 129–133
- Delauney AJ and Verma DPS (1993) Proline Biosynthesis and Osmoregulation in Plants. *Plant J* 4: 215–223
- Deutch CE and Winicov I (1995) Post-transcriptional regulation of a salt-inducible alfalfa gene encoding a putative chimeric proline-rich cell wall protein. *Plant Mol Biol* 27: 411–418
- Doehlert DC (1987) Ketose reductase in developing maize endosperm. *Plant Physiol* 84: 830–834
- Drossopoulos JB and Niavis CA (1988) Seasonal changes of the metabolites in the leaves, bark and xylem tissues of olive tree (*Olea europaea* L.). II. Carbohydrates. *Ann Bot* 62: 321–327
- Evans RD, Black RA, Loescher WH and Fellows RJ (1992)

- Osmotic relations of the drought-tolerant shrub *Artemisia tridentata* in response to water stress. *Plant Cell Environ* 15: 49–59
- Everard JD, Franceschi VR and Loescher WH (1993) Mannose-6-phosphate reductase, a key enzyme in photoassimilate partitioning, is abundant and located in the cytosol of photosynthetically active cells of celery (*Apium graveolens* L.) source leaves. *Plant Physiol* 102: 345–356
- Everard JD, Gucci R, Kann SC, Flore JA and Loescher WH (1994) Gas exchange and carbon partitioning in the leaves of celery (*Apium graveolens* L.) at various levels of root zone salinity. *Plant Physiol* 106: 281–292
- Everard JD, Cantini C, Grumet R, Plummer J and Loescher WH (1997) Molecular cloning of mannose 6-phosphate reductase and its developmental expression in celery. *Plant Physiol* 113: 1427–1435
- Fellman JK and Loescher WH (1987) Comparative studies of sucrose and mannitol utilization in celery (*Apium graveolens*). *Physiol Plant* 69: 337–341
- Fer A, Simier P, Arnaud MC, Rey L and Renaudin S (1993) Carbon acquisition and metabolism in a root-hemiparasitic angiosperm, *Thesium humile* (Santalaceae) growing on wheat (*Triticum vulgare*). *Aust J Plant Physiol* 20: 15–24
- Ferraris JD, Williams CK, Martin BM, Burg MB and García-Pérez A (1994) Cloning, genomic organization, and osmotic response of the aldose reductase gene. *Proc Natl Acad Sci USA* 91: 10742–10746
- Flinn CL and Ashworth EN (1995) The relationship between carbohydrates and flower bud hardiness among three *Forsythia* taxa. *J Amer Soc Hort Sci* 120: 607–613
- Flora LL and Madore MA (1993) Stachyose and mannitol transport in olive (*Olea europaea* L.). *Planta* 189: 484–490
- Florencio FJ, Gadál P and Buchanan BB (1993) Thioredoxin-linked activation of the chloroplast and cytosolic forms of *Chlamydomonas reinhardtii* glutamine synthetase. *Plant Physiol Biochem* 31: 649–655
- Flowers TJ, Troke PF and Yeo AR (1977) The mechanism of salt tolerance in halophytes. *Annu Rev Plant Physiol* 28: 89–121
- Fox TC, Kennedy RA and Loescher WH (1986) Developmental changes in photosynthetic gas exchange in the polyol-synthesizing species, *Apium graveolens* L. (Celery). *Plant Physiol* 82: 307–311
- Galinski EA (1993) Compatible solutes of halophilic eubacteria—Molecular principles, water-solute interaction, stress protection. *Experientia* 49: 487–496
- Geigenberger P, Langenberger S, Wilke I, Heineke D, Heldt HW and Stitt M (1993) Sucrose is metabolised by sucrose synthase and glycolysis within the phloem complex of *Ricinus communis* L. seedlings. *Planta* 190: 446–453
- Gibson TS, Speirs J and Brady CJ (1984) Salt-tolerance in plants. II. In vitro translation of mRNAs from salt-tolerant and salt-sensitive plants on wheat germ ribosomes. Responses to ions and compatible organic solutes. *Plant Cell Environ* 7: 579–587
- Goldstein G and Nobel PS (1994) Water relations and low-temperature acclimation for cactus species varying in freezing tolerance. *Plant Physiol* 104: 675–681
- Gorham J, Hughes L and Wyn Jones RG (1981) Low-molecular-weight carbohydrates in some salt-stressed plants. *Physiol Plant* 53: 27–33
- Grant CR and ap Rees T (1981) Sorbitol metabolism by apple seedlings. *Phytochemistry* 20: 1505–1511
- Grimshaw CE and Lai C-J (1996) Oxidized aldose reductase: In vivo factor. *Arch Biochem Biophys* 327: 89–97
- Grimshaw CE, Shahbaz M, Jahangiri G, Putney CG, McKercher SR and Mathur EJ (1989) Kinetic and structural effects of activation of bovine kidney aldose reductase. *Biochemistry* 28: 5343–5353
- Gucci R, Tattini M and Everard JD (1996) Carbon assimilation and partitioning in olive leaves. *Plant Physiol Biochem* 122: S10–S20
- Gucci R, Aronne G, Lombardini L and Tattini M (1997a) Salinity tolerance in Phillyrea species. *New Phytol* 97: 227–234
- Gucci R, Lombardini L and Tattini M (1997b) Analysis of water relation parameters in leaves of two olive (*Olea europaea* L.) cultivars with different tolerance to salinity. *Tree Physiol* 17: 13–21
- Gucci R, Moing A, Gravano E and Gaudillière, J.-P. (1998) Partitioning of photosynthetic carbohydrates in leaves of salt-stressed olive plants. *Aust J Plant Physiol* 25: 571–579
- Guicherd P, Peltier JP, Gout E, Bligny Rand Marigo G (1997) Osmotic adjustment in *Fraxinus excelsior* L.: Malate and mannitol accumulation in leaves under drought conditions. *Trees* 11: 155–161
- Häfliger B, Kindhauser E and Keller F (1998) Metabolism of D-glycero-D-manno-heptitol, volémitol, in *Polyanthus*. Discovery of a novel ketose reductase. *Plant Physiol* 119: 191–198
- Hanson EJ (1991) Movement of boron out of tree fruit leaves. *HortScience* 26: 271–273
- Harloff HJ and Wegmann K (1993) Evidence for a mannitol cycle in *Orobanche ramosa* and *Orobanche crenata*. *J Plant Physiol* 141: 513–520
- Henstra SA, Tolner B, Duurkens RHT, Konings WN and Robillard GT (1996) Cloning, expression, and isolation of the mannitol transport protein from the thermophilic bacterium *Bacillus stearothermophilus*. *J Bacteriol* 178: 5586–5591
- Hirai M (1981) Purification and characteristics of sorbitol-6-phosphate dehydrogenase from loquat leaves. *Plant Physiol* 67: 221–224
- Hirai M (1983) Seasonal changes in sorbitol 6-phosphate dehydrogenase in loquat leaf. *Plant Cell Physiol* 24: 925–931
- Holmström K-O, Mäntylä E, Welin B, Mandal A and Palva ET (1996) Drought tolerance in tobacco. *Nature* 379: 683–684
- Honegger R (1991) Functional aspects of the lichen symbiosis. *Annu Rev Plant Physiol Mol Biol* 42: 553–578
- Hsiao TC (1973) Plant responses to water stress. *Annu Rev Plant Physiol* 24: 519–570
- Hu H, Penn SG, Lebrilla CB and Brown PH (1997) Isolation and characterization of soluble boron complexes in higher plants. *Plant Physiol* 113: 649–655
- Huber SC and Huber JL (1996) Role and regulation of sucrose-phosphate synthase in higher plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 431–444
- Huber SC, Huber JL, Liao P-C, Gage DA, McMichael RW, Chourey PS, Hannah LC and Koch KE (1996) Phosphorylation of serine-15 of maize leaf sucrose synthase. Occurrence in vivo and possible regulatory significance. *Plant Physiol* 112: 793–802
- Ichiki S and Yamaya H (1982) Sorbitol in tracheal sap of dormant apple (*Malus domestica* Borkh) shoots as related to cold hardiness. In: Li PH and Sakai A (eds) *Plant Cold Hardiness and Freezing Stress*, pp 181–187. Academic Press, New York

- Ikawa T, Watanabe T and Nisizawa K (1972) Enzymes involved in the last steps of the biosynthesis of mannitol in brown algae. *Plant Cell Physiol* 13: 1017–1029
- Jang JC, León P, Zhou L and Sheen J (1997) Hexokinase as a sugar sensor in higher plants. *Plant Cell* 9: 5–19
- Jennings DH and Burke RM (1990) Compatible solutes—the mycological dimension and their role as physiological buffering agents. *New Phytol* 116: 277–283
- Kador PF and Kinoshita JH (1985) Role of aldose reductase in development of diabetes-associated complications. *Amer J Med* 79: 8–12
- Kanayama Y and Yamaki S (1993) Purification and properties of NADP-dependent sorbitol-6-phosphate dehydrogenase from apple seedlings. *Plant Cell Physiol* 34: 819–823
- Kanayama Y, Mori H, Imaseki H and Yamaki S (1992) Nucleotide sequence of a cDNA encoding NADP-sorbitol-6-phosphate dehydrogenase from apple. *Plant Physiol* 100: 1607–1608
- Kanayama Y, Sakanishi K, Mori H and Yamaki S (1995) Expression of the gene for NADP-dependent sorbitol-6-phosphate dehydrogenase in apple seedlings. *Plant Cell Physiol* 36: 1139–1141
- Kaarsen U, Barrow KD, Nixdorf O and King RJ (1996) The compatibility with enzyme activity of unusual organic osmolytes from mangrove red algae. *Aust J Plant Physiol* 23: 577–582
- Keller F (1991) Carbohydrate transport in discs of storage parenchyma of celery petioles. 2. Uptake of mannitol. *New Phytol* 117: 423–429
- Keller F and Ludlow MM (1993) Carbohydrate metabolism in drought-stressed leaves of pigeonpea (*Cajanus cajan*). *J Exp Bot* 44: 1351–1359
- Keller F and Matile P (1989) Storage of sugars and mannitol in petioles of celery leaves. *New Phytol* 113: 291–299
- Keller F and Pharr DM (1996) Metabolism of carbohydrates in sinks and sources: Galactosyl-sucrose oligosaccharides. In: Zamski E and Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops*, pp 157–183. Marcel Dekker, New York
- Keller JD and Loescher WH (1989) Nonstructural carbohydrate partitioning in perennial parts of sweet cherry. *J Amer Soc Hort Sci* 114: 969–975
- Kelly GJ and Gibbs M (1973a) Nonreversible D-glyceraldehyde 3-phosphate dehydrogenase of plant tissues. *Plant Physiol* 52: 111–118
- Kelly GJ and Gibbs M (1973b) A mechanism for the indirect transfer of photosynthetically reduced nicotinamide adenine dinucleotide phosphate from chloroplasts to the cytoplasm. *Plant Physiol* 52: 674–676
- Kirst GO (1990) Salinity tolerance of eukaryotic marine algae. *Annu Rev Plant Physiol Plant Mol Biol* 41: 21–53
- Kishor PBK, Hong Z, Miao G-H, Hu C-AA and Verma DPS (1995) Overexpression of **δ^1 -pyrroline-5-carboxylate synthetase** increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol* 108: 1387–1394
- Kiyosue T, Yoshioka Y, Yamaguchi-Shinozaki K and Shinozaki K (1996) A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis*. *Plant Cell* 8: 1323–1335
- Koch KE (1996) Carbohydrate-modulated gene expression in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 509–540
- Koch KE, Nolte KD, Duke ER, McCarty DR and Avigne WT (1992) Sugar levels modulate differential expression of maize sucrose synthase genes. *Plant Cell* 4: 59–69
- Krömer S (1995) Respiration during photosynthesis. *Annu Rev Plant Physiol* 46: 45–70; 45–70
- Kuo TM, Doehlert DC and Crawford CG (1990) Sugar metabolism in germinating soybean seeds. Evidence for the sorbitol pathway in soybean axes. *Plant Physiol* 93: 1514–1520
- Lambers H, Blacquière T and Stuiver B (1981) Interactions between osmoregulation and the alternative respiratory pathway in *Plantago coronopus* as affected by salinity. *Physiol Plant* 51: 63–68
- Lee SP and Chen THH (1993a) Molecular cloning of abscisic acid-responsive mRNAs expressed during the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss) suspension culture. *Plant Physiol* 101: 1089–1096
- Lee SP and Chen THH (1993b) Expression of an aldose reductase-related gene during the induction of freezing-tolerance in bromegrass cell suspension cultures. *J Plant Physiol* 142: 749–753
- Lewis DH (1984) Physiology and metabolism of alditols. In: Lewis DH (ed) *Storage Carbohydrates in Vascular Plants*, pp 157–179. Cambridge University Press, Cambridge
- Lewis DH and Smith DC (1967a) Sugar alcohols (polyols) in fungi and green plants. II. Methods of detection and quantitative estimation in plant extracts. *New Phytol* 66: 185–204
- Lewis DH and Smith DC (1967b) Sugar alcohols (polyols) in fungi and green plants. I. Distribution, physiology and metabolism. *New Phytol* 66: 143–184
- Loescher WH and Everard JD (1996) Metabolism of carbohydrates in sinks and sources: Sugar alcohols. In: Zamski E and Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships*, pp 185–207. Marcel Dekker, New York
- Loescher WH, Marlow GC and Kennedy RA (1982) Sorbitol metabolism and sink-source interconversions in developing apple leaves. *Plant Physiol* 70: 335–339
- Loescher WH, McCamant T and Keller J (1990) Carbohydrate reserves, translocation and storage in woody plant roots. *HortScience* 25: 274–281
- Loescher WH, Tyson RH, Everard JD, Redgwell RJ and Bielecki RL (1992) Mannitol synthesis in higher plants: Evidence for the role and characterization of a NADPH-dependent mannose 6-phosphate reductase. *Plant Physiol* 98: 1396–1402
- Loescher WH, Everard JD, Cantini C and Grumet R (1995) Sugar alcohol metabolism in source leaves. In: Madore MA and Lucas WJ (eds) *Carbon Partitioning and Source-Sink Interactions in Plants*, pp 170–179. Amer Soc Plant Physiol, Rockville
- Manetas Y, Petropoulou Y and Karabourniotis G (1986) Compatible solutes and their effects on phosphoenolpyruvate carboxylase of **C₄ halophytes**. *Plant Cell Environ* 9: 145–151
- Mariño G and Peltier JP (1996) Analysis of the diurnal change in osmotic potential in leaves of *Fraxinus excelsior* L. *J Exp Bot* 47: 763–769
- Mateos MI and Serrano A (1992) Occurrence of phosphorylating and non-phosphorylating **NADP⁺-dependent** glyceraldehyde-3-phosphate dehydrogenases in photosynthetic organisms. *Plant Sci* 84: 163–170
- Merlo L and Passera C (1991) Changes in carbohydrate and enzyme levels during development of leaves of *Prunus persica*,

- a sorbitol synthesizing species. *Physiol Plant* 83: 621–626
- Michels S, Scagliarini S, Della Steta F, Carles C, Riva M, Trost P and Branlant G (1994) Arguments against a close relationship between non-phosphorylating and phosphorylating glyceraldehyde-3-phosphate dehydrogenases. *FEBS Lett* 339: 97–100
- Moing A, Carbonne F, Rashad MH and Gaudillère J-P (1992) Carbon fluxes in mature peach leaves. *Plant Physiol* 100: 1878–1884
- Moing A, Escobar-Gutiérrez AJ and Gaudillière J-P (1994) Modeling carbon export out of mature peach leaves. *Plant Physiol* 106: 591–600
- Moing A, Carbonne F, Zipperlin B, Svanella L and Gaudillière J-P (1997) Phloem loading in peach: Symplastic or apoplastic? *Physiol Plant* 101: 489–496
- Moore BD, Talbot JN and Seemann JR (1997) Function of leaf hamamelitol as a compatible solute during water-stress treatment of *Hedera helix* L. *Plant Cell Environ* 20: 938–944
- Moriguchi T, Sanada T and Yamaki S (1990) Seasonal fluctuations of some enzymes relating to sucrose and sorbitol metabolism in peach fruit. *J Amer Soc Hort Sci* 115: 278–281
- Morjana NA, Lyons C and Flynn TG (1989) Aldose reductase from human psoas muscle. Affinity labeling of an active site lysine by pyridoxal 5'-phosphate and pyridoxal 5'-diphospho-5'-adenosine. *J Biol Chem* 264: 2912–2919
- Morton N, Dickerson AG and Hammond JBW (1985) Mannitol metabolism in Agaricus bisporus: Purification and properties of mannitol dehydrogenase. *J Gen Microbiol* 131: 2885–2890
- Munns R (1988) Why measure osmotic adjustment? *Aust J Plant Physiol* 15: 717–726
- Munns R (1993) Physiological processes limiting plant growth in saline soils—some dogmas and hypotheses. *Plant Cell Environ* 16: 15–24
- Munns R and Weir R (1981) Contribution of sugars to osmotic adjustment in elongating and expanded zones of wheat leaves during moderate water deficits at two light levels. *Aust J Plant Physiol* 8: 93–105
- Munns R, Schachtman DP and Condon AG (1995) The significance of a two-phase growth response to salinity in wheat and barley. *Aust J Plant Physiol* 22: 561–569
- Negm FB (1986) Purification and properties of an NADPH-aldoze reductase (aldehyde reductase) from Euonymus japonica leaves. *Plant Physiol* 80: 972–977
- Negm FB and Loescher WH (1979) Detection and characterization of sorbitol dehydrogenase from apple callus tissue. *Plant Physiol* 64: 69–73
- Negm FB and Loescher WH (1981) Characterization and partial purification of aldose-6-phosphate reductase (alditol-6-phosphate:NADP 1-oxidoreductase) from apple leaves. *Plant Physiol* 67: 139–142
- Negm FB and Marlow GC (1985) Partial purification and characterization of D-ribose-5-phosphate reductase from *Adonis vernalis* L. leaves. *Plant Physiol* 78: 758–761
- Niehaus WG, Jr. and Jiang W (1989) Nitrate induces enzymes of the mannitol cycle and suppresses versicolorin synthesis in *Aspergillus parasiticus*. *Mycopathologia* 107: 131–137
- Niimi T, Yamashita O and Yaginuma T (1993) A cold-inducible *Bombyx* gene encoding a protein similar to mammalian sorbitol dehydrogenase—yolk nuclei-dependent gene expression in diapause eggs. *Eur J Biochem* 213: 1125–1131
- Nolte KD and Koch KE (1993) Companion-cell specific localization of sucrose synthase in zones of phloem loading and unloading. *Plant Physiol* 101: 899–905
- Nomura M, Ishitani M, Takabe T and Rai AK (1995) *Synechococcus* sp PCC7942 transformed with *Escherichia coli* bet genes produces glycine betaine from choline and acquires resistance to salt stress. *Plant Physiol* 107: 703–708
- Paleg LG, Douglas TJ, van Daal A and Keech DB (1981) Proline, betaine and other organic solutes protect enzymes against heat inactivation. *Aust J Plant Physiol* 8: 107–114
- Patrick JW (1993) Osmotic regulation of assimilate unloading from seed coats of *Vicia faba*—role of turgor and identification of turgor-dependent fluxes. *Physiol Plant* 89: 87–96
- Penn SG, Hu HN, Brown PH and Lebrilla CB (1997) Direct analysis of sugar alcohol borate complexes in plant extracts by matrix-assisted laser desorption/ionization Fourier transform mass spectrometry. *Anal Chem* 69: 2471–2477
- Petrasch JM, Harter TM, Devine CS, Loins PO, Bhatnagar A, Liu S and Srivastava SK (1992) Involvement of cysteine residues in catalysis and inhibition of human aldoze reductase. *J Biol Chem* 267: 24833–24840
- Pharr DM, Stoop JMH, Williamson JD, Studer-Feusi ME, Massel MO and Conkling MA (1995) The dual role of mannitol as osmoprotectant and photoassimilate in celery. *HortScience* 30: 1182–1188
- Pieniazek J, Holubowicz T, Machnik B and Kasprzyk M (1978) Apple stem callus frost tolerance and growth modification by adding sorbitol and some growth regulators to the medium. *Acta Hortic* 81: 91–95
- Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeuken MJW, Weissebeek PJ and Smeekens SCM (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol* 107: 125–130
- Pollard A and Jones RGW (1979) Enzyme activities in concentrated solutions of glycinebetaine and other solutes. *Planta* 144: 291–298
- Popp M (1984) Chemical composition of Australian mangroves. II. Low molecular weight carbohydrates. *Z Pflanzenphysiol* 113: 411–421
- Popp M (1995) Salt resistance in herbaceous halophytes and mangroves. *Prog Bot* 56: 416–429
- Popp M and Smirnoff N (1995) Polyol accumulation and metabolism during water deficit. In: Smirnoff N (ed) *Environment and Plant Metabolism*, pp 199–215. Bios Scientific Publications Ltd, Oxford
- Prata RTN, Williamson JD, Conkling MA and Pharr DM (1997) Sugar repression of mannitol dehydrogenase activity in celery cells. *Plant Physiol* 114: 307–314
- Preiss J and Sivak MN (1996) Starch synthesis in sinks and sources. In: Zamski E and Schaffer AA (eds) *Photoassimilate Distribution in Plants and Crops*, pp 63–96. Marcel Dekker, New York
- Ranney TG, Bassuk NL and Whitlow TH (1991) Osmotic adjustment and solute constituents in leaves and roots of water-stressed cherry (*Prunus*) trees. *J Amer Soc Hort Sci* 116: 684–688
- Rentsch D, Hirner B, Schmelzer E and Frommer WB (1996) Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. *Plant Cell* 8: 1437–1446
- Rhodes D and Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu Rev*

- Plant Physiol Plant Mol Biol 44: 357–384
- Richter A and Popp M (1992) The physiological importance of accumulation of cyclitols in *Viscum album* L. New Phytol 121: 431–438
- Richter DFE and Kirst GO (1987) D-Mannitol dehydrogenase and D-mannitol-1-phosphate dehydrogenase in *Platymonas subcordiformis*: Some characteristics and their role in osmotic adaptation. Planta 170: 528–534
- Roncarati R, Salamini F and Battels D (1995) An aldose reductase homologous gene from barley: Regulation and function. Plant J 7: 809–822
- Ruepp B, Bohren KM and Gabbay K.H (1996) Characterization of the osmotic response element of the human aldose reductase gene promoter. Proc Natl Acad Sci U S A 93: 8624–8629
- Ruffner HP, Rast D, Tobler H and Karesch H (1978) Purification and properties of mannitol dehydrogenase from *Agaricus bisporus* sporocarps. Phytochemistry 17: 865–868
- Rumpho ME, Edwards GE and Loescher WH (1983) A pathway for photosynthetic carbon flow to mannitol in celery leaves: Activity and localization of key enzymes. Plant Physiol 73: 869–873
- Sakai A (1960) Relation of sugar content to frost-hardiness in plants. Nature 185: 698–699
- Sakai A (1966) Seasonal variations in the amounts of polyhydric alcohol and sugar in fruit trees. J Hortic Sci 41: 207–213
- Salmon S, Lemoine R, Jamai A, Bouché-Pillon S and Fromont JC (1995) Study of sucrose and mannitol transport in plasma-membrane vesicles from phloem and non-phloem tissues of celery (*Apium graveolens* L.) petioles. Planta 197: 76–83
- Sauter JJ and Ambrosius (1986) Changes in the partitioning of carbohydrates in the wood during bud break in *Betula pendula* Roth. J Plant Physiol 124: 31–43
- Scagliarini S, Trost P, Valenti V and Pupillo P (1990) Glyceraldehyde 3-phosphate:NADP+ reductase of spinach leaves. Plant Physiol 94: 1337–1344
- Scagliarini S, Trost P, Pupillo P and Valenti V (1993) Light activation and molecular-mass changes of NAD(P)-glyceraldehyde 3-phosphate dehydrogenase of spinach and maize leaves. Planta 190: 313–319
- Scheibe R (1991) Redox-modulation of chloroplast enzymes. Plant Physiol 96: 1–3
- Scheibe R (1994) [Light regulation of chloroplast enzymes] Lichtregulation von Chloroplastenenzymen. Naturwissenschaften 81: 443–448
- Schilling N, Dittrich P and Kandler O (1972) Formation of L-quebrachitol from D-bornesitol in leaves of *Acer pseudoplatanus*. Phytochemistry 11: 1401–1404
- Schobert B (1977) Is there an osmotic regulatory mechanism in algae and higher plants? J Theor Biol 68: 17–26
- Seemann JR, Downtown WJS and Berry JA (1986) Temperature and leaf osmotic potentials as factors in the acclimation of photosynthesis to high temperature in desert plants. Plant Physiol 80: 926–930
- Sharp RE, Hsiao TC and Silk WK (1990) Growth of the maize primary root at low water potentials. Plant Physiol 93: 1337–1346
- Sheen J (1994) Feedback control of gene expression. Photosynth Res 39: 427–438
- Shen B, Jensen RG and Bohnert HJ (1997) Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiol 113: 1177–1183
- Sheveleva EV, Marquez S, Chmara W, Zegeer A, Jensen RG and Bohnert HJ (1998) Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco. Plant Physiol 117: 831–839
- Simier P, Fer A and Renaudin S (1993) Identification of the main osmotically active solutes in the unstressed and water-stressed root-hemiparasitic angiosperm *Thesium humile* and its host *Triticum vulgare*. Aust J Plant Physiol 20: 223–230
- Simier P, Renaudin S and Fer A (1994) Characteristics of the mannitol pathway in a root hemiparasitic species, *Thesium humile* Vahl. (Santalaceae). J Plant Physiol 143: 33–38
- Simier P, Robert S and Fer A (1998) Mannitol metabolism in darkness in the leaves of the hemiparasitic angiosperm, *Thesium humile*. Plant Physiol Biochem 36: 237–245
- Singh M, Scrutton NS and Scrutton MC (1988) NADPH generation in *Aspergillus nidulans*: Is the mannitol cycle involved? J Gen Microbiol 134: 643–654
- Smirnoff N (1993) Tansley Review 52—The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125: 27–58
- Smirnoff N and Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057–1060
- Smirnoff N and Stewart GR (1985) Stress metabolites and their role in coastal plants. Vegetatio 62: 273–278
- Srivastava SK, Ansari NH, Brown JH and Pettrash MJ (1982) Formation of sorbitol 6-phosphate by bovine and human lens aldose reductase, sorbitol dehydrogenase and sorbitol kinase. Biochim Biophys Acta 717: 210–214
- Stewart GR and Press MC (1990) The physiology and biochemistry of parasitic angiosperms. Annu Rev Plant Physiol Plant Mol Biol 41: 127–151
- Stitt M (1990) Fructose-2,6-bisphosphate as a regulatory molecule in plants. Annu Rev Plant Physiol Plant Mol Biol 41: 153–185
- Stitt M and Sonnewald U (1995) Regulation of metabolism in transgenic plants. Annu Rev Plant Physiol Plant Mol Biol 46: 341–368
- Stitt M, Huber S and Kerr P (1987) Control of photosynthetic sucrose formation. In: Hatch MD and Boardman NK (eds) The Biochemistry of Plants, Vol 10, pp 327–409. Academic Press, New York
- Stitt M, Krapp A, Klien D, Röper-Schwarz U and Paul M (1995) Do carbohydrates regulate photosynthesis and allocation by altering gene expression? In: Madore MA and Lucas WJ (eds) Carbon Partitioning and Source-Sink Interactions in Plants, pp 68–77. Amer Soc Plant Physiol, Rockville
- Stoop JMH and Pharr DM (1992) Partial purification and characterization of mannitol:mannose 1-oxidoreductase from celeriac (*Apium graveolens* var. *rapaceum*) roots. Arch Biochem Biophys 298: 612–619
- Stoop JMH and Pharr DM (1993) Effect of different carbon sources on relative growth rate, internal carbohydrates, and mannitol 1-oxidoreductase activity in celery suspension cultures. Plant Physiol 103: 1001–1008
- Stoop JMH and Pharr DM (1994a) Mannitol metabolism in celery stressed by excess macronutrients. Plant Physiol 106: 503–511
- Stoop JMH and Pharr DM (1994b) Growth substrate and nutrient salt environment alter mannitol-to-hexose partitioning in celery petioles. J Amer Soc Hort Sci 119: 237–242
- Stoop JMH, Williamson JD, Conkling MA, Pharr DM and Williamson JD (1995) Purification of NAD-dependent mannitol dehydrogenase from celery suspension cultures. Plant Physiol

- 108: 1219–1225
- Stoop JMH, Williamson JD and Pharr DM (1996) Mannitol metabolism in plants: A method for coping with stress. *Trends Plant Sci* 1: 139–144
- Storey KB and Storey JM (1991) Biochemistry of cryoprotectants. In: Lee REJ and Denlinger DL (eds) *Insects at Low Temperature*, pp 64–93. Chapman and Hall, New York
- Stoop JMH, Williamson JD, Conkling MA, MacKay JJ and Pharr DM (1997) Characterization of NAD-dependent mannitol dehydrogenase from celery as affected by ions, chelators, reducing agents and metabolites, characterization and metabolite regulation of NAD-dependent mannitol dehydrogenase from celery suspension culture. *Plant Sci* 131: 43–51
- Sutherland MW (1991) The generation of oxygen radicals during host plant responses to infection. *Physiol Mol Plant Pathol* 39: 79–93
- Swedlund B and Lacy RD (1993) Sorbitol as the primary carbon source for the growth of embryogenic callus of maize. *Plant Physiol* 103: 1339–1346
- Tao R, Uratsu SL and Dandekar AM (1995) Sorbitol synthesis in transgenic tobacco with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. *Plant Cell Physiol* 36: 525–532
- Tarczynski MC, Jensen RG and Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* 259: 508–510
- Tattini M, Gucci R, Romani A, Baldi A and Everard JD (1996) Changes in non-structural carbohydrates in olive (*Olea europaea*) leaves during root zone salinity stress. *Physiol Plant* 98: 117–124
- Taylor CB (1996) Proline and water deficit: Up, downs, ins, and outs. *Plant Cell* 8: 1221–1224
- Thomas JC, Sepahi M, Arendall B and Bohnert HJ (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. *Plant Cell Environ* 18: 801–806
- Timasheff SN (1993) The control of protein stability and association by weak interactions with water: How do solvents affect these processes. *Annu Rev Biophys Biomol Struct* 22: 67–97
- Tripp P, Krotkov G and Nelson CD (1963) Biosynthesis of mannitol- C^{14} from C^{14}O_2 by detached leaves of white ash and lilac. *Can J Bot* 41: 1005–1010
- Tripp P, Krotkov G and Nelson CD (1964) Metabolism of mannitol in higher plants. *Am J Bot* 51: 828–835
- Trost P and Pupillo P (1993) Inhibition of spinach D-glyceraldehyde 3-phosphate: NADP+ oxidoreductase (nonphosphorylating) by adenylate compounds: The effect of dead-end inhibitors on a steady state random reaction mechanism. *Arch Biochem Biophys* 306: 76–82
- Turgeon R (1989) The sink-source transition in leaves. *Annu Rev Plant Physiol Plant Mol Biol* 40: 139–168
- Turgeon R and Beebe DU (1991) The evidence for symplastic phloem loading. *Plant Physiol* 96: 349–354
- van Bel AJE, Gamalei YV, Ammerlaan A and Bik LPM (1992) Dissimilar phloem loading in leaves with symplasmic or apoplasmic minor-vein configurations. *Planta* 186: 518–525
- Vander Jagt DL, Robinson B, Taylor K and Hunsaker LA (1990) Aldose reductase from human skeletal and heart muscle: Interconvertible forms related by thiol-disulfide exchange. *J Biol Chem* 265: 20982–20987
- Verbruggen N, Villarroel R and Vanmontagu M (1993) Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. *Plant Physiol* 103: 771–781
- von Schaewen A, Langenkamper G, Graeve K, Wenderoth I and Scheibe R (1995) Molecular characterization of the plastidic glucose-6-phosphate dehydrogenase from potato in comparison to its cytosolic counterpart. *Plant Physiol* 109: 1327–1335
- Wang Z, Quebedeaux B and Stutte GW (1995) Osmotic adjustment: Effect of water stress on carbohydrates in leaves, stems and roots of apple. *Aust J Plant Physiol* 22: 747–754
- Wang Z, Quebedeaux B and Stutte GW (1996) Partitioning of [$\text{^14}\text{C}$] glucose into sorbitol and other carbohydrates in apple under water stress. *Aust J Plant Physiol* 23: 245–251
- Webb KL and Burley JWA (1962) Sorbitol translocation in apple. *Science* 137: 766
- Webb SJ and Bhorjee JS (1968) Infrared studies of DNA, water, and inositol associations. *Can J Biochem* 46: 691–695
- Weng QP and Jacobson GR (1993) Role of a conserved histidine residue, His-195, in the activities of the *Escherichia coli* mannitol permease. *Biochemistry* 32: 11211–11216
- Wermuth B (1985) Aldo-keto reductases. In: Flynn TG and Weiner H (eds) *Enzymology of Carbonyl Metabolism 2: Aldehyde Dehydrogenase, Aldo-Keto Reductase, and Alcohol Dehydrogenase*, pp 209–230. Ian R. Liss, New York
- Whetter JM and Taper CD (1963) Note on the seasonal occurrence of sorbitol (D-glucitol) in buds and leaves of *Malus*. *Can J Bot* 41: 175–177
- Williams MW and Raese JT (1974) Sorbitol in tracheal sap of apple as related to temperature. *Physiol Plant* 30: 49–52
- Williamson JD, Stoop JMH, Massel MO, Conkling MA and Pharr DM (1995) Sequence analysis of a mannitol dehydrogenase cDNA from plants reveals a function for the pathogenesis-related protein ELI3. *Proc Natl Acad Sci U S A* 92: 7148–7152
- Wilson DK, Bohren KM, Gabbay KH and Quijano FA (1992) An unlikely sugar substrate site in the 1.65 Å structure of the human aldose reductase holoenzyme implicated in diabetic complications. *Science* 257: 81–84
- Wyn Jones RG (1984) Phytochemical aspects of osmotic adaptation. *Recent Adv Phytochem* 18: 55–78
- Yamaki S (1980a) Properties and functions of sorbitol-6-phosphate dehydrogenase, sorbitol dehydrogenase and sorbitol oxidase in fruit and cotyledon of apple (*Malus pumila* Mill. var. domestica Schneid.). *J Jpn Soc Hort Sci* 49: 429–434
- Yamaki S (1980b) A sorbitol oxidase that converts sorbitol to glucose in apple leaf. *Plant Cell Physiol* 21: 591–599
- Yamaki S (1982) Localization of sorbitol oxidase in vacuoles and other subcellular organelles in apple cotyledons. *Plant Cell Physiol* 23: 891–899
- Yamaki S (1984) NADP+-Dependent sorbitol dehydrogenase found in apple leaves. *Plant Cell Physiol* 25: 1323–1327
- Yamaki S (1987) ATP-promoted sorbitol transport into vacuoles isolated from apple fruit. *Plant Cell Physiol* 28: 557–564
- Yamaki S and Asakura T (1988) Energy coupled transport of sorbitol and other sugars into the protoplast isolated from apple fruit flesh. *Plant Cell Physiol* 29: 961–967
- Yamaki S and Ishikawa K (1986) Roles of four sorbitol related enzymes and invertase in the seasonal alteration of sugar metabolism in apple tissue. *J Amer Soc Hort Sci* 111: 134–137
- Yancey PH, Clark ME, Hand SC, Bowles RD and Somero GN

- (1982) Living with water stress: evolution of osmolyte systems. *Science* 217: 1214–1222
- Zamski E, Yamamoto YT, Williamson JD, Conkling MA and Pharr DM (1996) Immunolocalization of mannitol dehydrogenase in celery plants and cells. *Plant Physiol* 112: 931–938
- Ziegler DM (1985) Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu Rev Biochem* 54: 305–329
- Zimmermann MH and Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmerman MH and Milburn JA (eds) *Transport in Plants, I. Phloem Transport. Encyclopedia of Plant Physiology*, Vol 1, New Series, pp 408–503. Springer-Verlag, New York
- Zrenner R and Stitt M (1991) Comparison of the effect of rapidly and gradually developing water-stress on carbohydrate metabolism in spinach leaves. *Plant Cell Environ* 14: 939–946

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Chapter 13

Fructans: Synthesis and Regulation

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Summary

Roughly ten percent of higher plant species possess a mechanism for reserve carbon allocation based on soluble fructose polymers (vacuolar fructan), which differs markedly in its enzymology, subcellular compartmentation and regulation from the more common starch-based carbon economy. This includes many economically important species, especially the temperate grasses and cereals. This chapter describes these novel elements associated with fructan metabolism, with particular emphasis on fructan synthesis in photosynthetic tissue.

Fructan structures, although based on variations in a few characters (polymer size, glycosidic linkage, etc.) are shown to be both varied and complex, differing markedly between species but possessing a consistency within species which argues for a biosynthetic mechanism with a high degree of specificity. The enzymological mechanisms currently thought to be involved are discussed, with particular reference to the involvement of multifunctional enzymes and the strong effects of both substrate and enzyme concentration on the chemical nature of the products in vitro. The enzymatic polymerization of authentic grass fructan has been achieved, but the conditions required in vitro do not coincide with those expected in the vacuole, the currently accepted site of synthesis. The properties of fructosyl transferases in general are shown to be unusual and we emphasize the need to reconcile the characteristics of enzymes in vitro with the patterns of metabolism and conditions observed in the tissue.

The regulation of fructan synthesis is discussed in relation to the pivotal role of sucrose as the sole substrate and as the key element in the coarse control of fructan accumulation, apparently acting at the level of gene expression and de novo enzyme synthesis. Sucrose mediated feedback inhibition of starch metabolism via phosphate transport does not apparently occur in grass leaves. This isolation of chloroplast metabolism from cytosolic sucrose accumulation indicates a fundamental difference in the fine control of central carbon metabolism between fructan and starch accumulators.

Recently, non-fructan, starch-accumulating plants such as maize, spinach and tobacco have been transformed with bacterial genes for fructan synthesis and shown to accumulate fructan. The current value of such transgenics is in terms of what they may tell us about the regulation of primary carbon metabolism in the *recipient* plants. Transgenics currently provide little insight into the nature or control of endogenous fructan metabolism. Some neglected aspects of the physiology these transgenics are considered by comparison with endogenous reserve carbon metabolism in untransformed plants.

I. Introduction

A number of recent reviews have considered the structure, metabolism and physiology of fructans (oligo- and poly-fructosyl sucrose) in higher plants (Wiemken et al., 1995; Pollock et al., 1996; Pollock and Kingston-Smith, 1997). There is interest in these compounds in terms of their agricultural or industrial potential (Fuchs, 1993a,b), and their metabolism is of general scientific relevance because of a) the discontinuous occurrence of the syndrome within the plant Kingdom; b) its unusual cellular location; c) its distinctive mechanism of synthesis and d) its close interrelationship with sucrose metabolism. The metabolic analysis of fructan metabolism in higher plants began in the 1950s, culminating in the publication of a generic theory of fructan enzymology by Edelman and Jefford in 1968. Subsequently, this theory has been tested in a range of accumulating species and modifications proposed. Sucrose has been confirmed as the major substrate but in addition, appears to have significant regulatory roles (Pollock et al., 1996). This chapter considers the current status of models for both the metabolism of fructans and its regulation, with particular reference to leaves, where synthesis and breakdown are linked closely to photosynthetic carbon metabolism. Comparisons are drawn with the fine control of sucrose metabolism in non-fructan plants.

Enzymes and genes associated with bacterial fructan synthesis have been available for many years and have now been expressed in a number of non-

fructan accumulating plants to provide alternative sinks for the perturbation of whole plant carbon balance (Ebskamp et al., 1994; van der Meer et al., 1994; Röber et al., 1996). Genes for a few plant fructosyl transferases have recently become available (Sprenger et al., 1995). This chapter considers the ways in which such novel materials have increased our knowledge of both fructan and sucrose metabolism in plants.

II. The Distribution and Structure of Fructan

Roughly 10% of the higher plant flora exhibit fructan metabolism (Hendry and Wallace, 1993). Most of the experimental work has concentrated upon the economically important groups: the Asterales (e.g. Jerusalem artichoke and chicory); the Gramineae (mainly C₃ temperate members of the Poales including wheat, barley and temperate forages); and the Liliales (e.g. onion, leek and garlic). Within these families comparatively few species have been studied in great detail, but it has become very clear, following the development of modern separative and analytical techniques, that the range of diversity within fructan structures is very large. The most common form of fructan in higher plants is based upon the sequential addition of fructosyl residues to a sucrose motif, leading to a polydisperse, water soluble, non-reducing polymer containing one glucose residue per molecule of fructan (Pollock et al., 1996, and references therein). There have been reports of fructan series which do not contain glucose residues, but these constitute only a minor component *in vivo*. (Ernst et al., 1996). The complexity of fructan structures arises in four ways via; a) variation in polymer length, b) variation in the glycosidic linkage between adjacent

Abbreviations: DP – degree of polymerization (number of hexose moieties); FFT – fructan:fructan fructosyl transferase (EC 2.4.1.99); Mr – molecular mass; PEG – polyethylene glycol; SST – sucrose:sucrose fructosyl transferase (EC 2.4.1.100); TLC – thin layer chromatography

fructan residues, c) branching of linear chains, and d) through the position of the sucrose motif in the sugar chain. At its simplest, common in members of the Asterales, the sucrose motif is terminal and the polymer is an homologous series with the type structure G-1,2F-1,2-(F)_n with adjacent fructose residues linked in the β orientation (Edelman and Jefford, 1968). Where n = 1, the trisaccharide is known as 1-kestose. This trisaccharide appears to be present in all species which accumulate fructans, even in species such as the forage grasses, where the predominant polymer is of β -2,6 conformation. In Jerusalem artichoke, the fructan (also termed inulin) chain is linear, every member of the series between n = 1 and n = ca 40 can be isolated, and there is approximately the same mass of fructans in each separable oligosaccharide (i.e. molar abundance declines with increasing size; Edelman and Jefford, 1968).

In other species the situation is more complex. Two other trisaccharides have been isolated; 6-kestose (G-1,2-F-6,2-F) and neokestose (F-2,6-G-1,2F). In the latter, chain elongation from both fructose residues with both linkage patterns is observed, leading to structures which are branched, mixed-linkage or contain an included glucose residue (Fig. 1). The variation in mean polymer size between species is very large (Table 1) but tends to be constant within species. Size may also be affected by the extent to which environmental conditions promote fructan accumulation. What is also consistent within species is the relative abundance of specific oligosaccharide isomers. Not all grasses accumulate all the possible structural variants and the 'fingerprints' produced by thin-layer chromatography (compare tissue extracts in Fig. 2) or by high-performance anion-exchange chromatography are very distinctive (Pollock and Cairns, 1991; Chatterton et al., 1993; Slaughter and Livingston, 1994). In temperate grasses, the high molecular weight polymer (which will not pass through dialysis membrane: also termed levan) is almost entirely a linear β -2,6 linked fructan with a terminal glucose (Pollock et al., 1979; Figure 3), but analysis of the smaller fructan oligosaccharides in *Lolium temulentum* showed a progressive decline in the proportion of included glucose residues and 2:1 linkages as the mean size of the oligosaccharides increased (Table 2).

Isotopic tracer studies (Cairns and Pollock, 1988a; Sims et al., 1993) have demonstrated unequivocally that sucrose is the major donor of fructose residues

to growing fructan chains in vivo and that 1-kestose is the first labeled intermediate. Subsequently it appears that fructan residues are transferred to a range of oligosaccharide acceptors, most, if not all, of which are in isotopic equilibrium. The pattern of these acceptors is species-specific and changes radically with increasing size to give a much simpler 'final' structure.

A fuller discussion of this complexity can be found in Pollock et al., (1996), but the brief outline given above raises a number of interesting issues in terms of the regulation of the process and the species-specificity of the structures synthesized. Fructans appear unique among storage polymers in terms of the precision of the biosynthetic process within species and its variability between them. This species-specificity can be reproduced *in vitro* (Figs. 2 and 3) and implicates structural differences in the enzymes from different species as the primary determinant of the structure of the fructan product (Cairns and Ashton 1993). Any complete mechanistic hypothesis for the enzymology of fructan synthesis will need to explain these properties.

III. Physiology and Enzymology of Fructan Metabolism

A. Fructan Synthesis in Storage Roots

A previous review (Cairns, 1993) summarized earlier physiological and enzymological studies in roots, tubers and bulbs. These studies, particularly those of inulin metabolism in tubers of *H. tuberosus* by Edelman and co-workers (summarized in Edelman and Jefford 1968) provided both the core model and terminology currently used for the enzymes of fructan synthesis in plants. Briefly, this model designates two monofunctional enzymes: a) sucrose:sucrose fructosyl transferase (SST) which forms 1-kestose by fructosyl transfer from donor to acceptor sucrose, and b) fructan: fructan fructosyl transferase (FFT) which reversibly elongates acceptor fructans by the transfer of one fructosyl residue, at the expense of the equivalent shortening of a donor fructan, to produce a linear, β -2,1 linked polymer (i.e. FFT catalyses no increase in the number of glycosidic bonds and hence no net synthesis of fructan). The energy conserved in the glycosidic bond of sucrose is nearly as high as that in uridine diphosphoglucose (28 vs. 32 kJ mol⁻¹; Dey, 1980) and is sufficient to

Disaccharide	G↔F	Sucrose(ubiquitous)	
Trisaccharides	$G \leftrightarrow F1F$	$F \leftrightarrow G \leftrightarrow F$	$G \leftrightarrow F$
	1-kestose	neokestose	6 F
	all accumulators	Poales Liliales	6-kestose
			Poales Liliales
Tetrasaccharides	$G \leftrightarrow F1F1F$	$F1F \leftrightarrow G \leftrightarrow F$	$G \leftrightarrow F$
	1,1 nystose		6
		Asparagus	F
	all accumulators		6
			F
		$F \leftrightarrow G \leftrightarrow F$	Wheat
		6	
		F	
			<i>L. temulentum</i>
Pentasaccharides	$G \leftrightarrow F1(F)_21F$	$F \leftrightarrow G \leftrightarrow F1F1F$	$G \leftrightarrow F$
		$F1F \leftrightarrow G \leftrightarrow F1F$	6
	all accumulators		(F) ₂
		Asparagus	6
		<i>L. temulentum</i>	F
		Oat	
			<i>L. temulentum</i>
			Wheat
$G \leftrightarrow F1F1F$	$F \leftrightarrow G \leftrightarrow F$	$F \leftrightarrow G \leftrightarrow F$	
6	6 6		6
F	F F		F
			6
Wheat	<i>L. temulentum</i>		F
Oat	Oat		
		<i>L. temulentum</i>	
$G \leftrightarrow F1F$	$G \leftrightarrow F$	$G \leftrightarrow F1F$	
6	6		6
F1F	F1F		F
	6		6
Wheat	F		F
			Wheat
			Wheat

Fig. 1. The complexities of structure observed in tri-, tetra-, and pentasaccharides isolated from various monocot species. The 'minimalist' structural conventions suggested by Pollock are adopted:

(G↔F = Sucrose; F↔G↔F = Neokestose;

F1F = two adjacent residues linked β 2,1;

F = two adjacent residues linked β 2,6 (from Lewis, 1993).

6

F

Table 1. Variation in mean molecular size of fructans from different species

Species	Mean molecular size (kDa)	Method employed
Gramineae		
<i>Phleum pratense</i>	42	Size exclusion chromatography
<i>Phleum pratense</i>	46	End group analysis
<i>Lolium perenne</i>	12	Size exclusion chromatography
<i>Festuca pratensis</i>	12	Size exclusion chromatography
<i>Dactylis glomerata</i>	20	Size exclusion chromatography
<i>Bromus inermis</i>	5	End group analysis
Liliales		
<i>Allium sativa</i>	9	Size exclusion chromatography
Asterales		
<i>Helianthus tuberosus</i>	5	End group analysis

Source: Pollock and Cairns 1991.

Table 2. Linkage analysis of pooled high degree of polymerization fructans from *Lolium temulentum* following fractionation by GPC

	Fraction		
	A	B	C
Mean Degree of Polymerization (DP) ^a	7.2	12.8	30.1
Ratio of 2,6- to 2,1-linkages ^b	2.4:1	12.6:1	40.1:1
Ratio of terminal to linkage Glc residues ^c	2.6:1	7.4:1	^d

^a Mean DP was estimated from the Glc:Fru ratio.^b The ratio of 2,1- to 2,6-linkages was estimated from the relative peak intensities of m/z = 161, 190 and 162, 189, respectively.^c The ratio of terminal glucose to linkage glucose was estimated from relative peak areas following GC separation of partially methylated alditol acetates.^d Linkage glucose peak too small for estimation.

Source: Sims et al. 1992

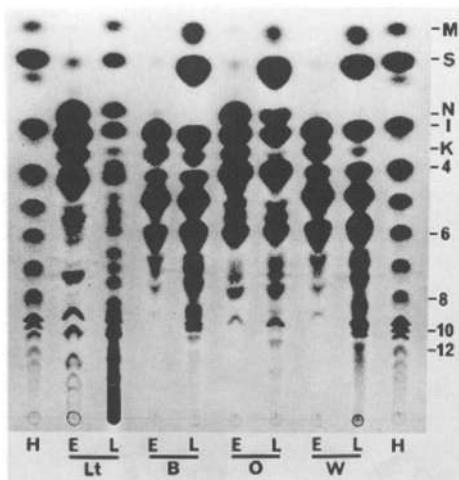


Fig. 2. Qualitative analysis by TLC of the fructan products of a 10h incubation of sucrose with enzyme from leaves of *Lolium temulentum*, (Lt), barley (B), oat (O) and wheat (W). Products of the enzyme (E) are compared with the native water soluble carbohydrate of the source leaves (L). Grass fructans are compared with standard oligoinulin standards from *Helianthus tuberosus*. Markers M, S, N, I and K refer to the mobilities of standard monosaccharide, sucrose, neokestose, 1-kestose, and 6-kestose respectively. Numerals 4–12 mark the mobilities of oligoinulins of degree of polymerization 4–12. Sugars were visualized with urea-phosphoric acid stain. (Source: Cairns and Ashton, 1993).

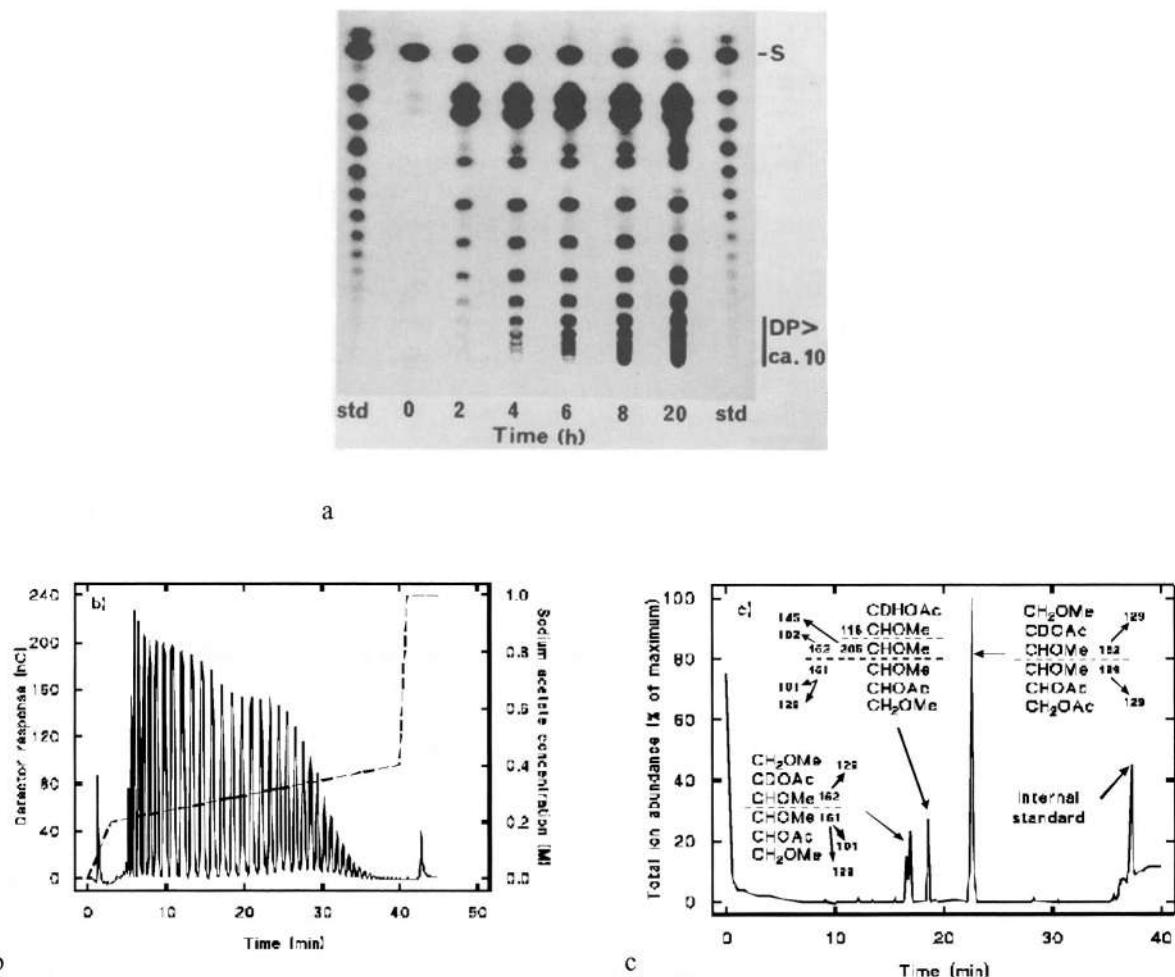


Fig. 3. Polymerization of fructan by a partially purified enzyme preparation from leaves of *Phleum pratense* L. a) Time course of formation of total fructan products (oligo- and poly saccharides) separated on TLC. Excess sucrose substrate was removed by HPLC prior to analysis. S and DP>10 respectively, designate sucrose and fructans of degree of polymerization greater than 10. b) Analysis of polysaccharide products (DP>10: ethanol precipitated) by anion exchange chromatography-pulsed amperometry. c) Structural determination of polysaccharide products. Fructan was methylated, hydrolyzed, reduced with sodium borodeuteride and acetylated. Derivatives were separated by GC on a high polarity BPX70 column and their mass spectra determined. Deduced structures and predominant fragments are shown, indicative of terminal fructose, terminal glucose and 2,6 linkage fructose (in order of elution). The internal standard was myo-inositol hexa-acetate. (Source: Cairns et al., 1999)

fuel the process without the involvement of other cofactors. The integration of inulin synthesis and degradation is thought to be mediated by SST, which is detectable in tubers during synthesis, but not during remobilization. Because the FFT reaction is reversible, the poise of the reaction towards or away from chain elongation would depend on the input of carbon via SST, favoring depolymerization when its activity is absent. A third enzyme, fructan exohydrolase (FEH) was implicated in the mobilization of fructan, releasing free fructose for subsequent

resynthesis into sucrose for export.

With the recent purification of SST and FFT enzymes from *H. tuberosus* tubers and chicory roots (Koops and Jonker, 1996; Lüscher et al., 1996; van den Ende and van Laere, 1996) this model for synthesis has been largely confirmed with two modifications: a) The SST is not monofunctional, since it also forms oligoinulins of DP > 3 from sucrose. b) In vitro reconstitution of SST and FFT allows the synthesis of inulin up to DP ≤ 15 from sucrose. The original model required separate

compartmentation of FFT (vacuole) and sucrose plus SST (cytoplasm) because FFT-mediated chain elongation in vitro was markedly inhibited by sucrose (Edelman and Dickerson, 1966). The recent demonstrations of FFT activity in the presence of sucrose and SST activity obviates this necessity. The general model for fructan synthesis in *Asparagus* roots and onion bulbs is thought to be analogous to that of *H.tuberosus*, though a third enzyme, a δ^G -fructosyltransferase is implicated to explain the occurrence of the neokestose series of fructan in these species. The enzymological data for these species is not as full or as conclusive as the recent data for *H.tuberosus* and chicory (Cairns, 1992a, 1993)

B. Fructan Synthesis in Leaves of Temperate Gramineae

Experimental perturbation of carbon partitioning in grass leaves is straightforward and has provided an excellent system for the study of fructan metabolism and its enzymology and by contrast with root sink systems, the physiology of the grass leaf system is well characterized (Wagner et al., 1983; Housley and Pollock 1985; Cairns and Pollock, 1988a,b; Simpson et al, 1991; Cairns et al., 1997). Leaves manipulated to contain low levels of photoassimilates fully degrade sucrose and fructan, and loose both the capacity for in vivo fructan synthesis and extractable fructosyl transferase activity (Wagner et al., 1986; Cairns and Pollock, 1988a,b; Obenland et al., 1991; Cairns, 1992b). When such tissues are subsequently placed into positive carbon balance, sucrose accumulates until a threshold concentration of 15–20 mg g⁻¹ fresh mass is reached, at which point tissue sucrose concentration stabilizes, conversion of sucrose into fructan is initiated and extractable fructosyl transferases are induced (the regulation of this process is discussed below). Synthesis of fructan occurs de novo, with sucrose as the sole precursor and with no requirement for (detectable) fructan primers. Fructan can accumulate at instantaneous rates similar to those of photosynthetic sucrose synthesis (e.g. 1.6 mg g⁻¹ h⁻¹ at 250 μ E m⁻² s⁻¹) and can reach tissue concentrations of 20–40 mg g⁻¹ fresh mass. Fructan synthesis thus represents a massive component of primary carbon metabolism, contributing substantially to resource allocation within the plant.

Most early enzymological studies in grasses detected 1-kestose as the sole transferase product

from sucrose. These data were interpreted as evidence for a monofunctional SST, and cited as evidence for the applicability of the SST/FFT scheme, though little evidence was presented for further polymerization by FFT or otherwise. By implication, the only route for carbon into grass fructan was through 1-kestose, consistent with the model of Edelman and Jefford (1968). However, the emerging enzymological evidence in grasses is equivocal in its support for this model since the isolated fructosyltransferase activities are generally multifunctional and/or also catalyze hydrolytic reactions. In addition, all the enzymes so far described have properties which are difficult to reconcile with the physiology of fructan synthesis in the source tissue. There are four areas in particular where behavior in vivo conflicts with in vitro observations. a) Kinetics: Fructosyl transferases from grasses (and other genera, e.g. Koops and Jonker, 1996; Van den Ende and Van Laere, 1996), universally require high substrate concentrations for function in vitro. Their kinetic response generally approximates to linearity in the range 50–500 mM sucrose and concentrations for half maximal activity are in the range 100–300 mM. b) Rates of synthesis: In the few instances where direct comparisons have been made, the in vitro sucrose fructosyl transferase activity of grass leaves engaged in fructan synthesis are adequate to support the rates of flux of fructose into fructan in vivo (Cairns and Pollock, 1988a,b; Cairns, 1992b). However, the sucrose concentrations required to cause physiologically significant rates of fructosyl transfer markedly exceed those thought to exist in the vacuoles of grass mesophyll cells, the currently accepted site of fructan synthesis (Wagner et al., 1983). c) Fidelity in reproducing representative natural structures: The products of fructosyl transferases assayed in vitro often differ in linkage structure, and especially in size, from the native complement of fructans in the source tissue and, d) Absence of large fructans: Polymerization of the large β 2,6 linked fructans characteristic of grass tissues has been difficult to demonstrate in vitro, despite the magnitude of the process in vivo.

Reports of purified fructosyl transferases from grasses which have appeared recently illustrate some of the difficulties described above: in barley, two enzymes have been isolated, both exhibiting a requirement for high sucrose concentration and neither fully saturating at 600 mM. The first, described as 1-SST, synthesizes 1-kestose as the predominant product from sucrose, though higher oligosaccharides

of DP ≤ 5 were also formed (Simmen et al., 1993). Consistent with the recently reported data for SST from *Helianthus* and chicory, barley 1-SST is at least bifunctional, having fructan-sucrase or FFT-like activity in addition to SST activity. Therefore, the current terminology is not wholly consistent with the range of activities exhibited by this enzyme in many different plant species. The second barley enzyme, termed 6-SFT (formerly 6-SST; Simmen et al., 1993; Duchateau et al., 1995), is also a multifunctional enzyme which, in the absence of 1-kestose, synthesizes 6-kestose directly from sucrose, providing an alternative route for carbon into fructan. 6-SFT also transfers single fructosyl residues from sucrose to carbon 6 of fructose residues in oligosaccharides of the inulin series, generating the branched fructans which occur naturally in barley leaves. Independently, the partial reactions of 1-SST and 6-SFT can explain the synthesis of barley oligofructans up to ca. DP5, but there is no evidence either that the rates are physiologically significant or that reconstitution of the two activities can generate oligosaccharides de novo. In addition, there is no direct evidence that they are involved in synthesis of the larger fructans which also occur naturally in barley leaves. The third activity of the 6-SFT preparation is as a β -fructofuranosidase (invertase). With 200 mM sucrose as sole substrate, invertase accounts for 78% of the total activity. In contrast to its low sucrose-affinity for trisaccharide synthesis ($K_m > 300$ mM), the value for sucrose hydrolysis is ca. 10 mM (Simmen et al., 1993). The detailed physiological consequences of this high-affinity hydrolytic activity, particularly in the early stages of de novo synthesis, remain to be explored. The properties of barley 1-SST and 6-SFT are clearly at variance with the SST/FFT model, suggesting as they do, potential roles in grasses for a) multifunctional transferases, b) direct sucrase-type fructosyl transfer, and c) an alternative route for fructosyl residues into fructan, via 6-kestose.

An enzyme analogous to the FFT activity from *H. tuberosus*, which catalyses non-synthetic transfer between pre-existing primer inulins to generate oligosaccharides of DP ≤ 6 , has been isolated recently from *Lolium rigidum* (St. John et al., 1997a). This enzyme did not saturate with increasing substrate concentration, even at 400 mM 1-kestose. These authors note that this species accumulates predominantly high Mr fructan with β 2,6-linkages. The predominant affinity of the isolated FFT, by contrast, was for low Mr, β 2,1-linked fructans. It showed no

detectable donor or acceptor activity against β 2,6 linkages and could not, therefore, account for their synthesis in the source tissue. When this FFT was incubated simultaneously with 100 mM sucrose and a partially purified, multifunctional preparation having SST, FFT and 6-kestose-forming activity, oligosaccharides of DP ≤ 6 were synthesized (St. John et al., 1997b). The only circumstances under which traces of larger fructans were generated were in experiments where sucrose was periodically added to the assay to maintain a concentration of 100 mM over a 10h reaction. These larger products contained both 2,6 and 2,1 linkages, which is interesting in the view of the absolute β 2,1 specificity of the FFT when assayed alone and the β 2,6 specificity of high Mr fructan synthesis in vivo. Clearly there is no simple relationship between the enzymological and physiological data. The evidence from *L. rigidum* supports the Edelman and Jefford model to the extent that a fraction active against sucrose is separable from an FFT active against fructan only. However, because the structure of the FFT product differs from the native fructan and because the kinetic parameters and rates of product formation have not been compared with the source tissue, the physiological significance of these observations is not fully resolved.

Since, for the reasons outlined above, it is often difficult to place incomplete enzymological data into a physiological context, we regard the demonstration of in vitro polymerization of authentic grass fructan at physiological rates as a prerequisite for the understanding of the process in vivo. We have shown that this can be achieved using a combination of high concentrations of partially purified enzyme and high sucrose concentration (Cairns, 1992b). The chromatographic fingerprints of the products are species-specific and strongly resemble the native fructan complement in the tissue used as the source of the enzyme (compare the species in Fig. 2). In the case of *Phleum pratense* we have established, using a combination of anion exchange chromatography-PAD and methylation/GC-MS analysis, the enzymatic synthesis of 2,6-linked polymers of high Mr (up to DP = ca. 50, Fig. 3). Because, a) large fructans representative of the native polymers are synthesized de novo from sucrose at physiological rates, b) the products are species-specific, and c) the pattern of sucrose-regulation and its sensitivity to inhibitors of gene expression parallels that of the tissue (Cairns, 1992b; Cairns and Ashton 1993), we can be reasonably certain that the in vitro activities are

involved in fructan synthesis *in vivo*.

The enzyme concentration required for this high fidelity synthesis *in vitro*, (>ca. 5.0 g fr mass equivalent cm^{-3} ; 10–14 nkat cm^{-3}), is at least 10-fold greater than that generally employed in enzymological studies of fructan synthesis. The apparent K_m for polymerization in *L. temulentum* and *P. pratense* is in the range 200–300 mM sucrose, though the reactions typically tend to saturation only at above 1.0 M sucrose. To emulate *in vivo* rates of accumulation of polymeric fructan, the substrate concentrations required for enzymatic synthesis are in the range 1.0–1.5M. The pH optima for these polymerising reactions are 5.5–6.0 (Cairns and Ashton, 1994) and the polymerising fractions have M_r of 50–60 kD based on size exclusion chromatography (Cairns et al., 1997, 1999). K_m , M_r and pH optima for polymerization are similar to those generally reported for trisaccharide and oligosaccharide formation by fructosyl transferases (SST, SFT and FFT) from most plants.

It is clear that fructosyl transferases assayed at a range of enzyme concentrations exhibit different product specificities, higher enzyme concentrations producing larger fructans over the same incubation period and with otherwise identical conditions of reaction (Cairns, 1995). Given the strong similarity in general properties between extracts which produce large fructans and ones which will only synthesize oligosaccharides, it is possible to explain the absence of polymerization by the latter, in terms of a requirement for high enzyme and substrate concentration. Enzymes assayed at low concentration and reported as 'SST' and '6-SFT' may well polymerize larger fructans under different reaction conditions. The oligosaccharide synthesis ($DP > 3$) observed for *Helianthus* and chicory SST may also be enhanced at higher enzyme concentration.

All reported enzymes of fructan synthesis exhibit peculiarities in specificity and/or kinetics which need to be considered against the conditions *in vivo*. Our polymerization reactions are no exception. Whilst they make realistic fructans at realistic rates and the pattern of regulation parallels that *in vivo*, the physiological consequences of the *in vitro* properties of the polymerase demand closer examination. From the known *in vivo* and *in vitro* rates of synthesis in leaves of 1.4 nkat g^{-1} (Cairns and Pollock, 1988b; Cairns and Ashton, 1994), and estimates of mesophyll vacuolar volume at $441 \text{ mm}^3 \text{ g}^{-1}$ fresh mass (Cairns et al., 1989), the concentration of synthetic activity

in vivo could theoretically reach 3.2 nkat cm^{-3} , less than one third the concentration needed *in vitro* to sustain this rate of synthesis. But to achieve even this activity would require a substrate concentration of more than 1.0M (Cairns and Ashton, 1994). By mensuration (Cairns et al., 1989) and by direct measurement, (Koroleva et al., 1997) the vacuolar sap of fructan-accumulating mesophyll cells contain sucrose at 100–200 mM, which would result in an activity of only 10–15% of the necessary rates. If the properties of *in vitro* polymerization reflect the situation *in vivo*, there is both insufficient enzyme and insufficient substrate in the vacuole to explain the rates of accumulation within the tissue. Kaeser (1983) reported micro-vesicular localization of inulin synthesis in tubers of *H. tuberosus*. It remains a possibility that high substrate and enzyme concentration could co-exist in such vesicles in grasses, satisfying the requirements for enzymatic polymerization *in vivo*. This can be reconciled with findings localizing fructans and their enzymes in isolated vacuoles (Wagner et al., 1983; Frehner et al., 1984; Cairns et al., 1989) since presumably such vesicles would fuse with the tonoplast resulting in a final location of both dilute enzyme and product in the vacuole.

C. Fructan Metabolism in Other Tissues of Temperate Gramineae

In terms of mass, the developing stem of cereal and grass inflorescence accumulates more fructan than any other organ (Pollock and Jones, 1979). Although the upper internode and the peduncle do contain chlorophyll, the majority of the carbon stored in the stems comes from leaf photosynthesis (Austin et al., 1977). Photosynthate from lower leaves appears more likely to accumulate as fructan in stems, contrasting with photosynthate from flag leaves, most of which passes directly into the ear (Pearman et al., 1978). Fructan concentrations can reach up to 40% of dry weight around the time of anthesis.

In annual cereals, up to 50% of stem dry weight is lost during grain maturation (Bonnett and Incoll 1992) and fructan contents decline markedly. The proportion of mobilized fructan which promotes grain filling is not easy to determine and may depend upon both genotype and cultivation conditions (Schnyder 1993). There are also indications that mobilization of stem fructan is induced developmentally, rather than in direct response to increased

demand for assimilate by the ears. (Kühbauch and Thome, 1989; Bonnett and Incoll, 1992). There have been few biochemical studies on stem fructan metabolism because of the recalcitrance of the tissue. Dubois et al., (1990) showed that SST activity in spring wheat stems showed genotypic differences which correlated both with initial sucrose content and with the rate of subsequent fructan accumulation. Elevated CO_2 concentrations increased sucrose contents, extractable SST activity and final stem fructan content. This experiment was carried out under controlled environment conditions where total soluble carbohydrates accumulated throughout the experiment. Under field conditions (Bancal and Triboli, 1993) there was little correlation between SST activity and fructan content. These authors suggest that net accumulation was regulated by changes in FEH activity which were associated with mobilization of stored fructan during grain filling. Changes in FEH activity are also associated with mobilization of fructan from stems of *Dactylis glomerata* (Yamamoto and Mino, 1989). In these cases the assumption is that activity changes would be caused by changes in the amount of enzyme protein and are under developmental control as well as being sensitive to assimilate abundance.

The development by Silk (1984) of the continuity equation allowed measurements of composition and elemental growth rate to be used to calculate assimilate fluxes into meristematic and extending tissues undergoing 'linear' growth. This has led to the demonstration of a substantial involvement of fructan metabolism during the growth of grass and cereal leaves. The main contribution to studies of this kind has been by Schnyder, Nelson and co-workers (Schnyder, 1986; Schnyder et al., 1988; Schnyder and Nelson, 1987, 1989). They demonstrated very large fluxes of material into fructan within the extension zone. As the segment of tissue aged, and moved further up the developing leaf, there was an equivalent flux out of fructan and into structural materials. Our own studies have shown that these fluxes are sensitive to changes in growth rate (associated with chilling) and assimilate abundance (caused by increased photoperiod or elevated CO_2), but that fructans remain the major temporary sink within the extension zone. We have also demonstrated that elevated CO_2 strongly stimulates hexose accumulation and that increased carbohydrate contents are associated with increased rates of respiration, regardless of growth rate (Table 3).

Table 3. Respiration rate in extension zones of cereal leaves as related to growth treatment and soluble carbohydrate content

Growth treatment	Respiration rate nmol $\text{O}_2 \text{ mg}^{-1} \text{ h}^{-1}$	Carbohydrate content mg g^{-1} fresh wt
20 °C:350ppm CO_2	18	10.2
20 °C:500ppm CO_2	125	18.6
5 °C:350ppm CO_2	163	20.3

J. A. Harrison and C. J. Pollock, unpublished.

Because of the extremely stable gradient of tissue development along the leaf, this experimental system has considerable potential for the study of metabolic regulation. To date, enzymological studies have been constrained by the small mass of tissue involved.

Developing cereal grains also accumulate fructan. Final concentrations only reach 1–2% of the dry weight (MacLeod and McCorquodale, 1958) but the proportion in young developing grain is much higher (Escalada and Moss, 1976). Measurements of SST and FFT activities (Housley and Daughtry, 1987) suggest that active fructan accumulation occurs very early in grain development, but that the fructan pool becomes progressively less accessible. During the main phase of starch biosynthesis, fructan forms a static pool making up a progressively declining proportion of total grain carbohydrate. It is not known either how this process is regulated or what its physiological significance is, although it has been suggested that synthesis of sucrose into fructan would facilitate continued passive unloading from the phloem (Hendrix, 1983).

D. Fructan Hydrolysis in Leaves of Temperate Gramineae

In general, studies of fructan hydrolysis have concentrated on non-photosynthetic tissues such as storage organs, cereal stem interposed and the stubble remaining after defoliation. Recent reviews have summarized such studies (Housley and Pollock, 1993; Simpson and Bonnett, 1993). This section summarizes our current understanding of fructan hydrolysis, emphasizing more recent reports.

Fructan mobilization may be under developmental control (as in the case of inulin breakdown in sprouting tubers of *H. tuberosus*; Edelman and Jefford, 1968), but in many cases it appears to be sensitive to the balance between supply of and demand for fixed carbon. It is not clear how the process is regulated to prevent net degradation of fructan during periods of

assimilate surplus. Leaves manipulated to be in negative carbon balance can completely hydrolyze any fructan and sucrose present and export the carbohydrate to sinks (Cairns and Pollock, 1988a; Simpson et al., 1991). In general, sucrose and smaller fructans are degraded very quickly whilst higher M_r fructans tend to be more persistent. There is good evidence for exo-hydrolysis of single terminal fructose being the major pathway of breakdown (Pollock, 1982). The hexose products, fructose and a small amount of glucose (terminal sucrose will be hydrolyzed by invertase), are assumed to be transferred from the vacuolar site of hydrolysis to the cytoplasm where they are phosphorylated via hexokinase and resynthesized into sucrose.

Fructan hydrolase activities isolated from plants are universally exo-hydrolytic and are termed fructan exohydrolase (FEH). Thus, hydrolysis of fructan *in vitro* parallels the process *in vivo*. Exohydrolases from grasses exhibit pH optima in the range 4.5–5.5 and have M_r of 40–69 kD (Simpson and Bonnett, 1993). There is emerging evidence that 'FEH' is not one enzyme but a number of isoforms with differing molecular properties and specificities (Bonnett and Simpson, 1995). In common with synthesis, the enzymology of fructan hydrolysis has been difficult to reconcile with the known physiology of fructan mobilization. The main areas of discrepancy are: a) Kinetic: High substrate concentrations are required for *in vitro* function of FEH. Reports of K_m vary widely between 0.22 and 89% w/v fructan but are generally c. 20% w/v (Simpson and Bonnett, 1993). Given that maximal concentrations of fructan in leaf tissue are around 4% w/v, these enzymes are likely to function at well below maximal rates *in vivo*. In addition, the rate of activity will be sensitive to substrate concentration and will be further limited as hydrolysis proceeds, particularly since many of the preparations show inhibition by free fructose. b) Enzyme specificity: The majority of grass hydrolases are assayed with, and preferentially hydrolyze β 2,1 fructan, which is not the form predominantly accumulated in the source tissue. There have, however, been recent reports of activities with a preference for β 2,6 bonds (Bonnet and Simpson, 1995; Henson and Livingston, 1996; Marx et al., 1997). c) Timing: Unlike the inducible enzymes of synthesis, many tissues contain 'constitutive' FEH activity, regardless of carbohydrate status. During fructan synthesis in grass leaves, for example, there is enough extractable FEH activity to counteract observed rates of fructan

accumulation, particularly as small oligofructans produced early in accumulation are especially susceptible to attack (Simpson et al., 1991; Cairns et al., 1997). Because synthesis, accumulation and hydrolysis of fructan are all currently thought to be vacuolar, it is difficult to see how intermediate oligofructans persist for long enough to permit polymer building unless there is some form of compartmentation or *in vivo* inhibition of hydrolysis occurs. d) Regulatory: During fructan mobilization, extractable FEH activity often increases. However, the increase occurs after substantial fructan breakdown has already taken place (Simpson et al., 1991). One explanation for this may be that, because of the low affinity of FEH for the substrate, an increase in the absolute amount of enzyme is required to support hydrolysis at low substrate concentrations (Simpson and Bonnet, 1993).

In overview, studies of FEH are practically difficult and interpretation of the results complicated. Despite this, progress has been made with the recent purification of hydrolases with appropriate specificities for native grass fructan (Bonnet and Simpson, 1995; Henson and Livingston, 1996; Marx et al., 1997). However, the complexities of the process will make it difficult to unambiguously assign function *in vivo* until specific activities can be abolished either by mutation or by antisense technology.

IV. The Control of Fructan Metabolism

A. Induction by Sucrose Accumulation

The clearest picture of the regulation of fructan synthesis is provided by the well characterized grass leaf system. When grown at low irradiance under a short photoperiod, leaves will not accumulate fructans. Excision and continuous illumination leads, as indicated above, to an increase in the concentration of sucrose and to the progressive appearance of fructans of increasing size. Treatment of leaves at the time of excision with inhibitors of gene expression blocks the conversion of sucrose into fructan without altering the total amount of soluble carbohydrate accumulated in the tissue (Wagner and Wiemken, 1987; Cairns and Pollock, 1988b; Table 4). Application of inhibitors at different times after excision indicated that the ability to convert sucrose into fructan was acquired fully within six hours of excision and illumination. After this, applications of

Table 4. Quantitative analysis of water-soluble carbohydrate fractions from excised leaves illuminated in the presence of inhibitors of gene expression^a

Inhibitor	Concentration (μM)	Total water-soluble carbohydrate accumulated in 24 h (mg g ⁻¹ fresh mass)	Proportion with DP >2 (%)
Water control	–	43.3	58.4
L-MDMP	10	44.5	53.0
D-MDMP	10	40.8	1.0
Cycloheximide	100	40.5	2.5
Cordycepin	1000	44.9	2.8
α -Amanitin	1000	48.6	4.2

^aExcised leaves of *Lolium temulentum* were stood in aqueous solutions of metabolic inhibitors for the initial 3 h of a 24-h illumination period. At the end of this period tissue was extracted and water-soluble carbohydrate analysed by high-performance liquid chromatography. Source: Cairns and Pollock, 1988b.

cycloheximide or cordycepin had no effect (Winters et al., 1994). These observations led to two significant conclusions. The first was that regulation of fructan metabolism occurs at the level of coarse control, i.e. changes in the absolute amount of the enzyme(s) which synthesize fructan or, perhaps less likely, the amount of a strong activator of existing enzyme. The second is that sucrose concentrations per se do not appear to affect carbon fixation in the short term. The rate of carbohydrate accumulation is constant over the first 54 h of leaf excision (Housley and Pollock, 1985) and is insensitive to inhibitors which block the conversion of sucrose to fructan (Cairns and Pollock, 1988b). By this stage, soluble sugars can make up 40% of the dry weight of the leaf! Feeding exogenous sugar in the dark to excised leaves also leads to a similar induction of fructan biosynthesis (Wagner and Wiemken, 1987; Table 5), leading to the conclusion that it is the rise in sucrose or in some clearly related metabolite, which triggers the induction of fructan biosynthesis. (Wiemken et al., 1995).

B. Coarse Control by Altered Gene Expression

As indicated above, the convenience of the excised leaf system has meant that almost all detailed studies on the biochemistry, enzymology and molecular biology of fructan metabolism in Gramineae has been carried out using leaves. Studies on other tissues in grasses and cereals and on storage organs in the Liliaceae and the Asterales have not, however, suggested that any radically different mechanisms are operating elsewhere, so the current assumption is that the regulatory factors operating in leaves are probably of general significance.

Table 5. The effects of exogenous sugars on fructan biosynthesis and fructosyl transferase activity in detached barley leaves kept in the dark

Sugar	Fructosyl transferase activity nkat ml ⁻¹	Fructan content mg g ⁻¹ fresh mass
None	0.08	0.01
Sucrose	0.64	1.48
Maltose	0.99	2.07
Maltotriose	0.50	0.38
Trehalose	0.48	0.01
Fructose	0.47	1.08
Glucose	0.22	0.31
Cellobiose	0.33	0.43
Lactose	0.31	0.13

Sugars were supplied for 16 h by standing leaf blades in 0.5M solutions. Data from Wagner, Wiemken and Matile, 1986.

The regulation of fructan metabolism is intimately connected with the various roles of sucrose within the overall syndrome. Sucrose can act as both a fructosyl donor and as a fructosyl acceptor. There is also evidence that sucrose prevents the inactivation of fructan-synthetic enzymes (Obenland et al., 1991; Cairns and Ashton, 1994), and protects small fructan intermediates from the action of hydrolases during polymer building (Cairns et al., 1997). In many systems sucrose is known to modulate carbon metabolism via fine control of existing enzyme activities and via its influence on the patterns of gene expression. It seems probable that all of the factors integrate to regulate the flow of carbon into fructans within leaves.

Newly synthesized proteins can be monitored very effectively in excised leaves by administering ³⁵S methionine to the cut ends. Changes in mRNA can also be monitored by cell-free translation. In

both cases, excised leaves fed sucrose in the dark showed a relatively small number of novel polypeptides which were synthesized up to about 8 h after excision, suggesting that only a few genes were involved. Illuminated leaves showed larger changes in both protein synthesis and cell-free translation, suggesting that photosynthetic processes were affected even though this did not result in declining assimilation rates (Winters et al., 1994). Differential screening of cDNA libraries from induced leaves using probes derived from induced and uninduced leaves revealed a number of clones exhibiting increased expression. One of these was analyzed further and showed extremely strong sensitivity to both increases and decreases in sucrose concentration. This gene was also more highly expressed in lines of *Lolium perenne* which accumulate higher amounts of soluble carbohydrates (L. Skot, personal communication). Unfortunately, sequence analysis indicated that the DNA sequence coded for S-adenosyl methionine synthetase rather than for an enzyme capable of using sucrose as a substrate (Winters et al., 1995). Subsequently, however, heterologous screening of a similar library with probes derived from maize root invertases has identified a gene sequence with close homology to the invertase family and to the barley SFT (Sprenger et al., 1995). This gene is strongly up-regulated in the presence of sucrose (J. Gallagher and C. Pollock, unpublished). This suggests that there are genes in fructan accumulating tissues which are up-regulated in the presence of sucrose and which code for enzymes which metabolize that sucrose, potentially into fructan. The up-regulated clone from *L. temulentum* produces a protein which cleaves sucrose but which will also catalyze the synthesis of higher oligosaccharides (J. Gallagher and C. Pollock, unpublished). The clone isolated and sequenced by Sprenger et al. (1995) also codes for an enzyme which is capable of both hydrolysis and fructosyl transfer and which is more closely related to higher plant invertases than to bacterial fructosyl transferases. These authors argue that the fructan syndrome in plants has arisen polyphyletically through a range of modifications of various members of the acid β -fructofuranosidase (invertase) family (Wiemken et al., 1995). Presumably such modifications would have been of selective advantage when they occurred in genes which were up-regulated by high levels of assimilate.

There is less direct evidence that the enzymes of fructan hydrolysis are regulated in a similar manner.

Possible increases in assimilate abundance and fructan accumulation are associated with reductions in extractable fructan exohydrolase activity (Simpson et al., 1991) and in some cases these changes are sensitive to inhibitors of gene expression (Wagner et al., 1986). It is not known whether specific gene-products coding for enzymes which degrade fructan are down-regulated under such conditions, but the increasing availability of clones for enzymes of fructan synthesis and the likelihood that fructan hydrolases will also show sequence homology to invertases should lead to the development of specific probes to measure changes in message abundance, and of specific antibodies to estimate enzyme protein levels.

C. Fine Control via Metabolites in Photosynthetic Tissue

As indicated above, the rate of sugar accumulation in excised, illuminated grass leaves remains constant until chlorophyll degradation sets in (Housley and Pollock, 1985). The rates are similar in magnitude to the maximum rate of leaf photosynthesis measured by gas exchange (Natr, 1969). If one assumes that sucrose concentrations are similar in cytosol and vacuole and that the maximum sucrose content stabilizes at around 20 mg g⁻¹ fresh mass, this would equate to a uniform concentration change from zero to ca 60–100 mM during the course of the experiment. Any selectivity which would lead to high concentrations of sucrose in the cytosol (as suggested by Winter et al., 1993) would, of course, amplify the effect. In plants where chloroplast starch forms the major leaf carbohydrate reserve, much smaller increases in sucrose concentration are thought to feed back, via elevated concentrations of the regulating fructose 2,6 bisphosphate, to slow export of triose phosphate from the chloroplast and stimulate starch biosynthesis by the activation of ADPG pyrophosphorylase (Stitt, 1996). This feedback does not apparently occur in grass leaves when sucrose accumulation was stimulated by chilling the sink tissue (Table 6).

We investigated the possibility that the cytoplasmic fructose 1,6 bisphosphatase for *L. temulentum* was less sensitive to fructose 2,6 bisphosphate, but this was not the case (Collis and Pollock, 1991), suggesting that the inhibition observed in vitro is over-ridden in vivo, possibly via elevated triose phosphate concentrations. This ‘isolation’ of

Table 6. Contrasting effects of sucrose accumulation on starch synthesis and F2,6BP accumulation in leaves of spinach and *Lolium temulentum* (from Pollock et al., 1995)

Plant	Leaf sucrose ($\mu\text{mol mg}^{-1}$ chl)	F2,6 BP (nmol mg^{-1} chl)	Starch/sucrose ratio
Spinach	1	0.1	0.2
	5	0.2	0.4
	10	0.3	0.6
Lolium	5	0.1	0.1
	33	0.3	0.1
	50	0.3	0.1

chloroplast metabolism from large changes in cytosolic sucrose concentrations appears to be a significant element of the fructan syndrome in Gramineae and may be part of its selective advantage. Many grasses evolved in an environment where, through shading; a perennial growth habit or herbivory, there would be large and rapid changes in the balance between supply of and demand for fixed carbon. These would result in large changes in the fluxes through the competing pathways of primary carbon metabolism.

There is a further aspect of fine control of fructan metabolism which must be distinctive, although the evidence for its occurrence is circumstantial. The stoichiometry of fructan biosynthesis (via the accepted model of direct fructosyl transfer from sucrose) liberates one mole of glucose for every mole of fructose which is transferred. However, radiotracer experiments demonstrate unequivocally that almost all the radioactivity present in sucrose after feeding $^{14}\text{CO}_2$ is eventually accumulated in fructan (Pollock, 1979). Recycling of glucose must, therefore, occur, presumably via hexokinase and sucrose phosphate synthase. Such a recycling pathway would have two effects. Firstly, elevated flux through hexokinase could invoke the signaling responses associated with down-regulation of gene expression (Jang and Sheen, 1996) and secondly, the flux through sucrose phosphate synthase would increase dramatically in relation to that through cytoplasmic fructose 1,6 bisphosphatase (Collis and Pollock, 1992). Direct measurements of enhanced flux or of the activation state of sucrose phosphate synthase (Huber et al., 1995) have not yet been made under these conditions. There have been suggestions that there may be direct transfer of fructose residues from UDP fructose to the growing fructan chain (Pontis, 1995). If this

suggestion is substantiated, then it would reduce the flux through the pathways discussed above.

D. Compartmentation of Sucrose and Fructan Metabolism

1. Intracellular Compartmentation

Sachs (1894) was the first researcher to propose a role for the vacuole in the storage of carbohydrates. By using ethanol to precipitate fructans, he observed the resulting sphaerocrystals in the vacuoles of members of the Asterales. For many years after that, only indirect evidence was available to support the hypothesis, but the ability to prepare isolated vacuoles permitted a direct examination of distribution (Pollock and Kingston-Smith, 1997). Measurements on enzymatically-released protoplasts and vacuoles indicate unequivocally that both the putative enzymes of fructan metabolism and the substrates and products can be found in vacuoles (Table 7). Concerns still exist over the disparity between the catalytic constants of putative enzymes measured *in vitro* and the apparent sucrose concentrations within vacuoles, but there seems little doubt that the vacuole is the major site of storage (Pollock and Kingston-Smith, 1997). It has been proposed, however, that fructan synthesis (as opposed to storage) may occur in small vesicles which subsequently fuse with the vacuole (Kaeser, 1983). Final resolution of these disparities will depend upon the purification of all the enzymes involved and the use of specific antibodies to localize these within the cell.

2. Intercellular Compartmentation

Jellings and Leech (1982) estimated that photosynthetic mesophyll cells make up only 55% of the cell population in cereal leaves, whereas they would make up in excess of 90% of the cells used to prepare isolated vacuoles in the experiments described above. Histochemical analysis of starch in barley leaves has already demonstrated discontinuities in the distribution between mesophyll cells and the photosynthetic cells of the parenchymatous bundle sheath (Williams et al., 1989) and there is no a priori reason for discounting such discontinuities in the metabolism of sucrose. Histochemical localization of acid invertase has demonstrated high concentrations near the vasculature. (Kingston-Smith and Pollock, 1996).

Table 7. Sub-cellular distribution of enzymes and carbohydrates involved in fructan metabolism in leaves of barley

Metabolite/enzyme activity	Percentage in vacuoles from illuminated excised barley leaves
Fructose	107
Glucose	109
Sucrose	65
Fructan (DP3)	87
Fructan (DP>3)	86
SST	92
FFT	nd
Invertase	81
FEH	94 ^a

Vacuolar distribution was estimated using α -methylmannosidase as a vacuolar marker and comparing activities in isolated protoplasts and the purified vacuoles liberated from such protoplasts. nd: not determined. ^a protoplasts prepared from leaves of whole seedlings undergoing fructan turnover. From Wagner et al 1983; Wagner and Wiemken, 1986.

Using the techniques of single-cell sampling (Tomos et al., 1992) increases in fructan metabolism in mesophyll and bundle sheath have been shown to be clearly linked, with vacuolar invertase activity almost undetectable, leading to the conclusion that sucrose hydrolysis in leaves of temperate Gramineae is spatially separated from fructan biosynthesis (Koroleva et al., 1997). Once again, histochemical location of relevant proteins and mRNA species will help to resolve the magnitude of gradients in primary carbon metabolism within leaves, but any tissue-level compartmentation has very significant consequences for models of regulation based upon measurements of whole tissue extracts.

V. Fructan Biosynthesis in Transgenic Plants: A Physiological Perspective

Progress with the identification, purification, sequencing and cloning of plant enzymes with fructosyltransferase activity has been achieved only in the last two years, presumably due to the equivocal and ambiguous nature of much of the earlier enzymological data (Pollock and Cairns, 1991; Cairns, 1993). Hence, work with transgenics has largely employed the available, well characterized levansucrases from bacteria, mainly the Sac B gene from *Bacillus* spp and levansucrase from *Erwinia amylovora* (Röber et al., 1996). These levansucrases synthesize very large fructan (M_r c. 10^5 – 10^6 D, termed

levan) from sucrose as sole precursor. Stable transformation of fructan non-accumulators such as potato, maize and tobacco has been reported and significant accumulation of levan demonstrated, ranging from 2–19% of tissue mass (Table 8). The stated rationales for the production of such plants are: a) Experimental: to produce plants with altered source-sink balance for the experimental investigation of carbon partitioning (Röber et al., 1996). b) Industrial: to provide sources of fructan qualitatively and quantitatively improved by comparison with that from 'natural' sources (Ebskamp et al., 1994). c) Aimed at crop improvement: to improve drought tolerance, since enhanced fructan content is believed by some to mediate stress tolerance in endogenous accumulators (Pilon-Smits et al., 1995a,b).

The Sac B transformants provide a number of interesting enigmas for the physiologist. The fructan accumulated in Sac B transformants is cited as evidence for major alterations in reserve carbon partitioning. While the transformants clearly accumulate large amounts of levan of high Mr, we question the significance of this accumulation in terms of alterations to instantaneous carbon flux and partitioning; The fructan concentrations are cited as end-point values, without consideration of the accumulation period or the accumulation rate. Without such observations, comparisons with the magnitude of endogenous carbon fluxes cannot be made. From the few studies which do cite accumulation period, net rates of formation can be estimated and compared with rates of fructan synthesis in the leaves of an endogenous accumulator (*L. temulentum*) or with starch accumulation in sinks of untransformed starch accumulators (developing pea and maize seed). Such an analysis is summarized in Table 8.

The transformants accumulate fructan at rates which are markedly lower than those observed in the natural fructan accumulator. The expressed rates of net levansucrase activity are correspondingly small when compared to the magnitude of the fluxes through endogenous pathways of primary carbon metabolism in untransformed starch accumulators, as indicated by natural starch accumulation rates. These metabolic comparisons show that the proportion of current photosynthate flux directed into levan is very small in the Sac B transformants. The accumulation is, therefore, a result of minor leakage of carbon into fructan over long periods into a chemical compartment which cannot be remobilized by the plant.

Table 8. Comparison of rates of reserve polysaccharide accumulation in Sac B transformants, an endogenous fructan accumulator and untransformed, endogenous starch accumulators

Species/ Tissue/ transformant/ treatment	Polysaccharide	Accumulated polymer (mg g ⁻¹ fr mass)	Accumulation period (d)	Accumulation rate ¹ (nkat g ⁻¹ fresh mass)	Reference
Maize, kernel cytoplasm	Levan (Sac B)	7.5	55	0.010	Caimi et al., 1996
Maize, kernel vacuole	Levan (Sac B)	18	30	0.043	
Potato leaves: KP7	Levan (Sac B)	4.5	49	0.007	Pilon-Smits et al., 1996
Potato leaves: KP17	Levan (Sac B)	4.7	49	0.007	
Tobacco KP; drought control	Levan (Sac B)	0.02	11	0.0001	Pilon-Smits et al., 1995a
Tobacco KP -0.4kPa	Levan (Sac B)	0.05	8	0.0005	
Tobacco KP -0.8kPa	Levan (Sac B)	0.275	11	0.0018	
<i>L.temulentum</i> leaf	Fructan (endogenous)	20	1	1.4	Cairns and Pollock, 1988a
Maize, kernel	Starch (Endogenous)	319	28	0.8	Ozbun et al., 1973
Pea seed	Starch (endogenous)	167	18	0.7	Milthorpe and Moorby 1974

¹ Calculated as anhydrohexose Mr = 162.

The apparent alterations in reserve metabolism are also of interest in the context of the conditions of plant culture used. Some transformants have been grown at irradiances reported at 3000 lux (van der Meer et al., 1994), equivalent to less than 40 μE m⁻² s⁻¹ PAR: (converted according to McCree, 1972) and 42 μE m⁻² s⁻¹ (Pilon-Smits et al., 1995a). These values are low and close to the light compensation point for C3 plants (Milthorpe and Moorby, 1974). At these irradiance values it is, perhaps, surprising that the plants were even in positive carbon balance and involved in reserve synthesis. The rationale for the use of low irradiance was not reported. However, Sac B transformants of tobacco produced deleterious necrotic lesions associated with high carbohydrate status when grown at higher irradiance. (A. Snow and R. J. Simpson, personal communication). They may explain the choice of lower PPFD in the other experiments.

The transformants are consistently reported as *not* containing Sac B mRNA detectable by hybridization, the enzyme protein cannot be detected immuno-

ologically and the enzyme activities are at the limit of detection (Ebskamp et al. 1994; van der Meer et al., 1994; Pilon Smits et al., 1995). Sensitive ¹⁴C enzyme assays are required to detect levan formation in vitro (Ebskamp et al., 1994; van der Meer et al., 1994; Röber et al., 1996), whereas, for natural accumulators, fructan production in vitro is readily detectable without the use of radioisotope (e.g. in Figs. 2 and 3). These observations are in apparent contradiction to the large tissue concentrations of levan reported, but can be rationalized in the context of the low expression of levansucrase in the transformants demonstrated in Table 8.

The properties of the levansucrase enzymes, in the context of the physiology of the plant, have also been neglected in discussion of the transformed plants. For the bacterial enzymes, the Km for sucrose is between 20–60 mM (Han, 1990) and is 20 mM for the Sac B gene product (Dedonder, 1966). This is of particular physiological interest. In only a few instances have transformant tissue sucrose concentration and dry weight:fresh weight ratio been reported

(Ebskamp et al., 1994; Pilon Smits et al., 1995). This allows estimation of mean tissue, and hence vacuolar, sucrose concentration. The value in each case was less than 3 mM so the enzyme would be functioning at less than 7% of Vmax. This would, of course, contribute to the very low rates of expression observed and make accumulation rate extremely sensitive to changes in tissue sucrose concentration.

In relation to drought stress, Sac B transformants of tobacco contained increased levan concentration when subjected to PEG-induced osmotic stress (Pilon-Smits et al., 1995a). Fructan accumulation was correlated with improved drought tolerance (as measured by dry weight increment), despite being under the control of the constitutive (i.e. stress-insensitive) e35S CaMV promotor (Ebskamp et al., 1994). It is interesting that sucrose accumulation in the stressed transformants also increased by 13-fold relative to the (-)drought control. This corresponds to an estimated change of 0.2–2.8 mM sucrose in the tissue sap. The resulting theoretical substrate-dependent stimulation of the Sac B enzyme of ca. 16-fold could alone explain the difference in levan content between control and droughted plants. This leaves open the question of whether the increase in levan accumulation was a direct or indirect consequence of the stress treatment, a debate which is equally valid for natural fructan accumulators. There is currently no convincing evidence that fructan accumulation in response to low-temperature or drought stress directly mediates stress tolerance in natural fructan accumulators (Pollock, Eagles and Sims, 1988; Pollock and Cairns, 1991). In addition, the drought experiments were performed at $42 \mu\text{E m}^{-2} \text{s}^{-1}$ PPFD (discussed above), which further complicates the interpretation of these results.

In summary, the data from the bacterial transformants provide an exciting and interesting new departure for studies of assimilate partitioning, though some of the conclusions benefit from closer re-examination from a physiological perspective.

VI. Concluding Remarks

Natural fructan accumulators are unique in the sense that they possess a mechanism for reserve carbon allocation which differs grossly in its enzymology, compartmentation and regulation, from 90% of higher plant species. Endogenous fructan metabolism represents a distinctive extension of sucrose

metabolism, an understanding of which also serves to illuminate factors governing primary carbohydrate metabolism in other, non-fructan, systems. However, much of the evidence to support the major hypotheses for its metabolism and regulation remains circumstantial, since purified proteins and clones for higher plant fructan genes have only recently become available.

There have been attempts to modify the reserve carbohydrate metabolism of other species by introducing both bacterial and higher plant genes associated with fructan metabolism and these experiments have generated plants with altered patterns of carbon partitioning, though more rigorous physiological examination will be required to substantiate some of the claims made on the basis of the available data. In terms of physiology, the current value of such transgenics is in terms of what they may tell us about the regulation of primary carbon metabolism in the *recipient* plants. Fructans are, in this case, only an alternative sink for carbon which may compete with existing metabolic pathways. In our view, transgenics currently provide little insight into the nature or control of endogenous fructan metabolism.

What is needed for the study of the endogenous metabolism is fructan-accumulating plants which have an altered capacity to make fructans. We propose that such plants would be of the highest scientific value if grass leaf metabolism was altered. The grass system has a number of advantages for such work: (a) the primary flux rates into sucrose and fructan are highest in these tissues, (b) their detailed physiology is extensively characterized, (c) bacterial levan is not hydrolyzed by grass FEH and would accumulate as a terminal sink, (d) the tissue has evolved to cope with fructan accumulation without deleterious effects, and (e) there is evidence from mutants and from inhibitor studies, that loss of endogenous fructan synthesis and the resultant accumulation of sucrose, are not immediately deleterious. Now that transformation of temperate Gramineae is routine, such an approach is feasible, as is the screening of species for mutations which alter fructan synthesis. The integration of current studies on enzymology and tissue compartmentation with the availability of such material will, we believe, provide the next major advance in the study of the regulation of fructan metabolism.

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References

- Austin RB, Edrich JA, Ford MA and Blackwell RD (1977) The fate of the dry matter, carbohydrates and ^{14}C lost from the leaves and stems of wheat during grain filling. Ann Bot 41: 1309–1321
- Bancal P and Tribol E (1993) Temperature effect on fructan oligomer contents and fructan-related enzyme activities in stems of wheat (*Triticum aestivum* L.) during grain filling. New Phytol 123: 247–253
- Bonnett GD and Incoll LD (1992) The potential pre-anthesis and post-anthesis contributions of stem interposed to grain yield in crops of winter barley. Ann Bot 69: 219–225
- Bonnett GD and Simpson RJ (1993) Fructan hydrolase activities from *Lolium rigidum* Gaudin. New Phytol 123: 443–451
- Bonnett GD and Simpson RJ (1995) Fructan exohydrolase activities from *Lolium Rigidum* that hydrolyze beta-2,1-glycosidic and beta-2, 6-glycosidic linkages at different rates. New Phytol 131: 199–209
- Caimi PG, McCole LM, Klein TM and Kerr PS (1996) Fructan accumulation and sucrose metabolism in transgenic maize endosperm expressing a *Bacillus amyloliquefaciens* *sacB* gene. Plant Physiol 110: 355–363
- Cairns AJ (1992a) A reconsideration of fructan biosynthesis in storage roots of *Asparagus officinalis* L. New Phytol 120: 463–473
- Cairns AJ (1992b) Fructan biosynthesis in excised leaves of *Lolium temulentum* L. V. Enzymatic de novo synthesis of large fructans from sucrose. New Phytol 122: 253–259
- Cairns AJ (1993) Evidence for the de novo synthesis of fructan by enzymes from higher plants: A reappraisal of the SST/FFT model. New Phytol 123: 15–24
- Cairns AJ (1995) Effects of enzyme concentration on oligofructan synthesis from sucrose. Phytochemistry 40: 705–708
- Cairns AJ and Ashton JE (1993) Species-dependent patterns of fructan synthesis by enzymes from excised leaves of oat, wheat, barley and timothy. New Phytol 124: 381–388
- Cairns AJ and Ashton JE (1994) Fructan biosynthesis in excised leaves of *Lolium temulentum* L. VI Optimisation and stability of enzymatic fructan synthesis. New Phytol 126: 3–10
- Cairns AJ and Pollock CJ (1988a) Fructan biosynthesis in excised leaves of *Lolium temulentum* L. I. Chromatographic characterisation of oligofructans and their labelling patterns following $^{14}\text{CO}_2$ feeding. New Phytol 109: 399–405
- Cairns AJ and Pollock CJ (1988b) Fructan biosynthesis in excised leaves of *Lolium temulentum* L. II. Changes in fructosyl transferase activity following excision and application of inhibitors of gene expression. New Phytol 109: 407–413
- Cairns AJ, Winters AL and Pollock CJ (1989) Fructan biosynthesis in excised leaves of *Lolium temulentum* L. III A comparison of the in vitro properties of fructosyl transferase activities with the characteristics of in vivo fructan accumulation. New Phytol 112: 343–352
- Cairns AJ, Bonnett GD, Gallagher JA, Simpson RJ and Pollock CJ (1997) Fructan biosynthesis in excised leaves of *Lolium temulentum* VII Sucrose and fructan hydrolysis by a fructan-polymerising enzyme preparation. New Phytol 136: 61–72
- Cairns AJ, Nash R, Machado de Carvalho MA and Sims IM (1999) Characterisation of the enzymatic polymerisation of 2,6-linked fructan by leaf extracts from timothy grass (*Phleum pratense*). New Phytol 142: 79–91
- Chatterton NJ, Harrison PA, Thornley WR and Bennett JH (1993) Structures of fructan oligomers in orchard grass (*Dactylis glomerata* L.) J Plant Physiol 142: 552–556
- Collis BE and Pollock CJ (1991) The control of sucrose synthesis in leaves of *Lolium temulentum* L., a fructan-accumulating grass. New Phytol 119: 483–489
- Collis BE and Pollock CJ (1992) Cytoplasmic carbohydrate metabolism in leaf tissues undergoing fructan synthesis and breakdown. J Plant Physiol 140: 124–126
- Dedonder R (1966) Levansucrase from *Bacillus subtilis*. Meths Enzymol 8: 500–505
- Dey PM (1980) Biochemistry of α -D-galactosidic linkages in the plant kingdom. Adv Carbohyd Chem Biochem 37: 283–372
- Dubois D, Winzeler M and Nösberger J (1990) Fructan accumulation and sucrose:sucrose fructosyl transferase activity in stems of spring wheat genotypes. Crop Sci 30: 315–319
- Duchateau N, Borthik K, Simmen U, Wiemken A and Bancal P (1995) Sucrose-fructan 6-fructosyl transferase: A key enzyme for diverting carbon from sucrose to fructan in barley leaves. Plant Physiol 107: 1249–1255
- Ebskamp MJM, van der Meer IM, Spronk BA, Weisbeek PJ and Smeekens SCM (1994) Accumulation of fructose polymers in transgenic tobacco. Bio/Technology 12: 272–275
- Edelman J and Dickerson AG (1966) The metabolism of fructose polymers in plants. Transfructosylation in tubers of *Helianthus tuberosus* L. Biochem J 98: 787–794
- Edelman J and Jefford TG (1968) The mechanism of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus*. New Phytol 67: 517–531
- Ernst M, Chatterton NJ and Harrison PA (1996) Purification and characterisation of a new fructan series from species of Asteraceae. New Phytol 132: 63–66
- Escalada JA and Moss DN (1976) Changes in the non-structural carbohydrate fractions of developing spring wheat kernels. Crop Sci 16: 627–631
- Frehner M, Keller F and Wiemken A (1984) Localisation of fructan metabolism in vacuoles isolated from protoplasts of Jerusalem artichoke tubers. J Plant Physiol 116: 197–208
- Fuchs A (1993a) Production and utilisation of inulin. Part I. Utilisation of inulin. In: Suzuki M and Chatterton NJ (eds) Science and Technology of Fructans, pp 320–352. CRC Press, Boca Raton
- Fuchs A (ed) (1993b) Inulin and Inulin-Containing Crops. Elsevier, Amsterdam
- Han Y (1990) Microbial Levan. Adv Appl Microbiol 35: 171–194
- Hendrix JE (1983) Phloem function: An integrated view. What's New in Plant Physiology, Physiol Plant 14: 45–48
- Hendry GAF and Wallace RK (1993) The origin, distribution and evolutionary significance of fructans. In: Suzuki M and Chatterton NJ (eds). Science and Technology of Fructans, pp 119–139. CRC Press, Boca Raton

- Henson CA and Livingston DP (1996) Purification and characterization of an oat fructan exohydrolase that preferentially hydrolyzes beta-2,6-fructans. *Plant Physiol* 110: 639–644
- Housley TL and Daughtry CST (1987) Fructan content and fructosyl transferase activity during wheat seed growth. *Plant Physiol* 83: 4–7
- Housley TL and Pollock CJ (1985) Photosynthesis and carbohydrate metabolism in detached leaves of *Lolium temulentum* L. *New Phytol* 99: 499–502
- Housley TL and Pollock CJ (1993) The metabolism of fructan in higher plants. In: Suzuki M and Chatterton NJ (eds) *Science and Technology of Fructan*, pp 191–225. CRC Press, Boca Raton
- Huber SC, Bachmann M, McMichael RW and Huber JC (1995) Regulation of sucrose phosphate synthase by reversible protein phosphorylation: Manipulation of activation and inactivation in vivo. In: Pontis H, Salerno GL and Echeverria EJ (eds) *Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology*, pp 6–13. American Society of Plant Physiologists, Rockville
- Jang JC and Sheen J (1994) Sugar sensing in higher plants. *Plant Cell* 6: 1665–1679
- Jellings AJ and Leach RM (1982) The importance of quantitative anatomy in the interpretation of whole leaf biochemistry in species of triticum, hordeum and avena. *New Phytol* 92:39–48
- Kaeser W (1983) Ultrastructure of storage cells in Jerusalem artichoke tubers (*Helianthus tuberosus* L.). Vesicle formation during inulin synthesis. *Zeit Pflanz* 111: 253–260
- Koops AJ and Jonker HH (1996) Purification and characterisation of the enzymes of fructan biosynthesis in tubers of *Helianthus tuberosus* (Colombia). 2. Purification of sucrose-sucrose 1-fructosyl transferase and reconstitution of fructan synthesis in vitro with purified sucrose-sucrose 1-fructosyl transferase and fructan-fructan 1-fructosyl transferase. *Plant Physiol* 110: 1167–1175
- Koroleva OA, Farrar JF, Tomos, AD and Pollock CJ (1997) Patterns of solute in individual mesophyll, bundle sheath and epidermal cells of barley leaves induced to accumulate carbohydrate. *New Phytol* 136: 97–104
- Kingston-Smith AH and Pollock CJ (1996) Tissue level localisation of acid invertase in leaves: An hypothesis for the regulation of carbon export. *New Phytol* 134: 423–432
- Kühbauch W and Thome U (1989) Nonstructural carbohydrates of wheat stems as influenced by source-sink manipulations. *J Plant Physiol* 134: 243–250
- Lewis DH (1993) Nomenclature and diagrammatic representation of oligomeric fructans: A paper for discussion. *New Phytol* 123: 583–594
- Lüscher M, Erdin C, Sprenger N, Hochstrasser U, Boller T and Wiemken A (1996) Inulin synthesis by a combination of purified fructosyl transferases from tubers of *Helianthus tuberosus*. *FEBS Lett* 385: 39–42
- MacLeod AM and McCrorquodale H (1958) Water-soluble carbohydrates of seeds of the Gramineae. *New Phytol* 57: 168–182
- Marx SP, Nosberger J and Frehner M (1997) Hydrolysis of fructan in grasses: A beta-(2-6)-linkage specific fructan-beta-fructosidase from stubble of *Lolium perenne*. *New Phytol* 135: 279–290
- McCree KJ (1972) Test of current definitions of photosynthetically active radiation against leaf photosynthetic data. *Agric Meteorol* 10: 443–453
- Milthorpe FL and Moorby J (1974) *An Introduction to Crop Physiology*. Cambridge University Press, Cambridge
- Natr L (1969) Influence of assimilate accumulation on rate of photosynthesis of barley leaf segments. *Photosynthetica* 3: 120–126
- Obenland DM, Simmen U, Boller T and Wienken A (1991) Regulation of sucrose-sucrose fructosyl transferase in barley leaves. *Plant Physiol* 97: 811–813
- Ozbun JL, Hawker JS, Greenberg E, Lammel C and Preiss J (1973) Starch synthetase, phosphorylase, ADPglucose pyrophosphorylase and UDPglucose pyrophosphorylase in developing maize kernels. *Plant Physiol* 51: 1–5
- Pearman I, Thomas SM and Thome GN (1978) Effects of nitrogen fertiliser on the distribution of photosynthate during grain growth of spring wheat. *Ann Bot* 42: 91–99
- Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeukens MJW, Weisbeek PJ and Smeekens SCM (1995a) Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol* 107: 125–130
- Pilon-Smits EAH, Ebskamp MJ, Weisbeek PJ and Smeekens SCM (1995b) Frucan-accumulation in transgenic plants: Effect on growth, carbohydrate partitioning and stress resistance. In: Pontis HG, Salerno GO and Echeverria EJ (eds) *Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology*, pp 88–99. American Society of Plant Physiologists, Rockville
- Pilon-Smits EAH, Ebskamp MJM, Jeukens MJW, van der Meer IM, Visser RGF, Weisbeek PJ and Smeekens SCM (1996) Microbial fructan production in transgenic potato plants and tubers. *Indust Crops Prod* 5: 35–46
- Pollock CJ (1979) Pathway of fructan synthesis in leaf bases of *Dactylis glomerata*. *Phytochemistry* 18: 777–779
- Pollock CJ (1982) Patterns of turnover of fructans in leaves of *Dactylis glomerata* L. *New Phytol* 90: 645–650
- Pollock CJ and Cairns AJ (1991) Fructan metabolism in grasses and cereals. *Ann Rev Plant Physiol Plant Mol Biol* 42:77–101
- Pollock CJ and Jones T (1979) Seasonal patterns of fructan metabolism in forage grasses. *New Phytol* 83: 8–15
- Pollock CJ and Kingston-Smith AH (1997) The vacuole and carbohydrate metabolism. In: Leigh RA and Sanders D (eds) *Advances in Botanical Research* 25, pp 195–215. Academic Press, London
- Pollock CJ, Hall MA and Roberts DP (1979) Structural analysis of fructose polymers by gas-liquid chromatography and gel filtration. *J Chromatogr* 171: 411–415
- Pollock CJ, Eagles CE and Sims IM (1988) Effect of photoperiod and irradiance changes upon development of freezing tolerance and accumulation of soluble carbohydrate in seedlings of *Lolium perenne* grown at 2 °C. *Ann Bot* 62: 95–100
- Pollock CJ, Cairns AJ, Gallagher JA, Winters AL and Farrar J (1995) Cold affects partitioning. Does partitioning affect photosynthesis? In: Mathis P (ed) *Photosynthesis: From Light to Biosphere*, Vol IV, pp 783–788. Kluwer Academic Publishers, Dordrecht
- Pollock CJ, Cairns AJ, Sims IM and Housley TL (1996) Fructans as Reserve Carbohydrates in Crop Plants. In: Zamski E and Shaffer AA (eds) *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships*, pp 97–113. Marcel Dekker Inc, New York
- Pontis H (1995) A discussion on the present model of fructan

- biosynthesis. In: Pontis H, Salerno GL and Echeverria EJ (eds) Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology, pp 190–197. American Society of Plant Physiologists, Rockville
- Röber M, Geider K, Muller-Röber B and Willmitzer L (1996) Synthesis of fructans in tubers of transgenic starch-deficient potato plants does not result in an increased allocation of carbohydrates. *Planta* 199: 528–536
- Sachs J (1864) Über die Spharokristalle des Inulins und den mikroskopische Nachweisung in den Zellen. *Botanische Zeitung* 22: 77–81; 85–89
- Schnyder H (1986) Carbohydrate metabolism in the growth zone of tall fescue leaf blades. In: Randall DD, Miles CD, Nelson CJ, Blevins DG and Miernyk JA (eds) Current Topics in Plant Biochemistry and Physiology, pp. 47–58. University of Missouri, Columbia
- Schnyder H (1993) The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling—a review. *New Phytol* 123: 233–245
- Schnyder H and Nelson CJ (1987) Growth rates and carbohydrate fluxes within the elongation zone of tall fescue leaf blades at high and low irradiance. *Plant Physiol* 85: 548–553
- Schnyder H and Nelson CJ (1989) Growth rates and assimilate partitioning in the elongation zone of tall fescue leaf blades at high and low irradiance. *Plant Physiol* 90: 1201–1206
- Schnyder H, Nelson CJ and Spollen WG (1988) Diurnal growth of tall fescue leaf blades. II. Dry matter partitioning and carbohydrate metabolism in the elongation zone and adjacent expanded tissue. *Plant Physiol* 86: 1077–1083
- Silk WK. (1984) Quantitative descriptions of development. *Ann Rev Plant Physiol* 35: 479–518
- Simmen U, Obenland D, Boller T and Wiemken A (1993) Fructan synthesis in excised barley leaves. Identification of two sucrose-sucrose fructosyl transferases induced by light and their separation from constitutive invertases. *Plant Physiol* 101: 459–468
- Simpson RJ and Bonnett GD (1993) Fructan exohydrolase from grasses. *New Phytol* 123: 453–469
- Simpson RJ, Walker RP and Pollock CJ (1991) Fructan exohydrolase in leaves of *Lolium temulentum* L. *New Phytol* 119: 499–507
- Sims IM, Pollock CJ and Horgan R (1992) Structural analysis of oligomeric fructans from excised leaves of *Lolium temulentum*. *Phytochemistry* 31: 2989–2992
- Sims IM, Horgan R and Pollock CJ (1993) The kinetic analysis of fructan biosynthesis in excised leaves of *Lolium temulentum* L. *New Phytol* 123: 25–29
- Slaughter LH and Livingston DP (1994) Separation of fructan isomers by high-performance anion-exchange chromatography. *Carbohydr Res* 253: 287–291
- Sprenger N, Bortlik K, Brandt A, Boller T and Wiemken A (1995) Purification, cloning and functional expression of sucrose-fructan 6-transferase, a key enzyme of fructan synthesis in barley. *Proc Nat Acad Sci USA* 92: 11652–11656
- Stitt M (1996) Metabolic regulation of photosynthesis. In: Baker NR (ed) Photosynthesis and the Environment, pp 151–190.
- Kluwer Academic Publishers, Dordrecht
- St John JA, Bonnett GD, Simpson RJ and Tanner GJ (1997a) A fructan:fructan fructosyl transferase activity from *Lolium rigidum*. *New Phytol* 135: 235–247
- St John JA, Sims IM, Bonnett GD and Simpson RJ (1997b) The identification of products formed by a fructan:fructan fructosyl transferase activity from *Lolium rigidum*. *New Phytol* 135: 249–257
- Tomos AD, Leigh RA, Palta JA and Williams JHH (1992) Sucrose and cell water relations. In: Pollock CJ, Farrar, JF and Gordon AJ (eds) Carbon Partitioning Within and Between Organisms, pp 71–89. Bios, Oxford
- van den Ende W and van Laere, A (1996) De novo synthesis of fructans from sucrose in vitro by a combination of 2 purified enzymes (sucrose-sucrose 1-fructosyl transferase and fructan-fructan 1-frucosyl transferase from chicory roots (*Cichorium intybus*) L. *Planta* 200: 335–342
- van der Meer IM, Ebskamp MJM, Visser RGF, Weisbeek PJ and Smeekens SCM (1994) Fructan as a new carbohydrate sink in transgenic potato plants. *Plant Cell* 6: 561–570
- Wagner W and Wiemken A (1986) Properties and subcellular localisation of fructan hydrolase in the leaves of barley (*Hordeum vulgare* L. cv. Gerbel). *J Plant Physiol* 123: 429–439
- Wagner W and Wiemken A (1987) Enzymology of fructan synthesis in grasses. Properties of sucrose-sucrose fructosyl transferase in barley leaves (*Hordeum vulgare* L. cv. Gerbel). *J Plant Physiol* 85: 706–710
- Wagner W, Keller F and Wiemken A (1983) Fructan metabolism in cereals: Induction in leaves and compartmentation in protoplasts and vacuoles. *Zeit Pflanz* 112: 359–372
- Wagner W, Wiemken A and Matile PH (1986) Regulation of fructan metabolism in leaves of barley (*Hordeum vulgare* L. cv. Gerbel). *J Plant Physiol* 81: 444–447
- Wiemken A, Sprenger N and Boller T (1995) Fructan—an extension of sucrose by sucrose. In: Pontis HG, Salerno GL and Echeverria E.J (eds) Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology, pp 179–189. American Society of Plant Physiologists, Rockville
- Williams ML, Farrar JF and Pollock CJ (1989) Cell specialisation within the parenchymatous bundle sheath of barley. *Plant Cell Env* 12: 909–918
- Winter H, Robinson DG and Heldt HW (1993) Subcellular volumes and metabolite concentrations in barley leaves. *Planta* 191: 180–190
- Winters AL, Williams JHH, Thomas DS and Pollock CJ (1994) Changes in gene expression in response to sucrose accumulation in leaf tissue of *Lolium temulentum* L. *New Phytol* 128: 591–600
- Winters AL, Gallagher JA, Pollock CJ and Farrar JF (1995) Isolation of a gene expressed during sucrose accumulation in leaves of *Lolium temulentum* L. *J Exp Bot* 46: 1345–1350
- Yamamoto S and Mino Y (1989) Mechanism of phleinase induction in the stem base of orchard grass after defoliation. *J Plant Physiol* 134: 258–260

Chapter 14

Acquisition and Diffusion of CO₂ in Higher Plant Leaves

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Summary

Acquisition of CO_2 by higher plants involves CO_2 diffusion from the air into leaves and, ultimately, into chloroplasts. There, fixation of CO_2 into organic compounds creates the concentration gradient which drives CO_2 diffusion. CO_2 encounters many obstructions along its diffusion path toward chloroplasts. The diffusion resistances attributable to boundary layer and stomata are shared with the opposing flux of water leaving the leaf. Once in the substomatal cavities, however, CO_2 faces additional resistances since it has to cross walls and membranes to reach the chloroplasts. We follow the CO_2 molecule from the air through the boundary layer, stomata and, finally, the mesophyll. After providing diverse anatomical examples at each level, we review the current understanding about the subtle balance which plants maintain between water loss and CO_2 acquisition.

Stomatal responses to many environmental variables are well known, despite our lack of understanding of the underlying mechanisms. Techniques are available that allow accurate measurements of conductances through the boundary layer and stomata. The estimation of conductance through the mesophyll, however, has not been feasible until the recent development of rapid measurements of isotopic discrimination and chlorophyll fluorescence. We discuss the principles which allow the estimation of internal conductance based on these techniques and the data available. Both stomatal conductance and internal conductance correlate strongly with photosynthetic capacity and are of similar magnitude. However, stomatal conductance can vary within minutes in response to changes in the environment. Internal conductance, on the other hand, seems to be stable over several days and depends on anatomical properties of the leaf. We close considering the special case of C_4 photosynthesis where spatial compartmentation and biochemical mechanisms of CO_2 concentration add complexity to the estimation of internal conductance.

I. Introduction

Photosynthesis requires light, CO_2 and water as substrates. Since water is usually a limited resource, plants control water losses by covering their aerial surfaces with impermeable structures which are perforated by stomata. Barriers to the diffusion of water also block the diffusion of CO_2 . CO_2 and water share the same diffusion pathway from surrounding air to intercellular airspaces in the sub-stomatal cavities. Consequently, any change to restrictions along the shared path will affect both CO_2 and water. Once inside the leaf, CO_2 faces additional diffusion barriers through intercellular airspaces, cell walls

and cell matrix. In general, surrounding air is drier than air within leaves so that there is a net diffusion of water out of leaves. During photosynthesis, consumption of CO_2 inside mesophyll cells creates a partial pressure gradient that results in CO_2 diffusing into the plant. Thus the water and CO_2 fluxes are usually in opposition to one another.

Conventional gas exchange measures fluxes of water and CO_2 into and out of a leaf. The gradient in partial pressure of CO_2 from ambient air, p_a , to the substomatal cavities (usually referred to as the intercellular CO_2 partial pressure, p_i) is derived using Fick's law of diffusion which states that the gradient in partial pressure is equal to the flux divided by the conductance $p_a - p_i = A/g_s$, where A is the rate of CO_2 assimilation and g_s is the stomatal conductance to CO_2 . When considering fluxes, it is convenient to use conductances as these vary in proportion with the flux. However, for a pathway with a series of limitations, it is more convenient to use the reciprocal of conductance, resistance, as the total resistance for a pathway is simply the sum of the individual resistances (although for distributed sinks and mixed pathways, this is not strictly true (Parkhurst, 1994)).

This chapter explores the diffusion pathway from the air surrounding a leaf to the sites of CO_2 fixation within that leaf. Firstly, we consider the unstirred layer of air adjacent to the leaf surface which is

Abbreviations: Δ , Δ_i – carbon isotope discrimination, carbon isotope discrimination predicted from p_i ; Γ , Γ_c – CO_2 compensation point, CO_2 compensation point in the absence of respiration R; ϕ_{PSII} –photochemical efficiency of Photosystem II; A – rate of CO_2 assimilation; E – rate of transpiration; g_b , g_s , g_w – boundary layer conductance, stomatal conductance, internal conductance; J , J_F – rate of electron transport, rate of electron transport calculated from chlorophyll fluorescence; LMA – leaf dry mass per unit area; p_a , p_s , p_i , p_c – partial pressures of CO_2 in the air surrounding the leaf, at the leaf surface, in the substomatal cavities, at the sites of carboxylation, respectively; R – rate of non-photorespiratory CO_2 evolution (rate of respiration); S_c , S_{mes} – surface area of chloroplasts exposed to intercellular airspace per unit leaf area, surface area of mesophyll cells exposed to intercellular airspace per unit leaf area; VPD – leaf to air vapor pressure difference

called the boundary layer. This layer is influenced by wind speed and such things as leaf size and shape as well as smaller scale features such as grooves, crypts and hairs. Secondly, we discuss stomata, the pores in plant aerial surfaces that control resistance to diffusion in response to many environmental signals. After examining stomatal structure and distribution across leaf surfaces of diverse plants, we discuss how they respond to CO₂, leaf to air vapor pressure difference and irradiance. The objective in this chapter is to present the general features rather than provide a comprehensive review of stomata which can be found elsewhere (Farquhar and Sharkey, 1982; Zeiger et al., 1987; Willmer and Fricker, 1996). There is a striking correlation between stomatal conductance and CO₂ assimilation rate that differs between C₃ and C₄ plants. The mechanism behind these relationships is not yet understood, but it results in an empirical equation that can be used to predict stomatal conductance. Gas exchange measurements usually rely on an assumption that the leaf material enclosed in the cuvette behaves uniformly. In some cases this assumption has been shown to be false and we discuss patchy or heterogeneous stomatal closure.

The third section considers internal leaf structure where CO₂ encounters resistances not faced by water. There are several methods available now to determine internal resistance. Since these are relatively recent, we present the methods and their strengths and weaknesses in some detail. We then dissect the problem of internal resistance into its airspace and intracellular components. The fourth section examines C₄ photosynthesis where anatomical and biochemical specialization has enabled more efficient CO₂ acquisition relative to water loss to be achieved.

II. Boundary Layer—CO₂ Diffusion to the Leaf Surface

Adjacent to any surface is a layer of still air called the boundary layer. When air moves past a leaf, air nearest the leaf surface shears in parallel leading to laminar flow near the leading edge. As one moves away from the leading edge or away from the leaf surface, air movement eventually becomes turbulent. It turns out that the average thickness of this layer, t_b , can be approximated by $t_b = 0.004\sqrt{w/u}$ (m) where w (m) is the width of the leaf in the direction of the air movement and u (m s⁻¹) is the wind speed (Nobel, 1991). For example, the boundary layer thickness of

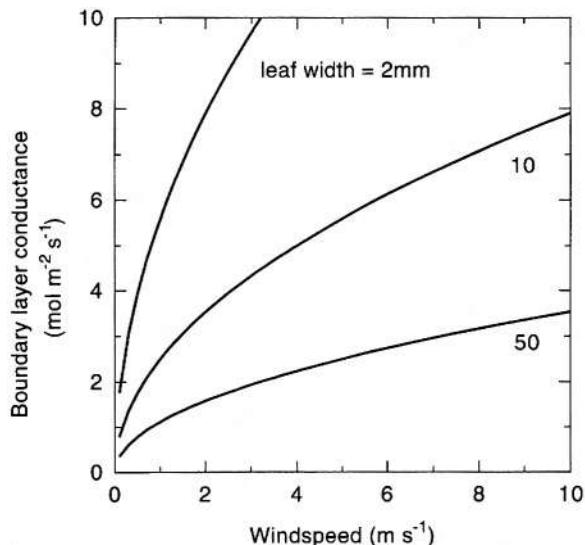


Fig. 1. Boundary layer conductance to water as a function of windspeed and leaf width at 25 °C. $g_b = 40D / (0.004\sqrt{w/u}) \text{ mol m}^{-2} \text{s}^{-1}$ where D is the diffusivity of water vapour in air at 25 °C ($2.5 \times 10^{-5} \text{ m}^2 \text{s}^{-1}$), w is the leaf width (m) and u is the windspeed (m s⁻¹) (Nobel, 1991).

a tobacco leaf 150 mm wide in a 2 m s⁻¹ wind would be 1 mm whereas for a wheat leaf 20 mm wide it would be only 0.4 mm thick. Boundary layer conductance, g_b , is then calculated as the ratio of the diffusivity of the molecule in air and boundary layer thickness. Boundary layer conductance is therefore a function of windspeed which depends on leaf width (Fig. 1). Narrow or dissected leaves have higher boundary layer conductances than broad leaves.

Boundary layer conductance can also be influenced by surface structures on the leaf and positioning of stomata. It is common to find stomata in positions away from the exposed leaf surface. They may be at the base of grooves or pits, or the leaf could be rolled. A dramatic illustration of this is seen with the desert dwelling C₄ grass, spinifex (*Triodia irritans*, Fig. 2A,B). The leaf is rolled up so that it becomes nearly circular in cross section. On both the exposed and enclosed surfaces run parallel grooves about 100 µm deep which are lined with hairs. Stomata are only found at the base of these grooves. The grooves are aligned across the leaf, separated by a cluster of large cells which by swelling or shrinking, can open and close the gap in the grooves. If stomata in the grooves around the outer surface were closed, CO₂ and water molecules would be required to travel an additional 600 µm along the cleft.

Hairs are commonly found on leaf surfaces and

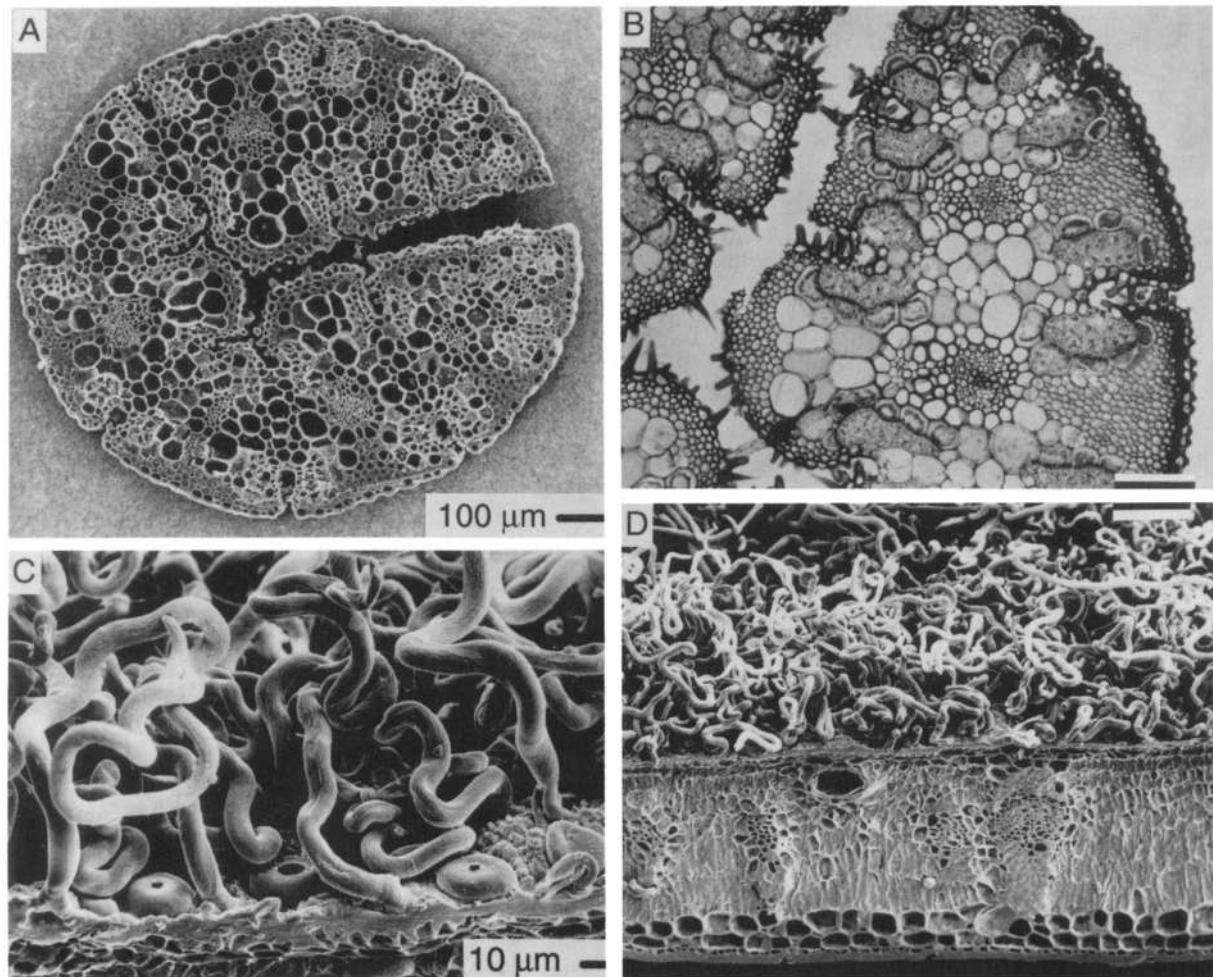


Fig. 2. Surface structures restricting CO_2 diffusion. The spinifex leaf (*Triodia irritans*) (A, Craig and Goodchild, 1977; B, McWilliam and Mison, 1974) is rolled with hair-lined grooves that have stomata at their base. The large cells between pairs of grooves allow the groove aperture to widen or close with changes in turgor. The New Zealand Christmas tree (*Metrosideros thomasi*) (C,D, S Craig) has a dense layer of twisted hairs covering the lower (abaxial) surface which has stomata. The upper surface (panel D, leaf shown upside down) has no stomata, a thick cuticle and a double layer of cells beneath the epidermis that contains no chloroplasts.

range considerably in density, length and shape. They play a clear role in increasing leaf reflectance during the summer in arid areas (Ehleringer and Björkman, 1978) or during leaf expansion (Ntefidou and Manetas, 1996). Hairs are also important for repelling water from the leaf surface during rain or dewfall (Brewer et al., 1991, Brewer and Smith, 1997). Since CO_2 diffusion through water is 10,000 times slower than through air, thin films of water on a leaf surface would effectively stop CO_2 assimilation. Hairs cause droplet formation on the leaf surface rather than a continuous film and can also raise the drops away from the leaf surface to further prevent

occlusion of stomata. In dry conditions, the hairs trap air and increase the thickness of the boundary layer. Some hypostomatous leaves (those with stomata on only one surface) have a dense trichome layer above the surface with stomata and a smooth thick cuticle on the other surface which is generally the upper (adaxial) surface. This is illustrated by the leaf of the coastal New Zealand Christmas tree (*Metrosideros thomasi*, Fig. 2C,D). The lower (abaxial) surface is covered by a dense mat of curly hairs around $200 \mu\text{m}$ thick. At the base of these hairs protrude numerous stomata. The other surface has a thick cuticle and two layers of large cells beneath the

epidermis devoid of chloroplasts.

The ratio of diffusivities of water and CO₂ are 1.37 in the boundary layer and 1.6 in stomata (von Caemmerer and Farquhar, 1981). Consequently, the ratio of conductances to CO₂ and water from surrounding air to sub-stomatal cavities depends to a small extent on the ratio of boundary layer to stomatal conductance. If boundary layer conductance is twice that of stomata, the ratio of conductance to CO₂ versus water is 0.66. This ratio increases to 0.71 if boundary layer conductance is only one quarter of stomatal conductance. That is, CO₂ uptake relative to water loss is favored when the boundary layer dominates the diffusion limitation, given equal leaf temperatures. However, since leaf temperature depends on boundary layer conductance and transpiration rate and transpiration rate depends on boundary and stomatal conductance, this is not a simple assumption.

When measuring gas exchange, leaf chambers are generally designed to have high windspeeds from either the flow rate or a fan so that boundary layer conductance is large relative to stomatal conductance.

III. Stomata—CO₂ Diffusion into the Leaf

A. Leaf Surface Structure

Stomata vary in size, shape and density over the surface. Primitive leaves have stomata only on the lower surface (Mott et al., 1982) and a survey of British flora revealed that woody species also tend to be almost exclusively hypostomatous, with stomata only on the lower surface (Peat and Fitter, 1994). CO₂ entering the lower surface must diffuse across the bulk of these leaves because light is mainly absorbed at the upper surface. Generally, leaves with greater photosynthetic capacities, such as those that exist in sunny habitats, have additional stomata on their upper epidermis (amphistomatous), thus reducing the diffusion pathlength (Mott et al., 1982). Given the flexible and dynamic aperture of stomata, stomatal conductance is not well predicted by stomatal density (Jones, 1987).

Diverse examples of leaf surfaces are shown in Fig. 3. In panel A, a tobacco leaf is shown which is typical of many dicot herbaceous leaves. There is a high density of regularly spaced stomata with occasional protruding hairs. The surface of *Kalanchoe* (Fig. 3B), a CAM plant which has low stomatal

conductance and nocturnal stomatal opening when water-stressed, clearly has a much lower stomatal density than tobacco. An apricot leaf is shown in Fig. 3C with a patch of stomata surrounded by veinlets. The veins have bundle sheath extensions reaching both epiderms and the epidermis above the veins is devoid of stomata. *Nerium* and *Banksia* leaves encrypt their stomata. *Nerium* leaves (Fig. 3D) have large pits scattered over the lower surface which are filled with hairs. Stomata are positioned near the base of the pits. *Banksia* (Fig. 3F, like *Metrosideros* Fig. 2C,D) has a dense array of hairs on the abaxial surface that obscure stomata from view. Wheat (Fig. 3E) is a typical monocot surface with stomata in linear files of cells, regularly spaced.

Another common feature is to place stomata at the base of an antechamber, such as in *Pinus* (Fig. 4). When viewed from the surface (Fig. 4C), the pores visible are not stomatal guard cells, but fixed lips of the antechamber. Inside the antechamber, wax structures place a further barrier to diffusion of water out and CO₂ in, before the stoma is reached (Fig. 4A). Beneath stomata are the sub-stomatal cavities (Fig. 4B) where CO₂ is free to diffuse away into the chlorenchyma through intercellular airspaces surrounding the highly lobed cells.

B. Stomatal Responses to CO₂, VPD and Irradiance

Stomata begin to respond within seconds to changes in several environmental variables, reaching a new steady state after about 20 min (Kirschbaum et al., 1988). After prolonged darkness, initial stomatal opening may take longer than once the leaf is actively photosynthesizing. Their role is to enable CO₂ uptake while minimizing water loss (Cowan, 1977). Therefore, stomatal conductance tends to relate to CO₂ assimilation rate such that with increasing irradiance or decreasing CO₂, stomatal conductance increases to maintain the supply of CO₂ needed for photosynthesis (Farquhar and Sharkey, 1982). On the other hand, stomatal conductance is also regulated in relation to leaf water status to control the rate of water loss. In the short-term, drier air surrounding the leaf which means a larger leaf to air vapor pressure difference, VPD, results in decreasing conductance. Stomatal responses of a given leaf are not fixed, but vary over the lifespan of the leaf (Constable and Rawson, 1980). If leaves have been water-stressed, stomatal sensitivity to CO₂ and VPD

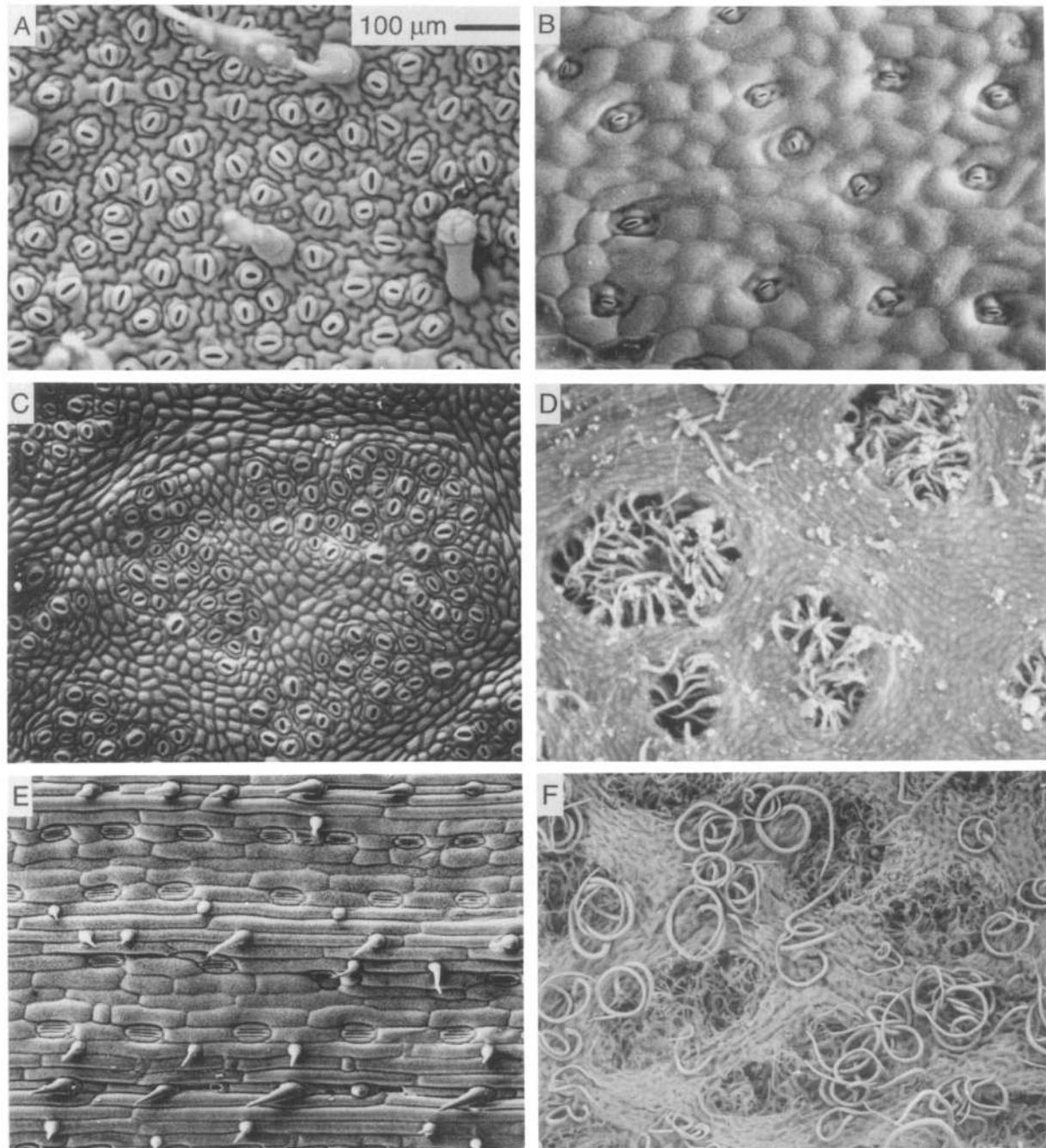


Fig. 3. Leaf surfaces viewed by SEM, all at the same magnification. A, Tobacco (*Nicotiana tabacum*); B, *Kalanchoe diagremontiana* a CAM plant; C, Apricot (*Prunus persica*)—a heterobaric leaf; D, *Nerium oleander*—stomata are in the base of the hair-filled pits; E, Wheat (*Triticum aestivum*); F, *Banksia serrata* (Photos by D Bussis (A), K Maxwell (B), J Santrucek (C,D,F) and S Craig (E)).

increases (Raschke, 1987).

Steady-state responses to CO_2 , VPD and irradiance are shown in Fig. 5. Because stomata respond to many signals, stomatal conductance is not uniquely

related to any single variable. For example, when there is only a small leaf to air VPD, stomatal conductance is much greater at any given CO_2 concentration. The mechanism behind CO_2 respon-

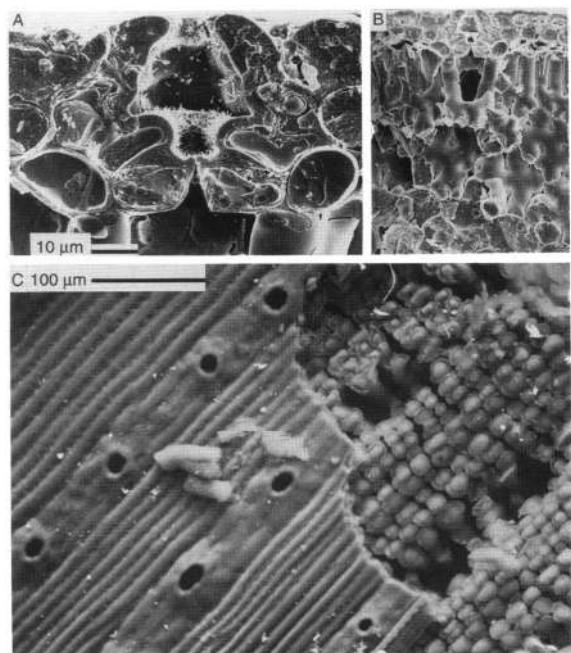


Fig. 4. Pine needle. A, Stomatal complex fractured in cross section showing the guard cell pair with wax structures in the base of the antechamber; B, Sub-stomatal cavity directly below the stoma with two layers of chlorenchyma; C, Surface view of the needle showing the fixed pores of the antechambers. On the right hand side, the epidermis has been broken away revealing the mesophyll and regularly spaced substomatal cavities. Multi-lobed cells in the mesophyll are lined with chloroplasts. (Photos by S Craig (A,B) and J Santrucek (C)).

siveness is as yet unknown. It appears that CO_2 is sensed via intercellular CO_2 rather than CO_2 in the stomatal pore or outside the leaf. Mott (1988) used amphistomatous leaves to examine the response of stomata on one surface where ambient and intercellular CO_2 could be independently varied. Stomatal conductance did not respond to changes in external CO_2 if intercellular CO_2 was kept constant, but did respond to changes in intercellular CO_2 when external CO_2 was kept constant.

Responses to VPD have been difficult to separate from those associated with evaporation rate and leaf temperature, since they all interact. However, in a clever experiment, Mott and Parkhurst (1991) substituted Helox (air in which N_2 is replaced by He) for air, which increases the rates of diffusion of water and CO_2 2.3-fold over that in air. They followed stomatal aperture during step changes to Helox in which either evaporation rate or VPD was held constant. Stomatal aperture was unaltered by switching to Helox when evaporation rate was kept

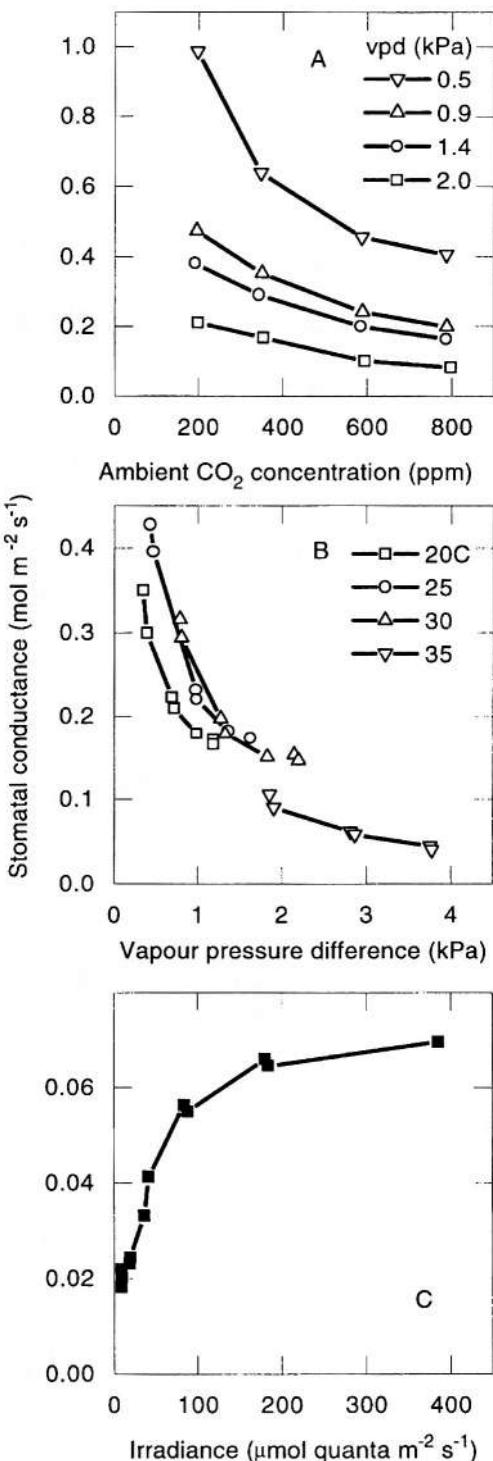


Fig. 5. Stomatal conductance as functions of (A) ambient CO_2 concentration, (B) leaf to air vapor pressure difference (vpd) and (C) irradiance. Data from (A) Morison and Gifford (1983) for rice at different vpd; (B) Leuning (1995) *Eucalyptus grandis* at different temperatures; (C) Kirschbaum et al. (1988) *Alocasia macrorrhiza*.

constant by reducing VPD from 14.5 mmol mol⁻¹ in air to 6 mmol mol⁻¹ in Helox. By contrast, stomatal aperture was reduced following a switch to Helox when VPD was held constant. Initially, evaporation was much greater in Helox, but stomatal aperture declined until evaporation returned to the rate previously found in air. Mott and Parkhurst (1991) therefore concluded that stomatal responses to humidity or VPD are based on the rate of water loss from the leaf rather than sensing water vapor concentration at the leaf surface. Interestingly, gender specific responses to VPD have been observed for Box Elder (*Acer negundo*). Dawson and Ehleringer (1993) found that male trees were much more sensitive to VPD than female trees which resulted in stomatal closure and consequently lower intercellular CO₂ partial pressures in the males. This was true for sapling and mature trees when measured by gas exchange and was confirmed by carbon isotope composition.

Stomatal responses to irradiance consist of several sensing mechanisms, including a blue light response (Sharkey and Raschke, 1981) and another light response linked to photosynthesis. Over a wide range of irradiance, stomatal conductance increases proportionately with CO₂ assimilation rate (Wong et al., 1985b) such that intercellular CO₂ partial pressure remains constant. Investigating the mechanism for this again utilized amphistomatous leaves. By giving different combinations of irradiances to the two leaf surfaces, it was possible to uncouple conductance from irradiance for one surface. For example, the most extreme case was when light was only given to one surface. Stomatal conductance of the surface in the dark increased nearly three-fold when irradiance given to the other surface increased from 0.5 to 2 mmol quanta m⁻² s⁻¹ (Wong et al., 1985b). While p_i for the abaxial surface increased slightly from 222 to 234 μbar , p_i calculated for the whole leaf remained unchanged at 227 μbar . Thus, in this example overall conductance for the leaf was closely coupled to CO₂ assimilation rate and not the distribution of light between the two surfaces. This ruled out the possibility of stomatal conductance being controlled directly by irradiance under high irradiance conditions.

C. Relationship Between Stomatal Conductance and CO₂ Assimilation Rate

While stomatal conductance is responsive to several environmental variables, the parameter that correlates

most closely with it is CO₂ assimilation rate. A close coupling has been found between stomatal conductance and rate of CO₂ assimilation regardless of whether CO₂ assimilation rate was varied by mineral nutrition, age or irradiance (Fig. 6, Wong et al., 1979, 1985a,b). Independent surveys have yielded striking correlations between A and g_s between species (Körner et al., 1979; Wong et al., 1979; Yoshie, 1986) with C₃ and C₄ species falling on two distinct lines.

The striking correlation between A and g_s (Fig. 6) for C₃ species implies a relatively constant ratio of intercellular to ambient CO₂ partial pressure, p_i/p_a . The ratio p_i/p_a is negatively related to the ratio A/g_s , which defines the instantaneous water-use efficiency of photosynthesis (mol CO₂ fixed per mol water transpired, $A/E = (A/g_s)/(1.6VPD)$). Therefore, knowledge of p_i/p_a has important ramifications in both agricultural and ecological work. Measurement of this ratio by conventional gas exchange techniques is labor intensive and it is difficult to resolve subtle differences between plants. However, with stable carbon isotope techniques (see Chapter 17, Brugnoli and Farquhar), it is now possible to screen plants using carbon isotopic composition, Δ , as an integrated signal of p_i/p_a . For example, 351 C₃ *Poa* species were sampled in Africa and $\delta^{13}\text{C}$ ranged from -22 to -34‰ (Vogel, 1993) which is equivalent to a range in p_i/p_a of nearly 0.5. More typically, Δ varies by around 2‰, equivalent to a range in p_i/p_a of 0.1. Therefore, while p_i/p_a is relatively conservative between C₃ species (0.7 ± 0.05), this implies greater variation in water-use efficiency as it is the same variation divided by 1 - p_i/p_a (0.3 ± 0.05).

The mechanism behind the correlation between A and g_s is still not known, but in explaining their results, Wong et al. (1979) proposed that stomata must respond to a photosynthetic metabolite produced in the leaf mesophyll. However, Sharkey and Raschke (1981) used cyanazine or DCMU to inhibit photosynthesis and showed that stomatal conductance still responded to blue light. With the advent of transgenic plants, it has been possible to disrupt photosynthesis in leaves through the specific reduction of proteins by antisense gene expression. When CO₂ assimilation rate has been reduced via lowering the content of Rubisco (Hudson et al., 1992), GAPDH (Price et al., 1995) or cytochrome *bf* complex (Price et al., 1998), stomatal conductance was unaltered except in the most severely affected plants. Since it has been possible to break the correlation between A and g_s , the role of a metabolite

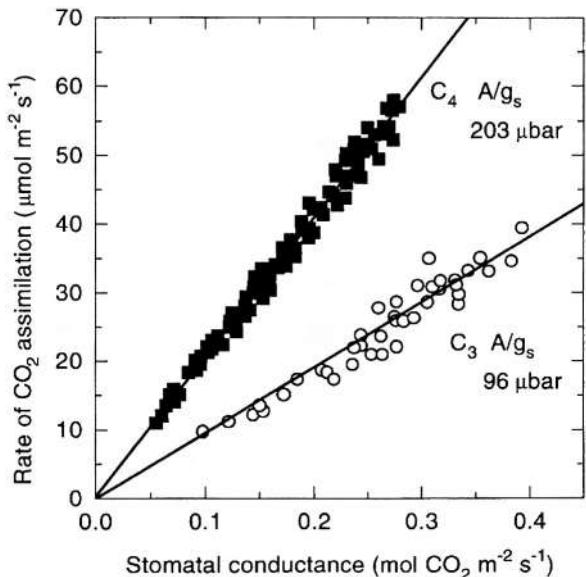


Fig. 6. Correlations between rate of CO₂ assimilation and stomatal conductance. Data from Wong et al. (1985a) for *Zea mays* (C₄) and *Gossypium hirsutum* (C₃) leaves measured with 2 mmol quanta m⁻² s⁻¹ and an ambient CO₂ partial pressure of 305 μbar. Photosynthetic capacity varied between leaves due to different mineral nutrition treatments. The average drawdown from ambient to intercellular CO₂ partial pressure (A/g_s) was 203 and 96 μbar for the C₄ and C₃ plants, respectively.

signal seems unlikely and the control mechanism remains a paradox.

Despite our inability to unravel the mechanism, the strong correlation between A and g_s still provides us with the best predictor of stomatal conductance. Ball et al. (1987) empirically derived an equation that encapsulated stomatal responses to CO₂, VPD and irradiance. It has subsequently been slightly refined (Leuning, 1995): $g_s = g_0 + a_1 A / [(p_s - \Gamma)(1 + D_s/D_0)]$ where g_0 is the conductance at zero irradiance, a_1 and D_0 are empirical coefficients, A is the rate of CO₂ assimilation, p_s is the partial pressure of CO₂ at the leaf surface, Γ is the CO₂ compensation point and D_s is the leaf to air VPD. The problem with this relationship is that to find g_s , one first needs to know A , which itself depends on g_s through its effect on intercellular CO₂. To obtain a solution therefore requires an iterative loop.

D. Stomatal Patchiness

Entire leaves or portions of a leaf are usually enclosed in cuvettes for gas exchange measurements. It is then assumed that the entire surface behaves uniformly when conditions are varied. Stomata may close in

one section of a leaf and remain open in another, forming a patchy pattern. A comprehensive and erudite review was written by Terashima (1992) following his pioneering work in this field. Many leaves have densely packed sclerenchymatous cells that extend above and below vascular strands that effectively prevent lateral diffusion of gases. When these bundle sheath extensions are associated with reticulate venation, small patches of leaf are isolated from one another and the leaf is termed heterobaric. An example of a heterobaric leaf is apricot, shown in Fig. 3C. If lateral diffusion is unimpeded, the leaf is described as homobaric.

Much of the interest in patchy closure has been due to the consequences it has on interpretation of photosynthetic CO₂ response curves. If gas exchange averages across patches, it will underestimate CO₂ assimilation rate for a given intercellular CO₂. ABA has been associated with stomatal closure during water stress, but when applied to leaves, lowers both conductance and photosynthesis. It had been suggested that ABA directly inhibited photosynthesis, but this was shown to be an artifact of patchy stomatal closure (Terashima et al., 1988). Water stress has been found to cause patchy stomatal closure (Beyschlag et al., 1992; Eckstein et al., 1996, although see Gunasekera and Berkowitz, 1992) as well as exposure to large VPD (Loreto and Sharkey, 1990). While gas exchange revealed a steady conductance, fluorescence imaging showed patches of leaf oscillating widely, which when averaged out over a larger area, appeared stable (Cardon et al., 1994). Oscillations induced by a sudden drop in irradiance gradually dampen out, first adjacent to veins and gradually spreading out into the mesophyll (Siebke and Weis, 1995b).

There exist several methods for detecting the existence of patchy stomatal closure. Carbohydrate accumulation reflects the preceding photosynthesis which will be reduced if stomatal conductance was lower than in adjacent areas of a heterobaric leaf. Terashima et al. (1988) visualized this by subsequent iodine staining. They combined the visualization of photosynthate with direct observation of stomatal aperture on epidermal replicas of the same leaf pieces. Areas with little starch staining corresponded to areas with closed stomata. Downton et al. (1988) used ¹⁴CO₂ labeling and autoradiography to visualize the spatial pattern of photosynthesis. More recently, chlorophyll fluorescence has been used to resolve the spatial and temporal patterns of photosynthesis

(Daley et al., 1989) and offers superb resolution. An alternative approach has been to assess stomatal conductance by measuring the infiltration of leaves by liquids of different viscosity (Beyschlag and Pfanz, 1990). Comparisons between conductance measured by gas exchange and quantity of water infiltrated into the same leaf yielded proportional linear relationships (Beyschlag and Pfanz, 1990; Beyschlag et al., 1992).

Clearly, heterogeneous stomatal closure is a widespread phenomenon that occurs for multiple reasons. However, this does not mean it is a common occurrence that always plagues gas exchange analysis. With unstressed plants not subject to abrupt changes in light or VPD, or for homobaric leaves, patchy stomatal closure is unlikely to be encountered.

IV. Internal—CO₂ Diffusion Within the Leaf

A. Leaf Mesophyll Structure

Just as there is great diversity in the surface of leaves in terms of hairs and patterns of stomata, so there is with mesophyll structure (Fig. 7, see also Bolhar-Nordenkampf and Draxler, 1993). The examples presented in Fig. 7 are all shown at the same magnification. Perhaps the most common feature is palisade tissue near the leaf surface. These cylindrical cells are closely packed in parallel to one another directly beneath the leaf surface. For bifacial leaves, palisade cells are usually beneath the adaxial surface, with spongy mesophyll near the abaxial surface (Fig. 7 A,B,C). Huge, densely packed cells fill the thick succulent tissue of the CAM plant *Kalanchoe* of which only a small fraction is shown in Fig. 7D. Monocots have cell shapes that depend on their position relative to vascular strands (Fig. 7E). For leaves that hang or grow vertically, isolateral anatomy is often found where palisade tissue is present on both surfaces with spongy tissue in the central part of the leaf (Fig. 7F). With the thick horizontally displayed leaf of *Metrosideros* (Fig. 7G), densely packed cells are present throughout the mesophyll.

It is difficult to fully appreciate intercellular airspaces from transverse fractures or sections, but clearly the airspaces of spongy mesophyll offer little resistance to diffusion. In palisade tissue, narrow pores extend up between adjacent cells and a cavernous gap can be seen near the top of Fig. 7D in

the densely packed succulent tissue of the CAM leaf. When the scanning electron microscopy was done with ‘nude’ (i.e. uncoated) samples, it is possible to see chloroplasts through the cell walls (Fig. 7 A,C,D). Chloroplasts cover the cell surface in tobacco and cotton (Fig. 7A,C) but are scattered diffusely over the surface of the large balloon-like cells of the CAM plant *Kalanchoe* (Fig. 7D, Maxwell et al., 1997). For coated samples, the chloroplasts are only evident when a cell is fractured open. Chloroplasts are then seen adjacent to the cell walls (Fig. 7E).

B. Methods for Determining Internal Conductance

Diffusion of CO₂ from substomatal cavities, p_i , to sites of carboxylation, p_c , must occur down a partial pressure gradient. Opinion as to the magnitude of this gradient has swung from one extreme (e.g. Gaastra, 1959) that $p_c = 0$ to the other (e.g. Farquhar and von Caemmerer, 1982) that p_c is approximately equal to p_i . The ease with which CO₂ can diffuse through the leaf to the sites of carboxylation can be defined by an internal conductance, g_w , using Fick's law: $g_w = A/(p_i - p_c)$. The subscript w was introduced by Evans (1983b) to emphasize the wall and liquid phase components of internal conductance. There exist several methods for determining g_w which are described below.

1. Stable Isotope Discrimination Combined with Gas Exchange

There are two naturally occurring stable isotopes of carbon, ¹²C and ¹³C. About 1.1% of atmospheric CO₂ contains the heavier isotope ¹³C. ¹³CO₂ diffuses more slowly than ¹²CO₂ and has slightly different chemical properties. Consequently, the carbon isotopic composition of plant material is depleted of ¹³C relative to atmospheric CO₂. Not only does ¹³CO₂ have a lower diffusivity in air than ¹²CO₂, but because Rubisco discriminates against ¹³CO₂, there is also a smaller difference in partial pressure. The preferential fixation of ¹²CO₂ during photosynthesis results in an enrichment of ¹³CO₂ in air surrounding the leaf.

Theory has been developed (Farquhar et al., 1982; Farquhar and Richards, 1984) that predicts net discrimination, Δ , by C₃ plants:

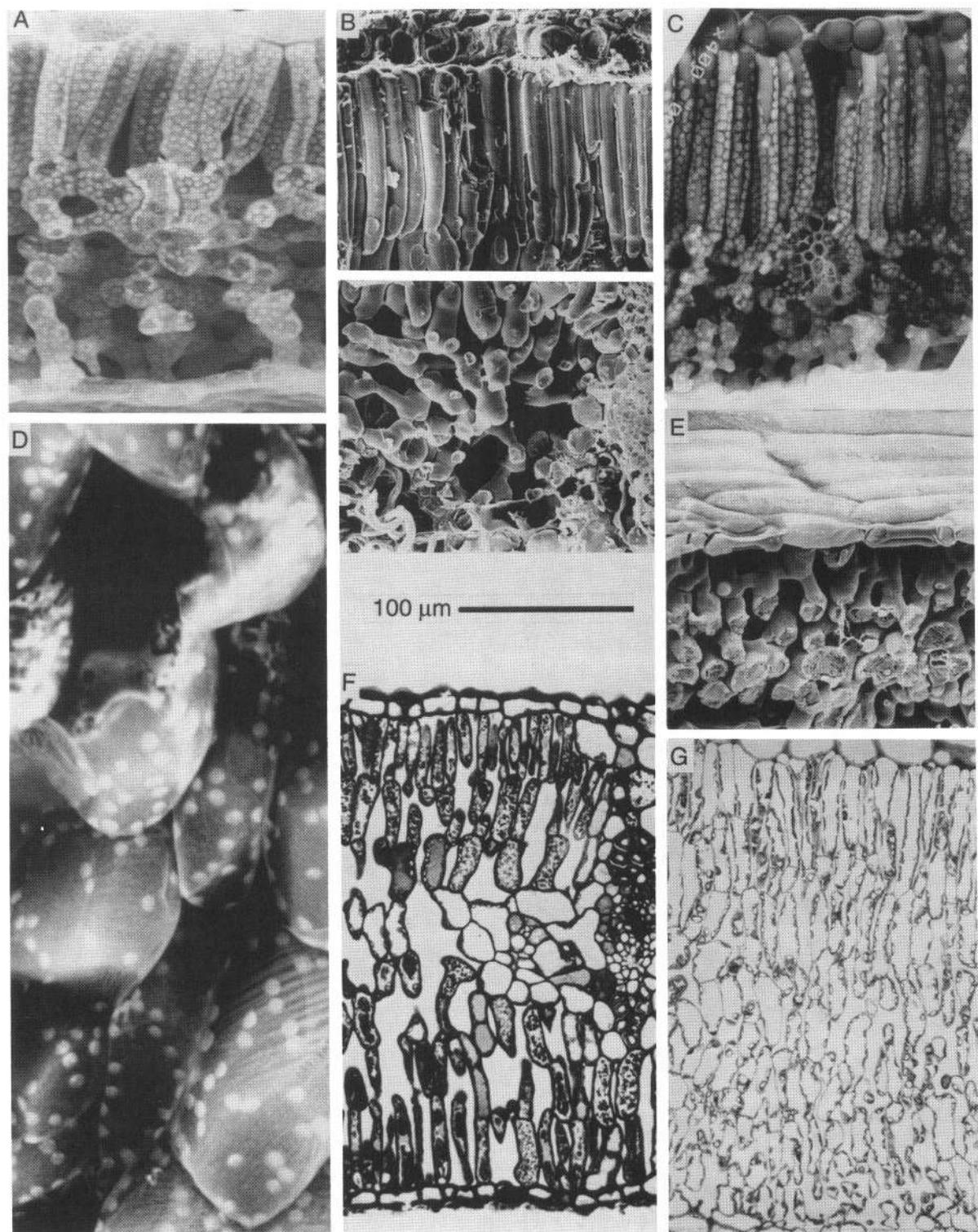


Fig. 7. Leaf mesophyll structure compared at the same magnification. A, Tobacco (*Nicotiana tabacum*); B, *Banksia marginata*; C, Cotton (*Gossypium hirsutum*); D, *Kalanchoe diagremontiana* – only a small portion as the leaf thickness was 1.2 mm; E, Wheat (*Triticum aestivum*); F, *Eucalyptus pauciflora*; G, *Metrosideros thomasii*. Photos by S Craig (B,E,G), J Santrucek (C) and K Maxwell (D).

$$\Delta = a_b \frac{(p_a - p_s)}{p_a} + a \frac{(p_s - p_i)}{p_a} + a_i \frac{(p_i - p_c)}{p_a} + b \frac{p_c}{p_a} - \frac{(eR/k + f\Gamma_*)}{p_a} \quad (1)$$

where a_b and a are the fractionations during diffusion through air in the boundary layer and stomata, respectively (2.9‰, 4.4‰), a_i is the combined fractionation due to dissolution and diffusion of CO₂ in water (1.8‰), b is the fractionation during carboxylation that occurs during photosynthesis, e and f represent fractionations associated with nonphotorespiratory CO₂ evolution, R , and photorespiration, respectively, k is the carboxylation efficiency of Rubisco and Γ_* is the CO₂ compensation point in the absence of dark respiration (Farquhar et al., 1982) and p_a , p_s , p_i , and p_c are the partial pressures of CO₂ in the surrounding air, leaf surface, intercellular airspace (strictly speaking, the substomatal cavity) and sites of carboxylation, respectively. The value of b has been determined in vitro (30 - 31.3‰, Roeske and O'Leary, 1984, Guy et al., 1993), but in the leaf a small proportion of CO₂ is fixed via phosphoenol pyruvate carboxylase which reduces the effective value of b in vivo (Farquhar and Richards, 1984). From concurrent measurements of gas exchange and discrimination in tobacco leaves that have an antisense RNA gene for Rubisco small subunit an estimate of 29‰ was obtained (Evans et al., 1994). Attempts to measure fractionation during respiration have suggested that e is close to zero (von Caemmerer and Evans, 1991). There appears to be significant fractionation during photorespiration ($f \approx 8\%$, Rooney, 1988; 3 - 12‰, Gillon and Griffiths, 1997).

It is usual to make some simplifications to Eq. (1) and present the relationship as

$$\Delta_i = a \frac{(p_a - p_i)}{p_a} + b \frac{p_i}{p_a} = a + (b - a) \frac{p_i}{p_a}. \quad (2)$$

This form of the equation distinguishes between fractionation during diffusion through air (a) and during carboxylation (b) with discrimination being linearly dependent on p_i/p_a . It is based on several assumptions. 1. The drawdown across the boundary layer and stomata are combined as this introduces little error and usually boundary layer conductance

is not routinely measured. 2. The drawdown from substomatal cavities to sites of carboxylation is negligible or constant. 3. Fractionations associated with dark respiration and photorespiration are negligible. One can rearrange Eq. (2) to highlight that Δ is simply related to the balance between CO₂ assimilation rate, A , and leaf conductance, g_s :

$$\Delta_i = b - (b - a)A/(g_s p_a). \quad (3)$$

During photosynthesis, CO₂ passing over the leaf changes its isotopic composition. By cryogenically trapping CO₂ from the source air and CO₂ from air that exits a gas exchange chamber ('on-line') and subsequently measuring their isotopic composition, it is possible to derive the net discrimination that occurred during that photosynthetic state (Evans et al., 1986, von Caemmerer and Evans, 1991). Multiple measurements can then be made on a leaf under a variety of conditions and subsequently be related to the photosynthetic measurements. This has enabled examination of the drawdown from substomatal cavity to the sites of carboxylation. By subtracting Eq. (1) from Eq. (2), one obtains:

$$\begin{aligned} \Delta_i - \Delta &= (b - a_i) \frac{(p_i - p_c)}{p_a} + \frac{eR/k + f\Gamma_*}{p_a} \\ &= (b - a_i) \frac{A}{g_w p_a} + \frac{eR/k + f\Gamma_*}{p_a} \end{aligned} \quad (4)$$

Equation 4 reveals that the difference between the discrimination predicted on the basis that $p_i = p_c$, Δ_i , and the measured Δ , should be linearly related to A/p_a with the slope proportional to the reciprocal of the internal conductance, g_w . The second term containing the respiration and photorespiration terms should appear as a non-zero intercept.

An example of this technique is shown in Fig. 8. A radish leaf was measured at a range of irradiances beginning at 1.35 mmol quanta m⁻²s⁻¹, decreasing to 0.11 mmol quanta m⁻²s⁻¹ and returning back to the starting irradiance. The insert shows the measured Δ as a function of p_i/p_a , with p_i/p_a increasing slightly at the lowest irradiances. The solid line represents Δ_i (Eq. 2) and the difference between Δ_i and Δ is replotted as a function of CO₂ assimilation rate in the main panel. The strong linear increase in difference between Δ_i and Δ gives clear support to the notion that there is a significant difference between p_i and p_c which

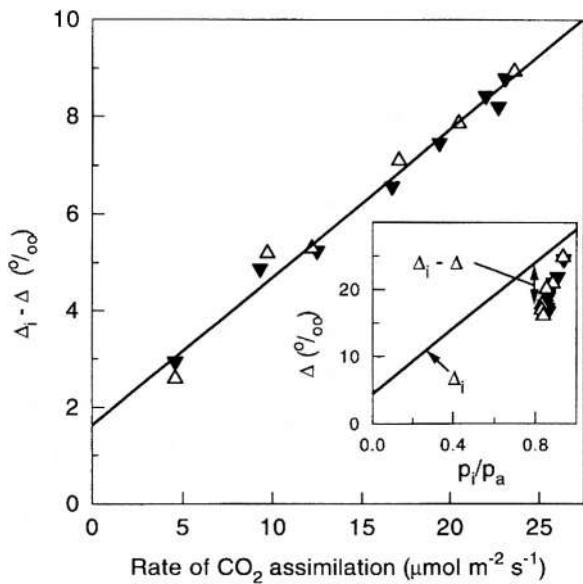


Fig. 8. Δ method for determining internal conductance. Carbon isotope discrimination is measured concurrently with gas exchange as a function of irradiance. The difference between discrimination calculated assuming that CO₂ partial pressure in the intercellular airspaces is the same as at the sites of carboxylation using $\Delta_i = 4.4 + 24.6 p/p_a$ and the measured Δ (see inset) is plotted against CO₂ assimilation rate. Internal conductance is proportional to the reciprocal of the slope: $\Delta_i - \Delta = (A/g_w) \times 27.2/p_a = (p_i - p_c) \times 27.2/p_a$. Data are from von Caemmerer and Evans (1991) for a radish leaf measured with irradiances decreasing from 1.35 to 0.11 mmol quanta m⁻² s⁻¹ (▼) then increasing again (△), slope yields $g_w = 0.25 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$.

depends on the rate of CO₂ assimilation. The data are consistent with a resistance that is fixed over the measurement time period for a given leaf. Repeated measurements on a tobacco leaf over the course of a week have shown that the internal conductance did not change (Evans and von Caemmerer, unpublished). The non-zero intercept reveals that the fractionations associated with respiration and photorespiration are occurring, but it also contains experimental error. Consequently, an estimate of CO₂ drawdown to the sites of carboxylation made from a single measurement is considerably less certain than that obtained by determining the slope as in Fig. 8 which is quite robust.

While this technique has been used to study internal conductance, it requires access to a ratio mass spectrometer with measurement precision an order of magnitude better than can be tolerated for plant dry matter samples. Since these machines are rare, the technique is not readily available for many people. The alternative is to determine Δ on soluble sugars

extracted from a leaf that has been held under constant conditions during photosynthesis and for which gas exchange parameters are known. One can then apply Eq. (4). The Δ of soluble sugars is known to closely reflect p_i/p_a (Brugnoli et al., 1988) and has been used to calculate g_w in rice (Sasaki et al., 1996) and sunflower, cotton and chestnut (Brugnoli et al., 1998). While the method is far less precise than on-line measurements, because it depends on the value assumed for b and assumes that the second term in Eq. (4) is zero, it should prove useful when a relative value is being sought. If access to ratio mass spectrometry is not possible, an alternative method is to combine gas exchange with chlorophyll fluorescence measurements.

2. Chlorophyll Fluorescence Combined with Gas Exchange

Development of the modulated chlorophyll fluorometer has enabled fluorescence to be measured on leaves during photosynthesis. All that is required are two measurements, namely steady state fluorescence, F , followed by fluorescence during a pulse of light (generally several times full sunlight) that closes all Photosystem II reaction centers, F_m . Photochemical efficiency of Photosystem II, that is, the proportion of quanta absorbed by Photosystem II that result in electron transport, is given by (Genty et al., 1989),

$$\phi_{PSII} = 1 - F/F_m. \quad (5)$$

Although this measurement appears simple, to use it to calculate the rate of electron transport requires important assumptions or calibration for a particular leaf. The rate of electron transport calculated from fluorescence, J_F , is given by,

$$J_F = \phi_{PSII} \cdot I \cdot a \cdot \beta, \quad (6)$$

where I is the irradiance, a is the leaf absorptance (generally around 0.8–0.85) and β is the fraction of absorbed quanta that reach PS II (generally 0.42–0.5). Equation (6) can be obtained by calibration. Fluorescence and gas exchange measurements are made at very low oxygen partial pressures that prevent photorespiration. The rate of electron transport, J , that is required to support a given rate of CO₂ assimilation, A , is given by (Farquhar and von Caemmerer, 1982),

$$J = (A + R)(4p_c + 8\Gamma_*) / (p_c - \Gamma_*), \quad (7)$$

where Γ_* is the CO_2 photocompensation point in the absence of nonphotorespiratory CO_2 evolution, R . Γ_* is linearly related to oxygen partial pressure, so in the limit when there is no oxygen present, Eq. (7) simplifies to $J = 4(A + R)$. Some uncertainty remains because R is partially suppressed in the light, although it is relatively constant over a broad range of irradiance above 0.2 mmol quanta $\text{m}^{-2} \text{s}^{-1}$ (Brooks and Farquhar, 1985). For a given leaf, J_F is measured over a range of irradiances and or CO_2 partial pressures to establish the relationship between J/I and ϕ_{PSII} . A linear relationship should be observed with the slope equivalent to α/β (Siebke and Weis, 1995a; Genty and Meyer, 1995). In some instances, a non-zero intercept is observed suggesting that not all electron transport is associated with photosynthesis (Laisk and Loreto, 1996), but this problem and the uncertainty in R become less important at higher irradiances.

Having established the relationship between J and ϕ_{PSII} in Eq. (6), the combined measurement of fluorescence and gas exchange measurements under 21% oxygen over a range of CO_2 partial pressures or irradiances is used to calculate p_c which is given by rearranging Eq. (7),

$$p_c = \Gamma_*(J_F + 8(A+R)) / (J_F - 4(A+R)). \quad (8)$$

Internal conductance can then be calculated for each point from the definition, $g_w = A / (p_i - p_c)$.

An example of the method is given for control and water-stressed spinach leaves (Fig. 9). Electron transport rate derived from fluorescence is shown as functions of irradiance (Fig. 9A squares). At higher irradiance, electron transport rate was calculated from gas exchange using Eq. (7) assuming $p_i = p_c$ (triangles) or calculating p_c as $p_i - A/g_w$ and assuming $g_w = 0.63$ or $0.18 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$ for control and water-stressed leaves, respectively, (circles). In Fig. 9B the rate of CO_2 assimilation is plotted as a function of the drawdown $p_i - p_c$, where p_i is obtained from gas exchange and p_c is calculated from chlorophyll fluorescence using Eq. (8). The data conform to the expectation that drawdown is proportional to the rate of CO_2 assimilation and the slope yields the estimate of internal conductance. Water stress clearly reduced internal conductance, as has been observed in other studies (Renou et al., 1990; Roupsard et al., 1996; Ridolfi and Dreyer, 1997; Brugnoli et al., 1998). Salt

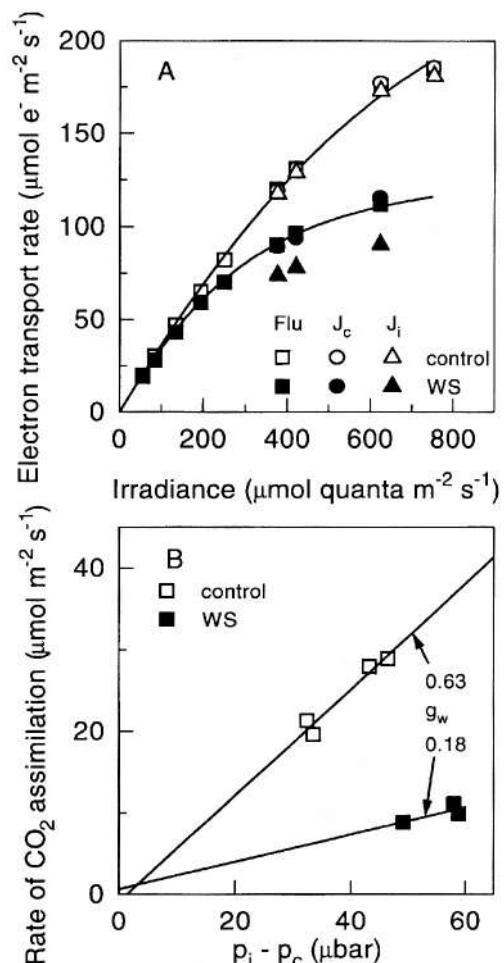


Fig. 9. Chlorophyll fluorescence method for determining internal conductance. A, Fluorescence was measured concurrently with gas exchange as a function of irradiance for a control (open) and water-stressed (WS, solid) leaf of *Spinacia oleracea*. Electron transport rate calculated from fluorescence (squares) or gas exchange assuming $p_i = p_c$ (triangles) or with $g_w = 0.63$ and 0.18 (circles) for control and water-stressed leaves, respectively (Loreto, unpublished). B, CO_2 assimilation rate, A , versus the draw-down between p_i (from gas exchange) and p_c (from Eq. 8), the slope of the line equals g_w .

stress has also been shown to reduce internal conductance in olive (Bongi and Loreto, 1989) and spinach leaves (Delfine et al., 1998).

The first to combine fluorescence with gas exchange to determine p_c was Di Marco et al. (1990). The method was subsequently refined by Harley et al. (1992) who proposed two modes of calculating internal conductance. The first, coined the 'variable J' method (Shown in Fig. 9), calculates p_c from Eq. (8) for a series of measurements where variation in either CO_2 or irradiance results in changes to the rate

Table 1. Γ_* values in the literature converted to 25 °C

(^Avalues were measured in vitro, all the others were measured in the leaf by gas exchange and in some cases with fluorescence as well)

Species	Γ_* (μbar)	Reference
<i>Spinacia</i>	47.7 ^A	Jordan and Ogren (1984)
	42.1	Brooks and Farquhar (1985)
	33.6	von Caemmerer et al. (1994)
	48.2–55.2 ^A	Kane et al. (1994)
<i>Nicotiana</i>	38.1 ^A	Parry et al. (1989)
	46.6	Peterson (1990)
	44.2 ^A	Delgado et al. (1993)
	38.6	von Caemmerer et al. (1994)
	48.4 ^A	Kane et al. (1994)
<i>Triticum</i>	39	Brooks and Farquhar (1985)
	33	Watanabe et al. (1994)
	44.1 ^A	Kane et al. (1994)
<i>Helianthus</i>	43.1–46.1	Laisk and Sumberg (1994)
	35.8	Epron et al. (1995)
	44.6	Laisk and Loreto (1996)
<i>Phaseolus, Populus, Viburnum</i>	43.8	Laisk (1977)
	36.9–43.2	Berry et al. (1994)
<i>Heteromeles, Lepechinia</i>	43.9, 43.4	Villar et al. (1994)
<i>Phaseolus</i>	37.4	Epron et al. (1995)
<i>Hordeum</i>	34.1	Hausler et al. (1996)
<i>Poa</i> (6 species)	33.1–42.6	Atkin (pers. com.)
<i>Quercus</i>	38.8, 42.5 ^A	Balaguer et al. (1996)

of electron transport calculated from chlorophyll fluorescence. The individual estimates are then averaged. In the second, coined the ‘constant J’ method, a series of measurements are made at an intermediate irradiance over a range of CO₂ partial pressures which do not alter the rate of electron transport calculated from chlorophyll fluorescence. A series of calculations are then made using Eq. (7) where different values for the internal conductance are assumed in order to calculate p_c . The minimum variance in the calculated J for the set of measurements is then sought and taken as the best estimate of internal conductance.

Harley et al. (1992) examined the limitations and potential errors associated with uncertainties in the various parameters. Perhaps the most crucial is Γ_* . Γ_* can either be measured by gas exchange techniques (Laisk, 1977, Brooks and Farquhar, 1985) or calculated from the specificity factor of Rubisco determined in vitro. There are now a considerable number of values reported for Γ_* in a range of species (Table 1). There is no consistent difference between species, given the scatter for each. In order to illustrate the significance of uncertainty in calculating g_w , the data presented in Fig. 9 has been analyzed using different values of Γ_* and R (Table 2). The two values chosen for Γ_* were 42.7 μbar (Brooks and Farquhar,

Table 2. The effect of varying Γ_* and R on the estimate of g_w in Fig. 9

Γ_* (μbar)	42.7	42.7	38.6
R (μmol m ⁻² s ⁻¹)	1	0.7	0.7
		g_w (mol m ⁻² s ⁻¹ bar ⁻¹)	
Control	0.88	0.63	0.36
Water-stressed	0.19	0.18	0.14

1985) and 38.6 μbar (von Caemmerer et al., 1994). For the water-stressed leaf, variation in R and Γ_* did not greatly affect the estimate of g_w . However, the estimate for the control leaf varied a great deal. This illustrates that this method becomes increasingly uncertain as g_w increases as was pointed out by Harley et al. (1992). When g_w is greater than 0.3 mol m⁻² s⁻¹ bar⁻¹, it would be advisable to measure Γ_* and R for the leaf. The normal protocol for obtaining the photocompensation point is the point of intersection of CO₂ response curves measured at different irradiances. The CO₂ partial pressure at this point, p_i^* and the rate of CO₂ evolution, R , are then used to calculate Γ_* as follows (von Caemmerer et al., 1994):

$$\Gamma_* = p_i^* + R/g_w \quad (9)$$

Γ_* is greater than p_i^* because mitochondrial

respiratory efflux increases CO_2 within the chloroplast above that in the substomatal cavities. Another uncertainty with the variable J method is the fraction of electrons that flow to sinks other than photosynthesis (Laisk and Loreto, 1996). If there are other sinks, fluorescence tends to overestimate the electron transport rate and consequently underestimates g_w .

3. ^{18}O Uptake in Combination with Gas Exchange

The uptake of O_2 by a leaf includes three processes: photorespiration, O_2 photoreduction (Mehler reaction) and dark respiration. The total O_2 uptake can be measured mass spectrometrically by using ^{18}O (Renou et al., 1990). Net CO_2 uptake, on the other hand, is gross photosynthesis less carbon losses in photorespiration and dark respiration and can be measured by CO_2 gas exchange.

This method for calculating g_w is based on the comparison of CO_2 and O_2 uptake and assumes that: 1. O_2 photoreduction is negligible and 2. nonphotorespiratory CO_2 evolution, R , is known. Oxygen uptake (U_o) is given by the sum of velocity of oxygenation (v_o), oxygen consumed in the oxidation of glycolate (0.5 v_o) and nonphotorespiratory CO_2 evolution, R :

$$\begin{aligned} U_o &= v_o + 0.5v_o + R \\ v_o &= 2/3(U_o - R). \end{aligned} \quad (10)$$

CO_2 assimilation rate, A , equals rate of carboxylation (v_c) minus half the rate of oxygenation (0.5 v_o) and R :

$$\begin{aligned} A &= v_c - 0.5v_o - R \\ v_c &= A + U_o/3 + 2R/3. \end{aligned} \quad (11)$$

Just as in the fluorescence method, it is necessary to use Γ_* . By definition (Farquhar and von Caemmerer, 1982),

$$\begin{aligned} p_c &= 2 \times \Gamma_* / (v_o/v_c) \\ &= \Gamma_* \times (3A + U_o + 2R) / (U_o - R). \end{aligned} \quad (12)$$

From the water and CO_2 exchange measurements, p_i is known, which enables g_w to be calculated from the definition, $g_w = A/(p_i - p_c)$.

This method has been successfully used to investigate the drawdown between p_i and p_c following

water stress in wheat (Renou et al., 1990) and to examine the role of carbonic anhydrase in *Commelina* (Peltier et al., 1995). A drawback of the method was that whole shoots were enclosed in the chamber leading to uncertainties in both leaf temperature (necessary for calculating p_i) and R . The use of ^{18}O and the need for a mass spectrometric system limits the availability of this method.

4. Comparison of the Methods for Measuring Internal Conductance

Three different techniques for measuring g_w have been described. Each requires a parameter to be measured in addition to conventional gas exchange in order to assess the restriction to CO_2 diffusion within the leaf. Loreto et al. (1992) have carried out what appears to be the only comparison between methods so far. They compared four leaves of both *Quercus rubra* and *Xanthium strumarium* using carbon isotope discrimination and chlorophyll fluorescence methods. For *Quercus*, which had a lower photosynthetic capacity than *Xanthium*, the different methods all yielded values of g_w around $0.15 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$. For *Xanthium*, isotope discrimination yielded a value of $0.49 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$ which was 20% less than the variable J method, while the constant J method failed to yield a minimum variance. To incorporate other leaves where just one method had been used, Loreto et al. (1992) also examined the relationship between g_w and photosynthetic rate at ambient CO_2 and high irradiance (see also below in section C). Again, there was close agreement between the methods for leaves with values of g_w below $0.3 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$ and the fluorescence methods yielded higher values than carbon isotope discrimination for leaves with g_w above $0.3 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$. The fact that independent methods yield similar values for g_w is strong evidence that the magnitude of the diffusion gradient within the leaf is accurate.

One can also compare the methods on the basis of their reliability and ease of use. If an absolute value is required, the carbon isotope discrimination method with measurements made over a range of irradiances is undoubtedly the most robust. Single measurements are to be avoided as they introduce uncertainty due to the discrimination associated with photorespiration and other experimental errors. Unfortunately, this method is not readily available as it requires access to a high precision mass spectrometer. Therefore, it is

more likely that one of the chlorophyll fluorescence methods would be chosen. These methods work best for leaves with values of g_w below $0.3 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$. The constant J method only uses chlorophyll fluorescence to indicate the range of CO_2 over which electron transport is constant. It is therefore more robust than the variable J method which requires that the chlorophyll fluorescence signal is calibrated and assumptions about alternative electron acceptors.

If one is only interested in comparison between cultivars or species, then measurement of the carbon isotopic composition of source air and of extracted sugars or dry matter in combination with gas exchange measurements to determine p_i , may be acceptable. It is important to sample source air when plant material is grown indoors, in glasshouses or with CO_2 supplementation. A simple method is to grow C_4 plants alongside the material of interest. The isotopic signal in extracted sugars is more likely to reflect current photosynthesis and the estimate of p_i than that in dry matter. While this technique is less accurate, it is more accessible because the carbon isotopic composition can be readily made in many laboratories.

C. Relationship Between Internal Conductance and CO_2 Assimilation Rate

There have now been a range of species where g_w has been measured either by Δ or fluorescence methods. What has emerged is a striking correlation between the photosynthetic capacity and internal conductance of a leaf (Fig. 10A). A line across the diagonal yields an average drawdown from p_i to p_c of $78 \mu\text{bar}$, only slightly less than the drawdown from p_a to p_i (Fig. 6). Mesophytic and sclerophyllous leaves are distinguished by open and solid symbols, respectively, to emphasize that they share a common relationship. In general, sclerophyllous leaves have lower photosynthetic capacities as well as lower internal conductances. Since the ratio A/g_w is similar for both sclerophyllous and mesophytic leaves, the drawdown from substomatal cavities to sites of carboxylation is similar in both leaf types (Fig. 10B). That is, low internal conductance does not mean that p_c will be low. If p_c is low, this may well be due to low stomatal conductance. It is time to correct the mistaken impression that has emerged in the literature that woody species are more disadvantaged by low internal conductances. This has been suggested by both Lloyd et al. (1992) and Epron et al. (1995), despite the fact that their data shows that the ratio of A/g_w for woody

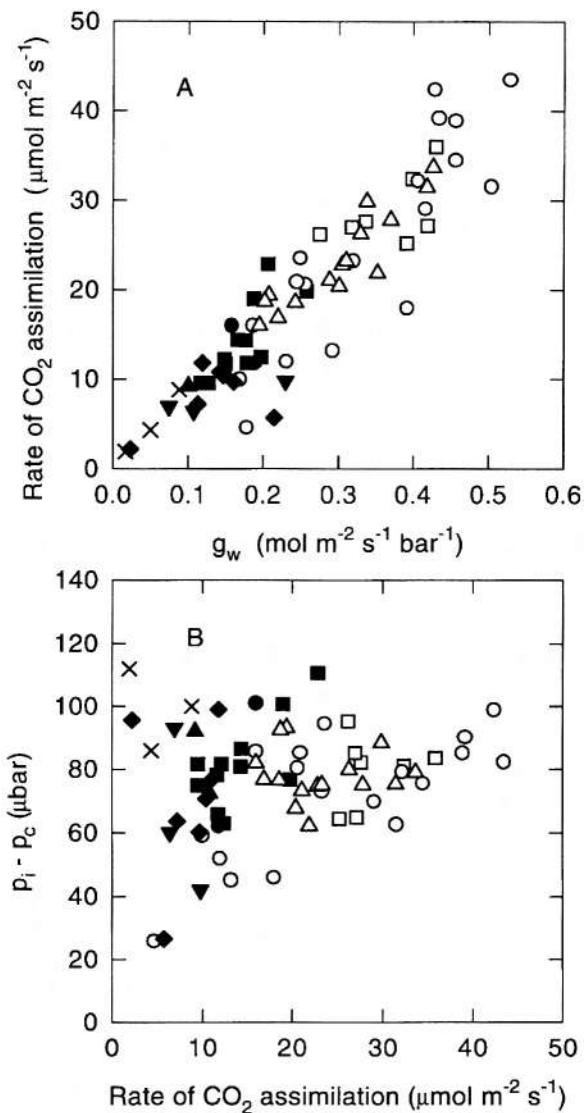


Fig. 10. A. Correlation between CO_2 assimilation rate and CO_2 transfer conductance. B. CO_2 drawdown from substomatal cavities to sites of carboxylation ($p_i - p_c$) versus CO_2 assimilation rate. Mesophytic (open) leaves are distinguished from sclerophyllous (solid) leaves. Data from: ○, ● – von Caemmerer and Evans (1991); □, ■ – Lloyd et al. (1992); ♦ – Loreto et al. (1992); ▲ – Epron et al. (1995); △ – Evans and Vellen (1996); ▼ – Roupard et al. (1996); × – Lauteri et al. (1997). Average drawdown in CO_2 partial pressure from intercellular airspaces to sites of carboxylation (A/g_w) is $83 \mu\text{bar}$.

species is similar to that for herbaceous leaves. While it is true that there was a trend for p_c to be lower for the leaves with lower photosynthetic rates (Lloyd et al., 1992), this was due to lower p_i because the difference between p_i and p_c was independent of photosynthetic capacity. Epron et al. (1995) stated

that 'high internal resistances to CO₂ transfer may account for the low net CO₂ assimilation rates that often characterize tree leaves'. However, low assimilation rate reflects the low biochemical capacity, not a high internal resistance. If high internal resistance was the cause of low CO₂ assimilation rates, $p_i - p_c$ would be much greater for sclerophytic leaves and this is not observed (Fig. 10B).

Over the lifespan of the leaf, both photosynthetic capacity and g_w change. Studies with wheat have shown that as leaves age, both photosynthetic capacity and g_w decline in parallel (Loreto et al., 1994; Evans and Vellen, 1996; Fig. 10 Δ). The changes are slow, occurring over periods of days to weeks, reflecting the remobilization of photosynthetic proteins such as Rubisco. The factors that may determine g_w are discussed below in section D, but it is not yet clear what factors are responsible for the decline in g_w as leaves age. The relative stability of g_w contrasts with that of stomatal conductance which can respond in seconds to minutes. Another intriguing observation is the suggestion that g_w might be sensitive to the growth temperature in rice (Makino et al., 1994), something which has yet to be followed up.

D. Determinants of Internal Conductance

CO₂ assimilation is distributed between chloroplasts and cells within the leaf. This means that the use of the Ohm's law analogy for resistances in series is prone to error. Parkhurst (1994) has examined in great detail how this analogy does not provide an adequate description of intercellular CO₂ partial pressure. The pattern of CO₂ uptake and distribution of photosynthetic capacity through the leaf can also bias the calculation of internal conductance (Lloyd et al., 1992). Bearing these problems in mind, it is still useful to consider the internal resistance as the sum of the intercellular airspace resistance, r_{ias} , and the liquid phase resistance, r_{liq} ,

$$r_w = r_{ias} + r_{liq} \text{ or } g_w = \frac{g_{liq}}{1 + \frac{g_{liq}}{g_{ias}}} \quad (13)$$

Chloroplasts cover the majority of the exposed cell surface area in tobacco and because diffusion through liquid in the absence of carbonic anhydrase is slow, CO₂ is unlikely to enter the chloroplast from the side unless it is derived from respiration and photorespiration. Therefore, conductance across the

cell wall and chloroplast can be scaled up to a projected leaf area basis by multiplying by the exposed chloroplast surface area per unit leafarea, S_c . That is,

$$g_{liq} = g'_{liq} \cdot S_c, \quad (14)$$

where g'_{liq} is the conductance across the cell wall and chloroplast per unit exposed chloroplast surface area. Substituting Eq. (14) into Eq. (13) we obtain

$$g_w = \frac{g'_{liq} \cdot S_c}{1 + \frac{g'_{liq} \cdot S_c}{g_{ias}}} \quad (15)$$

If $g_{ias} \gg g'_{liq}$, we can approximate Eq. (15) by,

$$g_w = g'_{liq} \cdot S_c. \quad (16)$$

Thus it can be seen that g_w is proportional to S_c if the airspace conductance is large relative to g'_{liq} or proportional to $g'_{liq} \cdot S_c$.

1. Intercellular Airspace Resistance

Having reached the sub-stomatal cavity of a leaf, CO₂ needs to diffuse throughout the intercellular airspaces to reach cell wall surfaces adjacent to chloroplasts. In bifacial leaves with spongy mesophyll tissue on the abaxial (lower) surface, intercellular airspaces are large (Fig. 7). This should offer little resistance to CO₂ movement apart from the distance traveled. Palisade tissue on the adaxial (upper) surface is much more ordered and can be tightly packed. Here, CO₂ diffusion must occur through narrow gaps alongside adjacent cells. Most CO₂ needs to reach this tissue as it is this part of the leaf that dominates CO₂ assimilation (Nishio et al., 1993).

Amphistomatous leaves which have stomata on both the upper and lower surfaces make it possible to independently measure the substomatal CO₂ partial pressure of each surface. Sharkey et al. (1982) measured leaves of *Xanthium* and *Gossypium* in order to validate the technique used to calculate p_i . The difference between p_i calculated on one surface and measured on the opposite surface ranged between 0 and 9 µbar. For wheat leaves, where p_i was calculated independently for each surface, the two values agreed to within experimental error (about 5 µbar, Evans, 1983a). In other studies with 350 µbar CO₂ outside the leaf, differences between the upper and lower surface p_i values amount to 0 to 20 µbar, or less than

10 μbar on average (Mott and O'Leary, 1984; Wong et al., 1985b; Parkhurst et al., 1988). Since CO_2 is being absorbed by cells throughout the leaf, these measurements are not simply related to conductance to diffusion across a leaf. A more direct method is to use an inert gas such as helium or N_2O . This has yielded values of intercellular conductance across the leaf of 0.33 mol $\text{m}^{-2} \text{s}^{-1}$ for *Xanthium* (Farquhar and Raschke, 1978; Mott and O'Leary, 1984) and 0.017 or 0.07 mol $\text{m}^{-2} \text{s}^{-1}$ for *Zea mays* (Long et al., 1989; Farquhar and Raschke, 1978, respectively). The lower value for *Zea mays* is probably associated with a very dense mesophyll layer through the center of leaves of some cultivars. As it is possible to determine intercellular conductance by this method only with amphistomatous leaves and since intercellular conductance to CO_2 diffusion involves lateral as well as vertical movement, these conductances are probably underestimates. Amphistomatous leaves with high rates of photosynthesis are probably unlikely candidates for a limiting conductance through intercellular airspace. It seems more likely that limiting intercellular airspace conductances would occur in leaves which have stomata only on one surface.

Another approach is to compare gas exchange in air with that in Helox (air in which nitrogen is replaced by helium) as rates of diffusion in Helox are 2.3 times faster than in air. Resistance to diffusion in intercellular airspaces can be reduced in Helox which could increase photosynthetic rate at a given substomatal CO_2 partial pressure. Helox increased photosynthetic rates by an average of 2 and 12% for amphistomatous and hypostomatous leaves, respectively (Parkhurst and Mott, 1990). Using the observed correlation between internal conductance and photosynthetic capacity shown in Fig. 10 ($g_w = 0.012A$) and taking values for the rate of CO_2 assimilation and p_i of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 249 μbar for amphistomatous and 9 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 256 μbar for hypostomatous leaves, one can calculate that p_c was 166 and 173, respectively, for the two leaf types. Knowing that Helox increased the rates of CO_2 assimilation by 2 and 12% by decreasing intercellular airspace resistance by a factor of 2.3, one can calculate that intercellular airspace resistance in normal air represents about 10 and 57% of the internal resistance in amphistomatous and hypostomatous leaves, respectively.

A large intercellular airspace resistance was inferred for the thick *Metrosideros* leaves up an

elevational transect (Vitousek et al., 1990; see Fig. 7G). A strong positive correlation was found between $\delta^{13}\text{C}$ and leaf mass per unit leaf area (i.e. CO_2 drawdown from surrounding air to inside the chloroplasts increased with increasing LMA). From gas exchange measurements, they were able to show that the ratio of intercellular to ambient CO_2 partial pressures did not differ greatly along the elevational transect which left internal conductance as the most likely source of variation. Estimating g_w from the data presented of photosynthetic rate, p_i/p_a and Δ of leaf dry matter reveals a negative relationship between g_w and leaf mass per unit leaf area, LMA, (Fig. 11). The increase in LMA may reflect an increase in leaf thickness as well as an increase in tissue density, both of which are consistent with the calculated lowering of g_w . This trend is not evident in other species for which data is available. For tobacco, increased g_w was found in leaves that had been grown under higher irradiance which resulted in higher LMA (Evans et al., 1994). Syvertsen et al. (1995) pooled their species (peach, citrus and *Macadamia*) to obtain a negative dependence of g_w on LMA, but such a dependence is not evident when each species

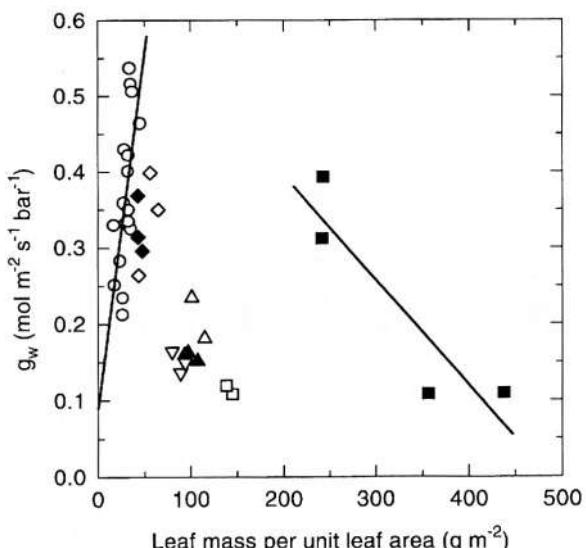


Fig. 11. Relationships between internal conductance and leaf mass per unit leaf area. Data are from Evans et al. (1994) tobacco – ○; Syvertsen et al. (1995) sun peach – ◇, shade peach – ◆, sun lemon – △, shade lemon – ▲, sun grapefruit – ▽, macadamia – □; Vitousek et al. (1990) *Metrosideros polymorpha* ■. If intracellular airspaces represent a significant resistance, internal conductance should decline with increasing LMA as seen for *Metrosideros*. By contrast, the increase in g_w in tobacco was associated with increased chloroplast surface area exposed to intercellular airspace which requires increased LMA.

is examined by itself. For both peach and citrus, growth in part shade resulted in lower LMA than for leaves in full sun as well as slightly lower g_w , although the differences were slight. Clearly more data is needed, especially for species which have dense or thick leaves. To resolve the separate contributions of intercellular and liquid components of the resistance pathway also calls for another approach, such as the combination of Helox with isotope discrimination or chlorophyll fluorescence. By examining Δ or chlorophyll fluorescence concurrently with gas exchange in both normal air and Helox, it would be possible to dissect the relative contribution of the two components. If intercellular airspaces constitute a significant resistance to CO_2 diffusion, then changing from air to Helox should result in a significant increase in Δ at a given p/p_a .

2. Intracellular Resistance

a) Surface Area of Chloroplasts Exposed to Intercellular Airspace, S_c

There is a striking analogy between CO_2 exchange across mesophyll cell walls in leaves and mammalian lungs. The resting exchange rate of CO_2 in mammals is $3 \mu\text{mol} (\text{m alveolar surface})^{-2} \text{s}^{-1}$, regardless of animal size over four orders of magnitude (Tenney and Remmers, 1963; Fig. 12). Wheat and tobacco photosynthesizing in sunlight have similar exchange rates per unit chloroplast surface area adjacent to intercellular airspace. Rapid exchange requires large surface areas to reduce gradients across the interfaces in both lungs and leaves. However, the similarity in CO_2 exchange rate per unit surface area is not due to similar conductances. In the lung, CO_2 partial pressure is, on average, 20 mbar while the partial pressure of CO_2 in the blood declines from 60 to 53 mbar as it passes through the lungs. The average partial pressure difference is thus 36 mbar. In wheat leaves, the difference from substomatal cavities to the sites of carboxylation is only 80 μbar . This suggests that the conductance to CO_2 from the air to the liquid phase is 450 times greater in leaves than in lungs. Allowing for the fact that CO_2 exchange rate increases ten-fold in an active mammal over the resting rate, the conductance in leaves is still about 45 times greater than in mammalian lungs. The reason for this difference is unclear given that the liquid pathlengths appear similar. Plants require a greater conductance to support the rapid exchange of CO_2 during

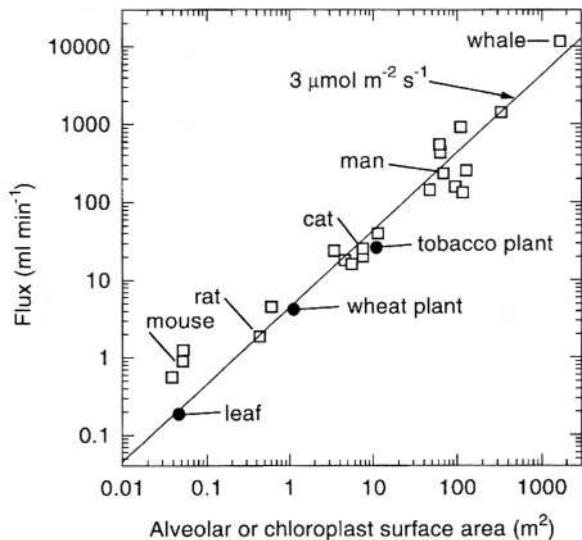


Fig. 12. Correlation between gaseous flux and alveolar or chloroplast surface area in mammals and plants. Solid line represents an exchange rate of $3 \mu\text{mol m}^{-2} \text{s}^{-1}$. Data for mammals from Tenney and Remmers (1963).

photosynthesis, but at present we do not understand exactly how they achieve this.

For leaves where intercellular airspace resistance plays a minor role, Eq. (16) suggests that g_w should be proportional to the surface area of chloroplasts exposed to intercellular airspace per unit leaf area, S_c , (Laisk et al., 1970). Mesophyll structure of leaves is diverse, as shown in Fig. 7. Palisade tissue is found in many dicot leaves near the adaxial surface. This structure allows close packing of cells with a high surface to volume ratio while still leaving regular intercellular airspaces. The arrangement is interrupted beneath stomata to form sub-stomatal cavities, but generally stomatal density is less on adaxial than abaxial surfaces. Spongy mesophyll adjacent to abaxial surfaces allows free and unrestricted diffusion away from stomata on this surface.

Cell size or volume is related to nuclear DNA content or ploidy. Wheat provides a nice example of this as cell volume increases with ploidy (Dunstone and Evans, 1974). Mesophyll cells of diploid, tetraploid and hexaploid wheat had volumes of 6710 , 13050 and $17860 \mu\text{m}^3$, respectively (1:1.9:2.7, Kaminski, 1984). Along with larger cells, Rubisco content increases in proportion with ploidy level (wheat, Dean and Leech, 1982; alfalfa Molin et al., 1982) so that Rubisco per cell increases from 1:2.1:3.2 for diploid:tetraploid:hexaploid wheat (Pyke and Leech, 1987). If the cells were spherical, this would

result in a decline in the surface area to volume ratio relative to diploid cells by 20 and 28% for the tetraploid and hexaploid wheat, respectively. However, the cells become progressively more lobed with increasing ploidy (Dunstone and Evans, 1974, Chonan et al., 1977, Parker and Ford, 1982, Sasahara, 1982) such that exposed mesophyll cell surface per unit leaf area, S_{mes} , is independent of ploidy (Kaminski et al., 1990). Mesophyll cell lobing was noted by Haberlandt (1914) to occur in many species and is illustrated in Fig. 13 for wheat (A,B,C), pine (E) and tobacco (F).

S_{mes} varies considerably between species and with growth irradiance. Turrell (1936) measured 11 species, finding S_{mes} ranged from 13.5 and 26.4 for shade and sun leaves of *Syringa vulgaris*, up to 62.5 for *Eucalyptus globulus*. While S_{mes} has been measured in quite a few studies, it is the proportion of S_{mes} that is covered by chloroplasts that is probably the more relevant measure that needs to be made. Interestingly, the proportion of cell wall covered by chloroplasts is not related to chloroplast number (Ellis and Leech, 1985). Mutants of *Ambidopsis* that have impaired chloroplast division have the same proportion of cell wall covered by chloroplasts as wildtype because the chloroplasts are larger (Pyke and Leech, 1992, 1994; Pyke et al., 1994). The proportion of cell wall covered varies between species, from 86–93% for tobacco (Evans et al., 1994), 76% for wheat (Evans, 1983a) and 73% for spinach (Honda et al., 1971) down to 26–34% for three shade species (Araus et al., 1986). Chloroplasts are generally adjacent to cell walls exposed to intercellular airspace in palisade tissue (Fig 13D; Psaras, 1986, Bolhar-Nordenkampf and Draxler, 1993, Psaras et al., 1996) as well as spongy tissue (Fig. 13F) where the absence of chloroplasts on walls shared by an adjacent cell is seen most readily.

The measurement of S_c is laborious and in the past it has been the total surface area of mesophyll cells exposed to airspace that has been measured (S_{mes} , Nobel et al., 1975). Consequently there are not many data sets to generalize from yet. The available data is presented in Fig. 14. Setting aside *Macadamia*, *Kalanchoe* and aging wheat leaves, there is a remarkable similarity between all the remainder. Tobacco, recently fully expanded wheat flag leaves and sun- and shade-grown peach and citrus leaves, all share a conductance of 24 mmol (m chloroplast surface)⁻² s⁻¹ bar⁻¹. This similarity is despite considerable variation in leaf thickness and porosity,

again highlighting that the intercellular airspace is a less important determinant of g_w . *Macadamia* has around half, and the thick CAM leaf of *Kalanchoe* around one quarter of the internal conductance of the other group, possibly indicating that these leaves do have a significant intercellular airspace resistance. The picture is further complicated by the change found in wheat leaves as they aged (Evans and Vellen, 1996). In the early stages of leaf aging, photosynthetic capacity and g_w were both declining in parallel without any change in exposed chloroplast surface area, presumably because the chloroplasts were becoming thinner. This suggests that the permeability of one or more component of the liquid diffusion pathway declined with increasing age. At present this is an intractable problem experimentally.

Haberlandt (1914, Fig. 107) observed ‘in the photosynthetic tissues of higher plants, the chloroplasts adhere exclusively, or in great part, to those walls which abut upon airspaces; by this means they evidently obtain the most favorable conditions for the absorption of carbon dioxide.’ The reason for this is that CO₂ diffuses 10,000 times more slowly in water than air so that short liquid pathways are essential if rapid CO₂ exchange is to occur. The other way to reduce resistance is to increase the surface area available for gas exchange. Both are employed by the leaf. Photosynthetic capacity of a leaf can be increased in many species by higher growth irradiance. Increases in photosynthetic capacity require extra Rubisco and thylakoid proteins so that the chloroplast volume per unit leaf area increases. At the same time, the internal leaf surface increases so that changes in the ratio of photosynthetic capacity to chloroplast surface area exposed to intercellular airspace are small (Nobel et al., 1975; Evans et al., 1994).

b) Liquid Phase Resistance

The resistances imposed by the cell wall and segments of the liquid phase can be roughly calculated. This requires assumptions about the effective diffusivities of CO₂ through the cell wall and cytosol and the permeabilities of the plasmalemma and chloroplast envelope (Evans et al., 1994). The resistance to CO₂ imposed by plant membranes is unknown. Measurements have been made on red blood cells (167 s m⁻¹, 4.17 m² s bar mol⁻¹; Solomon, 1974), lecithin-cholesterol lipid bilayers (286 s m⁻¹, Gutknecht et al., 1977) and the plasma membrane of *Chlamydomonas*

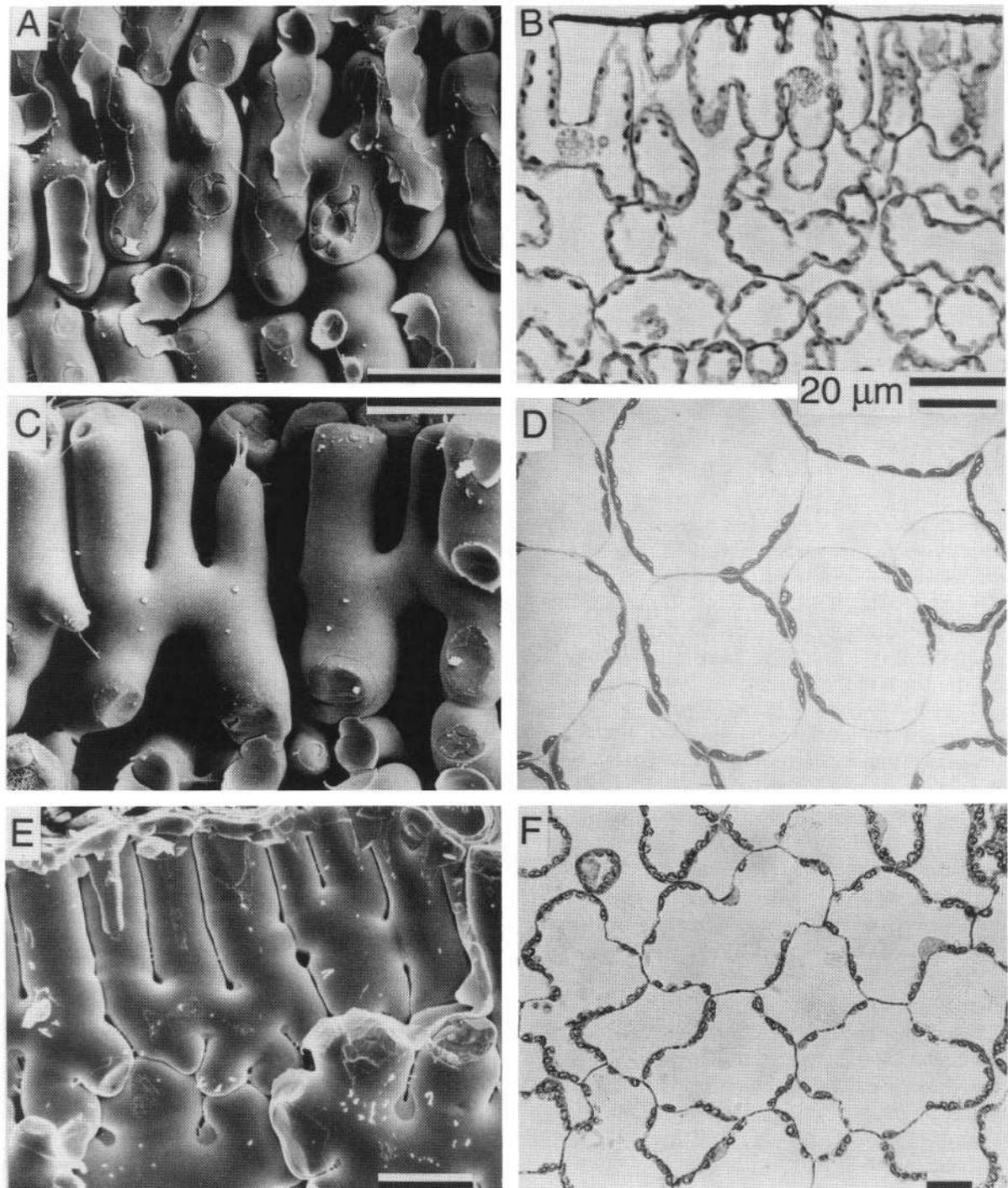


Fig. 13. Cell lobing and chloroplast positioning. In order to maximize surface area to cell volume, mesophyll cells of many species are lobed. Wheat (A,B,C longitudinal), tobacco (D,F paradermal) and *Pinus* (E). Chloroplasts are appressed against the cell surface adjacent to airspace and rarely against walls with neighboring cells. When viewed in cross-section, wheat mesophyll cells appear circular or elliptical. When viewed in longitudinal section, the lobing becomes apparent, although depending on where the section is cut, the lobes may appear like small circular cells.

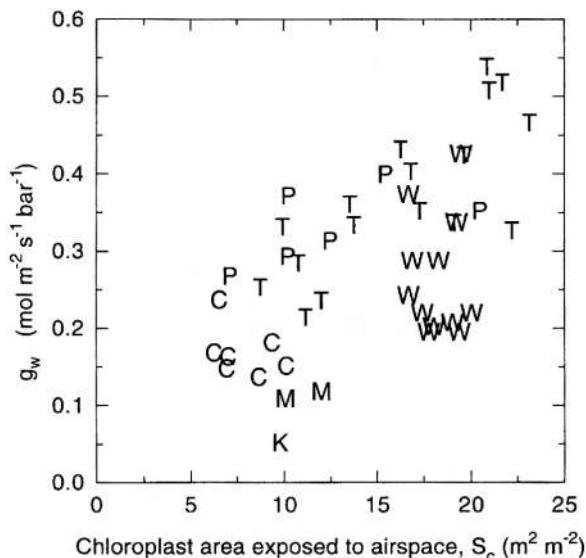


Fig. 14. Relationship between internal conductance and chloroplast surface area exposed to intercellular airspace. Data from Evans et al. (1994) tobacco – T; Syvertsen et al. (1995) peach – P, citrus – C, macadamia – M; Evans and Vellen (1996) wheat – W; Maxwell et al. (1997) Kalanchoe – K. Tobacco, young wheat, peach and citrus appear to share a common relationship. The wheat data represent an age sequence for flag leaves, showing that the decline in g_w with increasing leaf age that paralleled the decline in assimilation rate was not associated with a reduction in chloroplast surface area.

(56,000–67,000 sm^{-1} , Sultemeyer and Rinast, 1996). This last resistance value is so high that photosynthesis in higher plants would not be possible without an active CO_2 concentrating mechanism, whereas the first two estimates result in about 50% of the liquid resistance being imposed by the lipid membranes. Once inside the chloroplast, CO_2 diffusion across the chloroplast is facilitated by carbonic anhydrase, CA, which rapidly interconverts CO_2 and bicarbonate so that many more molecules are available for diffusion (at pH 8, the ratio of bicarbonate to CO_2 is 45). For tobacco, where intercellular airspace resistance is probably negligible, the internal resistance is 43 m^2 chloroplast s bar mol^{-1} (Evans et al., 1994). By subtracting from this the estimated resistances imposed by the cell wall (12.5), membranes (3×4.17) and cytosol (2.5), we are left with 16 m^2 chloroplast s bar mol^{-1} (38%) due to the resistance within the chloroplast.

It has been possible to reduce CA activity to 1% of wild-type activity in transgenic tobacco containing an antisense gene for CA which resulted in lowering the CO_2 partial pressure in the chloroplast by about 15 μbar and decreasing the internal conductance by

25–30% (Price et al., 1994; Williams et al., 1996). Another way to examine the role of CA has been to inhibit the activity with ethoxzolamide (Peltier et al., 1995). Working with *Commelina communis*, Peltier et al. found that 97% inhibition of CA activity reduced the CO_2 diffusion constant by 20%. While the authors stated that the ratio of p_c/p_a was not significantly different and therefore that the major part of CA was not involved in the transfer of CO_2 , one can calculate that internal conductance was actually reduced by nearly 20%, similar to the reduction in the CO_2 diffusion constant. Thus independent techniques and laboratories have all observed a similar effect following removal of 95% of CA activity, confirming that CA facilitates CO_2 diffusion across the chloroplast by reducing diffusion resistance within the chloroplast. CA plays a similar role in facilitating CO_2 diffusion and interconversion in red blood cells. Surprisingly, complete inhibition of CA in the blood by specific inhibitors did not noticeably affect CO_2 transport in the bloodstream (Schmidt-Nielsen, 1991).

In a study of transgenic tobacco having excess phytochrome, Sharkey et al. (1991) observed that the transgenic plants had a lower photosynthetic capacity than expected on the basis of their Rubisco content which was actually greater than the wild-type plants. The leaves were thicker and contained cup-shaped chloroplasts which, it was suggested, reduced the internal conductance because of the additional thickness of cytoplasm that CO_2 needed to traverse to reach the sites of carboxylation. Detailed measurements revealed that the average thickness of the cytoplasm at the middle of the chloroplast was increased from 0.25 to 0.55 μm in the leaves of the transgenic plants. This increase in cytoplasmic thickness seems insufficient to explain the lower photosynthetic capacity, but reflects the uncertainties in our current understanding. Although carbonic anhydrase is mainly located in chloroplasts, significant amounts (13%) have also been found in the cytoplasm of *Solanum tuberosum* (Rumeau et al., 1996) and 1 to 3% of CA in spinach and wheat has been localized to the plasmalemma (Utsunomiya and Muto, 1993). It is therefore possible that CA could be increasing the permeability of CO_2 through the plasmalemma and cytoplasm as well as the chloroplast stroma. Facilitation of CO_2 diffusion by CA in the cytoplasm would be less effective because the lower cytoplasmic pH results in a smaller ratio of bicarbonate to CO_2 than in the chloroplast.

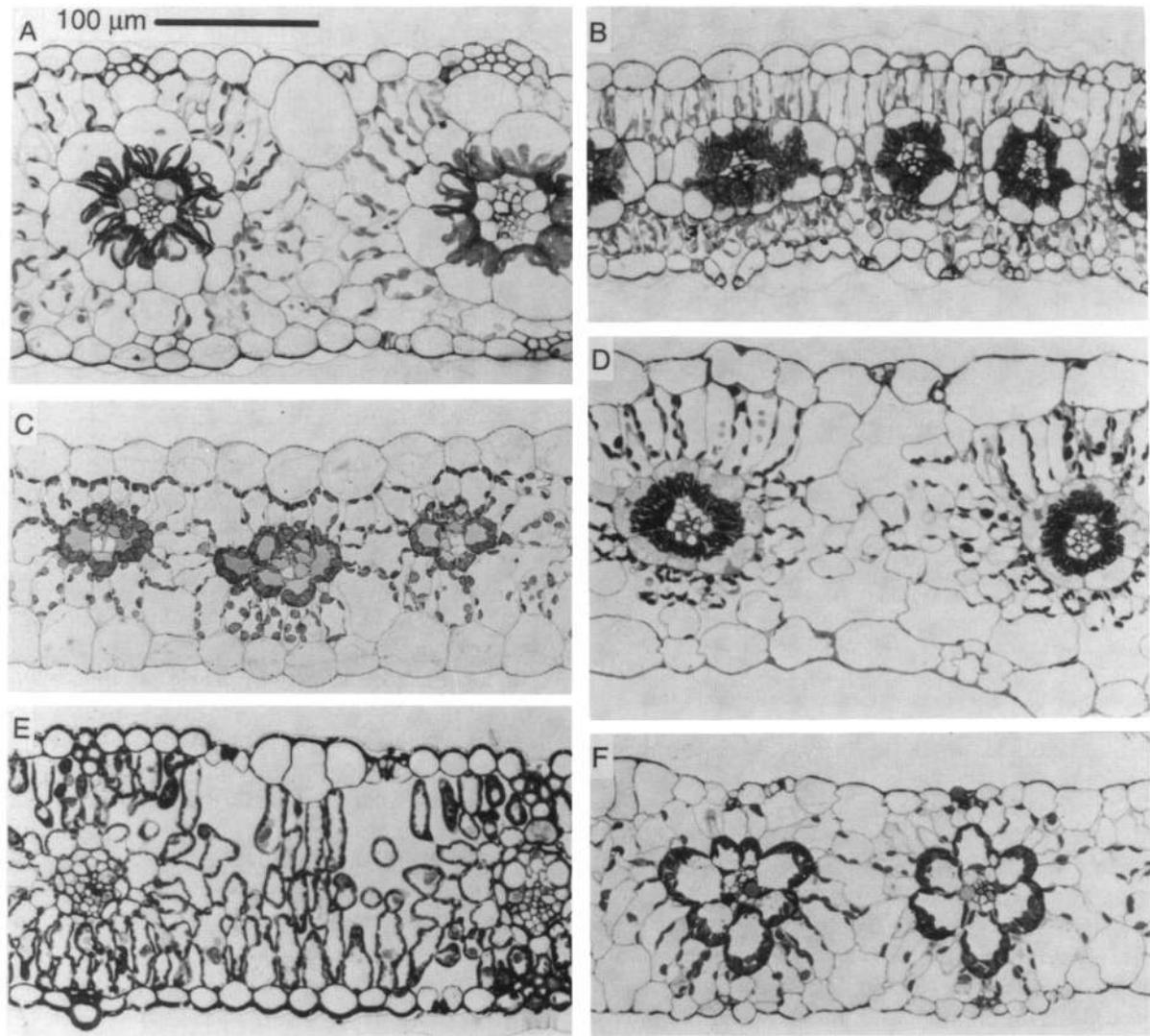


Fig. 15. C₄ leaf structures in cross section. A, *Eleusine coracana* NAD-ME monocot; B, *Amaranthus edulis* NAD-ME dicot; C, *Zea mays* NADP-ME monocot; D, *Gomphrena globosa* NADP-ME dicot; E, *Triticum aestivum* C₃; F, *Urochloa panicoides* PCK monocot. C₄ material from Henderson et al. (1992), wheat from L Vellen).

V.C₄

A. CO₂ Diffusion in C₄ Leaves

C₄ photosynthesis requires a specialized leaf anatomy where photosynthetic cells are organized in two concentric cylinders (Fig. 15). The 'Kranz' or wreath anatomy was recognized by Haberlandt (1914, Fig. 113) who speculated at the time 'It is uncertain whether this green inner sheath merely represents an unimportant addition to the chlorophyll-apparatus of the plant, or whether there exists some as yet

undiscovered division of labor between the chloroplasts in the sheath and those in the girdle-cells'. The C₄ photosynthetic pathway is characterized by a CO₂ concentrating mechanism that involves the co-ordinated functioning of mesophyll and bundle-sheath cells within a leaf. CO₂ is initially assimilated into C₄ acids by phosphoenol pyruvate (PEP) carboxylase in mesophyll cells. These acids then diffuse to the bundle-sheath cells where they are decarboxylated. This concentrates CO₂ in the bundlesheath which enhances RuBP carboxylation while inhibiting RuBP oxygenation (Hatch and Osmond, 1976). Thin-walled

mesophyll cells adjacent to intercellular airspace radiate from thick-walled bundle-sheath cells. The diffusion of CO_2 back out from the bundlesheath limits the efficiency of the CO_2 concentrating mechanism and many attempts have been made to quantify the CO_2 diffusion resistance across the bundlesheath (Jenkins et al., 1989; Brown and Byrd, 1993; Hatch et al., 1995). Limitations to CO_2 diffusion from intercellular airspaces to the mesophyll cells have received less attention (Longstreth et al., 1980).

B. From Intercellular Airspace to the Mesophyll

The necessity for metabolite transport between mesophyll and bundle-sheath cells requires intimate contact between these cells and limits the amount of mesophyll tissue which can be functionally associated with bundlesheath tissue. For example, the number of chlorenchymatous mesophyll cells between adjacent bundlesheaths is usually between two and four. Mesophyll surface area exposed to intercellular airspace per unit leaf area is slightly less in C_4 than in C_3 species (Longstreth et al., 1980; Pearcy et al., 1982; Dengler et al., 1994) and thus the surface area available for CO_2 diffusion is also less.

No robust techniques are presently available that allow the estimation of conductance to CO_2 diffusion from intercellular airspace to sites of PEP carboxylation. Carbon isotope discrimination cannot be used because of the low discrimination factor of PEP carboxylase and the confounding effects of CO_2 leakage from the bundlesheath (Henderson et al., 1992). Similarly, chlorophyll fluorescence signals are not suitable as bundlesheath and mesophyll cells have different chloroplast populations. In an approach analogous to Evans (1983b), Pfeffer and Peisker (1995) compared the change in the reciprocal of initial slope of a CO_2 response curve against PEP carboxylase activity for maize leaves grown under natural light or shade. By assuming that internal conductance was the same for all leaves, they derived an estimate of $0.8 \text{ mol m}^{-2} \text{s}^{-1}$ for maize (Pfeffer and Peisker, 1995). The value obtained for wheat was $0.49 \text{ mol m}^{-2} \text{s}^{-1} \text{ bar}^{-1}$ (Evans, 1983b) which is similar to the value obtained by the more rigorous stable carbon isotope method (von Caemmerer and Evans, 1991). Thus, on this limited evidence, maize internal conductance is greater than that of wheat.

For both C_3 and C_4 species one can calculate a minimum internal conductance (Evans and von Caemmerer, 1996). This limit is reached when p_c is

reduced to the CO_2 compensation point, Γ , ($g_{wmin} = A/(p_i - \Gamma)$). At 25°C , high light and ambient CO_2 , intercellular CO_2 is $\approx 100 \mu\text{bar}$ in C_4 species versus $250 \mu\text{bar}$ for C_3 species and the compensation point is close to zero in C_4 versus $\approx 50 \mu\text{bar}$ in C_3 species. Thus for the same CO_2 assimilation rate, g_{wmin} of C_4 species needs to be approximately twice that of C_3 species. Given that the exposed mesophyll surface is less in C_4 , this requires conductance across the cell wall through to the cytosol in C_4 species to be more than double that in C_3 species. There are several possible contributing factors. Firstly, mesophyll cell walls of C_4 species may be thinner than for C_3 species ($0.07 \mu\text{m}$ *Amaranthus retroflexus*, Longstreth et al., 1980; cf $0.3 \mu\text{m}$ *Nicotiana tabacum*, Evans et al., 1994) although a general survey is needed to confirm this. Secondly, in contrast to C_3 plants where CA is mainly located in the chloroplast to facilitate CO_2 diffusion, in C_4 plants CA is found in the cytosol alongside PEP carboxylase (Hatch and Burnell, 1990). Utsunomiya and Muto (1993) separated a plasmalemma fraction from a leaf extract and found that it contained 20–60% of CA activity in *Zea mays* leaves compared to 1–3% in wheat. CA in the plasmalemma may facilitate CO_2 movement across membranes, perhaps even delivering HCO_3^- into the cytosol. Thirdly, the initial carboxylation reaction by PEP carboxylase which utilizes HCO_3^- occurs in the cytosol, so the liquid diffusion path in C_4 plants may be considerably shorter and does not have to cross the chloroplast envelope. It is likely that CO_2 assimilation rate will be sensitive to reduction in CA activity in C_4 plants because PEP carboxylase would have to rely on the uncatalyzed rate of conversion of CO_2 to bicarbonate which is 10,000 times slower than the catalyzed rate. If the observed internal conductance of C_3 leaves (Fig. 10) was also assumed for C_4 leaves, this would result in values for the CO_2 partial pressure in C_4 mesophyll cytoplasm of only $17 \mu\text{bar}$ compared to $167 \mu\text{bar}$ in the chloroplasts of C_3 leaves.

C. Across the Bundlesheath

A low conductance to CO_2 diffusion across the bundlesheath is an essential feature of the C_4 pathway. It effectively limits CO_2 exchange with the atmosphere and C_4 acid decarboxylation is the major source of CO_2 in this compartment. Inhibiting PEP carboxylase activity reduced CO_2 assimilation rate by 80–98% (Jenkins, 1989). The conductance to CO_2 diffusion

across bundlesheath walls is considerably less than across mesophyll cell walls, with estimates ranging from 0.6–2.4 mmol m⁻² leaf s⁻¹ or 0.5–0.9 mmol (m bundle sheath)⁻² s⁻¹ for different C₄ species (Jenkins et al., 1989; Brown and Byrd, 1993; Brown, 1997) compared to 25 mmol m⁻² chloroplast s⁻¹ for tobacco (Evans et al., 1994). The absence of CA in the bundlesheath prevents the rapid conversion of CO₂ to bicarbonate which would increase the diffusion of CO₂ out of the bundlesheath, similar to the way it facilitates CO₂ diffusion across chloroplasts in C₃ plants. When CA from tobacco was expressed in transgenic *Flaveria bidentis* leaves, leakage of bicarbonate out of the bundle sheath was enhanced, as measured by concurrent carbon isotope discrimination and gas exchange measurements (von Caemmerer et al., 1997). The liquid pathlength is also long, imposing a considerable resistance. For NAD ME decarboxylation types, chloroplasts are centripetally arranged (Fig. 15 A,B) and decarboxylation occurs in the mitochondria that are interposed between the chloroplasts (Hatch et al., 1975, Brown and Hattersley, 1989). In the NADP ME type, decarboxylation occurs in the bundlesheath chloroplasts which are either located centrifugally in the monocots (*Zea* Fig. 15C) or centripetally in the dicots (*Gomphrena* Fig. 15D). Furthermore, in C₄ species that have either centrifugally arranged chloroplasts (eg Fig. 15C,F) or bundlesheaths with uneven cell outlines, the bundlesheath cell wall is lined by a suberised lamella, which may also help reduce conductance to CO₂. The PCK type has decarboxylation in the cytosol, so it makes sense for chloroplasts to be centrifugally located to catch the CO₂ as it diffuses away from the vascular strand (*Urochloa* Fig. 15F).

The C₄ cycle consumes energy and so leakage of CO₂ from the bundlesheath is an energy cost to the leaf which represents a compromise between keeping CO₂ in, letting O₂ out and letting metabolites diffuse in and out at rates fast enough for the rate of CO₂ fixation. The leakage depends upon the balance between the rates of PEP carboxylation and Rubisco activity and the conductance of the bundlesheath to CO₂. In transgenic *Flaveria bidentis* leaves where antisense reduction in Rubisco content altered the balance between the C₄ and C₃ cycles, leakiness increased from 24% in wild-type to 37% (von Caemmerer et al., 1997). Various estimates have been made of what proportion of CO₂ fixed by PEP carboxylase subsequently leaks out of the bundle-

sheath. This has been termed leakiness, and estimates have ranged from 8% to 50% (see Henderson et al., 1992; Hatch et al., 1995). Meinzer and Zhu (1998) found variation in dry matter Δ correlated with variation in quantum yield associated with both leaf nitrogen content and genotype in *Saccharum* species and suggested that this was most likely due to variation in leakiness. Leaves with higher N contents had an increased ratio of Rubisco: PEP carboxylase activity and lower calculated leakiness. Despite this and earlier suggestions that leakiness differed between C₄ decarboxylation types (being less in species with a suberised lamella, Hattersley, 1982), recent measurements of leakiness have found little variation (Henderson et al., 1992; 8–12% Hatch et al., 1995). Extensive concurrent measurements of Δ with gas exchange have revealed little variation in leakiness either between species, or with variation in photosynthetic rate due to short-term changes in CO₂, irradiance and temperature, or with longer term changes in leaf nitrogen content (average 21%, Henderson et al., 1992, 1994). Since leakiness is determined not only by the physical conductance of the bundlesheath, but also by the balance of the capacities of the C₄ and C₃ cycles, this suggests that the biochemistry of C₄ photosynthesis is highly regulated.

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References

- Araus JL, Alegre L, Tapia L, Calafell R and Serret MD (1986) Relationships between photosynthetic capacity and leaf structure in several shade plants. Amer J Bot 73: 1760–1770
- Balaguer L, Afif D, Dizengremel P and Dreyer E (1996) Specificity

- factor of ribulose carboxylase/oxygenase of *Quercus robur*. *Plant Physiol Biochem* 34:879–883
- Ball JT, Woodrow IE and Berry JA (1987) A model predicting stomatal conductance and its contribution to the control of photosynthesis under different environmental conditions. In: Biggins I (ed) *Progress in Photosynthesis Research*, pp 221–224. Martinus Nijhoff, The Netherlands
- Berry JA, Collate GJ, Guy RD and Fogel MD (1994) The compensation point: Can a physiological concept be applied to global cycles of carbon and oxygen? In: Tolbert NE and Preiss J (eds) *Regulation of Atmospheric CO₂ and O₂ by Photosynthetic Carbon Metabolism*, pp 234–248. Oxford University Press, Oxford
- Beyschlag W and Pfanz H (1990) A fast method to detect the occurrence of nonhomogeneous distribution of stomatal aperture in heterobaric plant leaves. *Oecologia* 82:52–55
- Beyschlag W, Pfanz H and Rye RJ (1992) Stomatal patchiness in Mediterranean evergreen sclerophylls. *Planta* 187:546–553
- Bolhar-Nordenkampf HR and Draxler G (1993) Functional leaf anatomy. In: Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC and Long SP (eds) *Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual*, pp 91–112. Chapman and Hall, London
- Bongi G and Loreto F (1989) Gas-exchange properties of salt-stressed Olive (*Olea europaea* L.) leaves. *Plant Physiol* 90:1408–1416
- Brewer CA and Smith WK (1997) Patterns of leaf surface wetness for montane and subalpine plants. *Plant Cell Env* 20: 1–11
- Brewer CA, Smith WK and Vogelmann TC (1991) Functional interaction between leaf trichomes, leaf wettability and the optical properties of water droplets. *Plant Cell Env* 14: 955–962
- Brooks A and Farquhar GD (1985) Effect of temperature on the CO₂/O₂ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* 165: 397–406
- Brown RH (1997) Analysis of bundle sheath conductance and C₄ photosynthesis using a PEP-carboxylase inhibitor. *Aust J Plant Physiol* 24: 549–554
- Brown RH and Byrd GT (1993) Estimation of bundle sheath cell conductance in C₄ species and O₂ insensitivity of photosynthesis. *Plant Physiol* 103: 1183–1188
- Brown RH and Hattersley PW (1989) Leaf anatomy of C₃–C₄ species as related to evolution of C₄ photosynthesis. *Plant Physiol* 91:1543–1550
- Brugnoli E, Hubick KT, von Caemmerer S, Wong SC and Farquhar GD (1988) Correlation between the carbon isotope discrimination in leaf starch and sugars of C₃ plants and the ratio of intercellular and atmospheric partial pressures of carbon dioxide. *Plant Physiol* 88: 1418–1424
- Brugnoli E, Scartazza A, Lauteri M, Monteverdi MC and Mágus C (1998) Carbon isotope discrimination in structural and non-structural carbohydrates in relation to productivity and adaptation to unfavourable conditions. In: Griffiths H (ed) *Stable Isotopes: Integration of Biological, Ecological and Geochemical Processes*, pp 133–146. BIOS, Oxford
- Cardon ZG, Mott KA and Berry JA (1994) Dynamics of patchy stomatal movements, and their contribution to steady-state and oscillating stomatal conductance calculated using gas-exchange techniques. *Plant Cell Env* 17: 995–1007
- Chonan N, Kawahara H and Matsuda T (1977) Ultrastructural development of mesophyll cells in rice seedlings. *Jpn J Crop Sci* 46: 147–156
- Constable GA and Rawson HM (1980) Effect of leaf position, expansion, and age on photosynthesis, transpiration and water use efficiency of cotton. *Aust J Plant Physiol* 7: 89–100
- Cowan IR (1977) Water use in higher plants. In: McKintyre AK (ed) *Water: Planets, Plants and People*, pp 71–107. Aust Acad Sci, Canberra
- Craig S and Goodchild DJ (1977) Leaf ultrastructure of *Triodia irritans*: A C₄ grass possessing an unusual arrangement of photosynthetic tissues.
- Daley PF, Raschke K, Ball JT and Berry JA (1989) Topography of photosynthetic activity of leaves obtained from video images of chlorophyll fluorescence. *Plant Physiol* 90: 1233–1238
- Dawson TE and Ehleringer JR (1993) Gender-specific physiology, carbon isotope discrimination, and habitat distribution in boxelder, *Acer negundo*. *Ecology* 74:798–815
- Dean C and Leech RM (1982) Genome expression during normal leaf development. 2. Direct correlation between ribulose bisphosphate carboxylase content and nuclear ploidy in a polyploid series of wheat. *Plant Physiol* 70:1605–1608
- Delfine S, Alvino A, Zacchini M and Loreto F (1998) Consequences of salt stress on conductance to CO₂ diffusion, Rubisco characteristics and anatomy of spinach leaves. *Aust J Plant Physiol* 25: 395–402
- Delgado E, Parry MAJ, Lawlor DW, Keys AJ and Medrano H (1993) Photosynthesis, ribulose-1,5-bisphosphate carboxylase and leaf characteristics of *Nicotiana tabacum* L. genotypes selected by survival at low CO₂ concentrations. *J Exp Bot* 44:1–7
- Dengler NG, Dengler RE, Donelly PM and Hattersley PW (1994) Quantitative leaf anatomy of C₃ and C₄ grasses (Poaceae): Bundle sheath and mesophyll surface area relationships. *Ann Bot* 73: 241–255
- Di Marco G, Manes F, Tricoli D and Vitale E (1990) Fluorescence parameters measured concurrently with net photosynthesis to investigate chloroplastic CO₂ concentration in leaves of *Quercus ilex* L. *J Plant Physiol* 136: 538–543
- Downton WJS, Loveys BR and Grant WJR (1988) Stomatal closure fully accounts for the inhibition of photosynthesis by abscisic acid. *New Phytol* 108:263–266
- Dunstone RL and Evans LT (1974) Role of changes in cell size in the evolution of wheat. *Aust J Plant Physiol* 1:157–165
- Eckstein J, Beyschlag W, Mott KA and Rye RJ (1996) Changes in photon flux can induce stomatal patchiness. *Plant Cell Env*, 19: 1066–1074
- Ehleringer JR and Björkman O (1978) A comparison of photosynthetic characteristics of *Encelia* species possessing glabrous and pubescent leaves. *Plant Physiol* 62: 185–190
- Ellis JR and Leech RM (1985) Cell size and chloroplast size in relation to chloroplast replication in light-grown wheat leaves. *Planta* 165: 120–125
- Epron D, Godard D, Comic G and Gentil B (1995) Limitation of net CO₂ assimilation rate by internal resistances to CO₂ transfer in the leaves of two tree species (*Fagus sylvatica* L. and *Castanea sativa* Mill.) *Plant Cell Env* 18: 43–51
- Evans JR (1983a) Photosynthesis and nitrogen partitioning in leaves of *Triticum aestivum* and related species. PhD thesis, ANU, Canberra
- Evans JR (1983b) Nitrogen and photosynthesis in the flag leaf of

- wheat (*Triticum aestivum* L.). *Plant Physiol.* 72: 297–302
- Evans JR and von Caemmerer S (1996) CO_2 diffusion inside leaves. *Plant Physiol.* 110: 339–346
- Evans JR and Vellen L (1996) Wheat cultivars differ in transpiration efficiency and CO_2 diffusion inside their leaves. In: Ishii R and Horie T (eds) *Crop research in Asia: Achievements and perspective*, pp 326–329. Asian Crop Science Association, Tokyo
- Evans JR, Sharkey TD, Berry JA and Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO_2 diffusion in leaves of higher plants. *Aust J Plant Physiol.* 13: 281–292
- Evans JR, von Caemmerer S, Setchell BA and Hudson GS (1994) The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. *Aust J Plant Physiol.* 21: 475–495
- Farquhar GD and Raschke K (1978) On the resistance to transpiration of sites of evaporation within the leaf. *Plant Physiol.* 61: 1000–1005
- Farquhar GD and Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Aust J Plant Physiol.* 11: 539–552
- Farquhar GD and Sharkey TD (1982) Stomatal conductance and photosynthesis. *Ann Rev Plant Physiol.* 33: 317–345
- Farquhar GD and von Caemmerer S (1982) Modelling of photosynthetic responses to environmental conditions. In: Lange OL, Nobel PS, Osmond CB and Ziegler H, (eds) *Physiological Plant Ecology II. Water Relations and Carbon Assimilation Encycl. Plant Physiol. (New Ser.)*, Vol 12B, pp 549–587. Springer Verlag, Berlin
- Farquhar GD, O'Leary MH and Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust J Plant Physiol.* 9: 121–137
- Gastra P (1959) Photosynthesis of crop plants as influenced by light, CO_2 , temperature and stomatal diffusion resistance. Mededelingen van de Landbouwhogeschool te Wageningen, Nederland 59: 1–68
- Genty B and Meyer S (1995) Quantitative mapping of leaf photosynthesis using chlorophyll fluorescence imaging. *Aust J Plant Physiol.* 22: 277–284
- Genty B, Briantais JM and Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta* 990: 87–92
- Gillon JS and Griffiths H (1997) The influence of (photo)respiration on carbon isotope discrimination in plants. *Plant Cell Env.* 20: 1217–1230
- Gunasekera D and Berkowitz GA (1992) Heterogeneous stomatal closure in relation to leaf water deficits is not a universal phenomenon. *Plant Physiol.* 98: 660–665
- Gutknecht J, Bisson MA and Tosteson FC (1977) Diffusion of carbon dioxide through lipid bilayer membranes. *J Gen Physiol.* 69: 779–794
- Guy RD, Fogel ML and Berry JA (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. *Plant Physiol.* 101: 37–47
- Haberlandt G (1914) *Physiological Plant Anatomy*. Translation of the fourth German edition by M. Drummond, reprint edition (1965) (Today and Tomorrow's Book Agency, New Delhi)
- Harley PC, Loreto F, Di Marco G and Sharkey TD (1992) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2 . *Plant Physiol.* 98: 1429–1436
- Hatch MD and Burnell JN (1990) Carbonic anhydrase activity in leaves and its role in the first step of C_4 photosynthesis. *Plant Physiol.* 93: 825–828
- Hatch MD and Osmond CB (1976) Compartmentation and transport in C_4 photosynthesis. In: Stocking CR and Heber U (eds) *Transport in Plants III. Intracellular Interactions and Transport Processes. Encyclopedia of Plant Physiology New Series*, Vol 3, pp 144–184. Springer-Verlag, Berlin
- Hatch MD, Agostino A and Jenkins CLD (1995) Measurement of the leakage of CO_2 from bundle-sheath cells of leaves during C_4 photosynthesis. *Plant Physiol.* 108: 173–181
- Hatch MD, Kagawa T and Craig S (1975) Subdivision of C_4 pathway species based on differing C_4 acid decarboxylating systems and ultrastructural features. *Aust J Plant Physiol.* 2: 111–128
- Hattersley PW (1982) $\delta^{13}\text{C}$ values of C_4 types in grasses. *Aust J Plant Physiol.* 9: 139–154
- Hausler RE, Bailey KJ, Lea PJ and Leegood RC (1996) Control of photosynthesis in barley mutants with reduced activities of glutamine synthetase and glutamine synthase. III. Aspects of glyoxylate metabolism and effects of glyoxylate on the activation state of ribulose-1,5-bisphosphate carboxylase oxygenase. *Planta* 200: 388–396
- Henderson SA, von Caemmerer S and Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C_4 species. *Aust J Plant Physiol.* 19: 263–285
- Henderson S, Hattersley P, von Caemmerer S and Osmond CB (1994) Are C_4 pathway plants threatened by global climatic change? In: Schulze ED, Caldwell MM (eds) *Ecophysiology of Photosynthesis*, pp 529–549. Springer-Verlag, Berlin
- Honda SI, Hongladarom-Honda T, Kwanyuen P and Wildman SG (1971) Interpretations on chloroplast reproduction derived from correlations between cells and chloroplasts. *Planta* 97: 1–15
- Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC and Andrews TJ (1992) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA lowers photosynthesis in transgenic tobacco plants. *Plant Physiol.* 98: 294–302
- Jenkins CLD (1989) Effects of the phosphoenolpyruvate carboxylase inhibitor 3,3-dichloro-2-(dihydroxyphosphinoylmethyl) propenoate on photosynthesis. C_4 selectivity and studies on C_4 photosynthesis. *Plant Physiol.* 89: 1231–1237
- Jenkins CLD, Furbank RT and Hatch MD (1989) Inorganic carbon diffusion between C_4 mesophyll and bundle sheath cells. *Plant Physiol.* 91: 1356–1363
- Jones HG (1987) Breeding for stomatal characters. In: Zeiger E, Farquhar GD and Cowan IR (eds) *Stomatal function*, pp 431–443. Stanford University Press, Stanford.
- Jordan DB and Ogren WL (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Dependence on ribulosebisphosphate concentration, pH and temperature. *Planta* 161: 308–313
- Kaminski A (1984) Effects of ploidy on photosynthesis and leaf structure among *Triticum* and related species of *Hordeum vulgare*. PhD thesis, Cambridge
- Kaminski A, Austin RB, Ford MA and Morgan CL (1990) Flag

- leaf anatomy of *Triticum* and *Aegilops* species in relation to photosynthetic rate. Ann Bot 66:359–365
- Kane HJ, Viil J, Entsch B, Paul K, Morell MK and Andrews TJ (1994) An improved method for measuring the CO₂/O₂ specificity of ribulosebisphosphate carboxylase-oxygenase. Aust J Plant Physiol 21: 449–461
- Kirschbaum MUF, Gross LJ and Pearcy RW (1988) Observed and modelled stomatal responses to dynamic light environments in the shade plant *Alocasia macrorrhiza*. Plant Cell Env 11: 111–121
- Körner C, Scheel JA and Bauer H (1979) Maximum leaf diffusive conductance in vascular plants. Photosynthetica 13:45–82
- Laisk AK (1977) Kinetics of photosynthesis and photorespiration in C₃ plants (In Russian) Nauka, Moscow
- Laisk A and Loreto F (1996) Determining photosynthetic parameters from leaf CO₂ exchange and chlorophyll fluorescence. Plant Physiol 110: 903–912
- Laisk A and Sumberg A (1994) Partitioning of the leaf CO₂ exchange into components using CO₂ exchange and fluorescence measurements. Plant Physiol 106: 689–695
- Laisk A, Oja V and Rahi M (1970) Diffusion resistance of leaves in connection with their anatomy. Fiziologiya Rastenii 17, 40–48
- Lauteri M, Scartazza A, Guido MC and Brugnoli E (1997) Genetic variation in photosynthetic capacity, carbon isotope discrimination and mesophyll conductance in provenances of *Castanea sativa* adapted to different environments. Funct Ecol 11: 675–683
- Leuning R (1995) A critical appraisal of a combined stomatal-photosynthesis model for C₃ plants. Plant Cell Env 18: 339–355
- Lloyd J, Syvertsen JP, Kriedemann PE and Farquhar GD (1992) Low conductances for CO₂ diffusion from stomata to the sites of carboxylation in leaves of woody species. Plant Cell Env 15: 873–899
- Long SP, Farage PK, Bolhar-Nordenkampf HR and Rohrboeck U (1989) Separating the contribution of the upper and lower mesophyll to photosynthesis in *Zea mays* L. leaves. Planta 177: 207–216
- Longstreth DJ, Hartsock TL and Nobel PS (1980) Mesophyll cell properties for some C₃ and C₄ species with high photosynthetic rates. Plant Physiol 48: 494–498
- Loreto F and Sharkey TD (1990) Low humidity can cause uneven photosynthesis in olive (*Olea europaea* L.) leaves. Tree Physiol 6:409–415
- Loreto F, Harley PC, Di Marco G and Sharkey TD (1992) Estimation of mesophyll conductance to CO₂ flux by three different methods. Plant Physiol 98: 1437–1443
- Loreto F, Di Marco G, Tricoli D and Sharkey TD (1994) Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. Photosyn Res 41: 397–403
- McWilliam JR and Mison K (1974) Significance of the C₄ pathway in *Triodia irritans* (spinifex), a grass adapted to arid environments. Aust J Plant Physiol 1:171–175
- Makino A, Nakano H and Mae T (1994) Effects of growth temperature on the responses of ribulose-1,5-bisphosphate carboxylase, electron transport components, and sucrose synthesis enzymes to leaf nitrogen in rice, and their relationships to photosynthesis. Plant Physiol 105: 1231–1238
- Maxwell K, von Caemmerer S and Evans JR (1997) Is a low internal conductance to CO₂ diffusion a consequence of Crassulacean Acid Metabolism? Aust J Plant Physiol 24: 777–786
- Meinzer FC and Zhu J (1998) Nitrogen stress reduces the efficiency of the C₄ CO₂ concentrating system, and therefore quantum yield, in *Saccharum* (sugarcane) species. J Exp Bot 49: 1227–1234
- Molin WT, Meyers SP, Baer GR and Schrader LE (1982) Ploidy effects in isogenic populations of alfalfa. II. Photosynthesis, chloroplast number, ribulose-1,5-bisphosphate carboxylase, chlorophyll, and DNA in protoplasts. Plant Physiol 70:1710–1714
- Morison JIL and Gifford RM (1983) Stomatal sensitivity to carbon dioxide and humidity. A comparison of two C₃ and C₄ grass species. Plant Physiol 71:789–796
- Mott KA (1988) Do stomata respond to CO₂ concentrations other than intercellular? Plant Physiol 86:200–203
- Mott KA and O'Leary JW (1984) Stomatal behaviour and CO₂ exchange characteristics in amphistomatic leaves. Plant Physiol 74: 47–51
- Mott KA and Parkhurst DF (1991) Stomatal responses to humidity in air and Helox. Plant Cell Env 14: 509–515
- Mott KA, Gibson AC and O'Leary JW (1982) The adaptive significance of amphistomatic leaves. Plant Cell Env 5: 455–460
- Nishio JN, Sun J and Vogelmann TC (1993) Carbon fixation gradients across spinach leaves do not follow internal light gradients. Plant Cell 5: 953–961
- Nobel PS (1991) Physicochemical and Environmental Plant Physiology. Academic Press, New York
- Nobel PS, Zaragoza LJ and Smith WK (1975). Relation between mesophyll surface area, photosynthetic rate and illumination level during development for leaves of *Plectranthus parviflorus* Henkel. Plant Physiology 55, 1067–70.
- Ntefidou M and Manetas Y (1996) Optical properties of hairs during the early stages of leaf development in *Platanus orientalis*. Aust J Plant Physiol 23: 535–538
- Parker ML and Ford MA (1982) The structure of the mesophyll of flag leaves in three *Triticum* species Ann Bot 49:165–176
- Parkhurst DF (1994) Diffusion of CO₂ and other gases in leaves. New Phytol 126: 449–479
- Parkhurst DF and Mott K (1990) Intercellular diffusion limits to CO₂ uptake in leaves. Plant Physiol 94: 1024–1032
- Parkhurst DF, Wong SC, Farquhar GD and Cowan IR (1988) Gradients of intercellular CO₂ levels across the leafmesophyll. Plant Physiol 86: 1032–1037
- Parry MAJ, Keys AJ and Gutteridge S (1989) Variation in the specificity factor of C₃ higher plant Rubiscoes determined by the total consumption of ribulose-P₂. J Exp Bot 40:317–320
- Pearcy RW, Osteryoung K and Randall D (1982) Carbon dioxide exchange characteristics of C₄ Hawaiian *Euphorbia* species native to diverse habitats. Oecologia 55: 333–341
- Peat HJ and Fitter AH (1994) A comparative study of the distribution and density of stomata in the British flora. Biol J Linnean Soc 52:377–393
- Peltier G, Courcier L, Despauv V, Dimon B, Fina L, Genty B and Rumeau D (1995) Carbonic anhydrase activity in leaves as measured in vivo by ¹⁸O exchange between carbon dioxide and water. Planta, 196: 732–739
- Peterson RB (1990) Effects of irradiance on the in vivo CO₂:O₂ specificity factor of tobacco using simultaneous gas exchange

- and fluorescence techniques. *Plant Physiol* 94:892–898
- Pfeffer M and Peisker M (1995) In vivo K_m for CO_2 (K_p) of phosphoenolpyruvatecarboxylase(PEPC) and mesophyll CO_2 transport resistance (r_m) in leaves of *Zea mays* L. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol V, pp 547–550. Kluwer Academic Publishers, Dordrecht
- Price GD, von Caemmerer S, Evans JR, Yu J-W, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A and Badger MR (1994) Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation. *Planta*, 193:331–340
- Price G D, Evans JR, von Caemmerer S, Yu J-W and Badger MR (1995) Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO_2 assimilation via a reduction in RuBP regeneration in transgenic tobacco plants. *Planta*, 195: 369–378
- Price GD, von Caemmerer S, Evans JR, Siebke K, Anderson JM and Badger M (1998) Photosynthesis is strongly reduced by antisense suppression of chloroplastic cytochrome *bf* complex in transgenic tobacco. *Aust J Plant Physiol* 25: 445–452
- Psaras GK (1986) Chloroplast arrangement along intercellular spaces in the leaves of a Mediterranean subshrub. *J Plant Physiol* 126: 189–193
- Psaras GK, Diamantopoulos GS and Makrypoulias CP (1996) Chloroplast arrangement along intercellular air spaces. *Israel J Plant Sci* 44: 1–9
- Pyke KA and Leech RM (1987) Cellular levels of ribulose 1,5-bisphosphate carboxylase and chloroplast compartment size in wheat mesophyll cells. *J Exp Bot* 38:1949–1956
- Pyke KA and Leech RM (1992) Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana*. *Plant Physiol* 99:1005–1008
- Pyke KA and Leech RM (1994) A genetic analysis of chloroplast division and expansion in *Arabidopsis thaliana*. *Plant Physiol* 104:201–207
- Pyke KA, Rutherford SM, Robertson EJ and Leech RM (1994) *arc6*, a fertile *Arabidopsis* mutant with only two mesophyll cell chloroplasts. *Plant Physiol* 106:1169–1177
- Raschke K (1987) Action of abscisic acid on guard cells. In: Zeiger E, Farquhar GD and Cowan IR (eds) Stomatal Function, pp 253–279. Stanford University Press, Stanford.
- Renou JL, Gerbaud A, Just D and Andre M (1990) Differing substomatal and chloroplastic CO_2 concentrations in water-stressed wheat. *Planta* 182:415–419
- Ridolfi M and Dreyer E (1997) Responses to water stress in an ABA-unresponsive hybrid poplar (*Populus koreana* x *trichocarpa* cv. Peace). III. Consequences for photosynthetic carbon assimilation. *New Phytol* 135:31–40
- Roeske CA and O'Leary MH (1984) Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose bisphosphate. *Biochemistry* 23: 6275–6284
- Rooney MA (1988) Short-term carbon isotope fractionation by plants. Ph.D. thesis, University of Wisconsin, Madison, WI.
- Roupsard O, Gross P and Dreyer E (1996) Limitation of photosynthetic activity by CO_2 availability in the chloroplasts of oak leaves from different species and during drought. *Ann Sci For* 53:243–254
- Rumeau D, Cuine S, Fina L, Gault N, Nicole M and Peltier G (1996) Subcellular distribution of carbonic anhydrase in *Solanum tuberosum* L. leaves. Characterisation of two compartment-specific isoforms. *Planta*, 199:79–88
- Sasahara T (1982) Influence of genome on leaf anatomy of *Triticum* and *Aegilops*. *Ann Bot* 50:491–497
- Sasaki H, Samejima M and Ishii, R (1996) Analysis by $\delta^{13}\text{C}$ measurement on mechanism of cultivar difference in leaf photosynthesis of rice (*Oryza sativa* L.). *Plant Cell Physiol* 37: 1161–1166
- Schmidt-Nielsen K (1991) Animal physiology: adaptation and environment. Cambridge University Press, Cambridge.
- Sharkey TD and Raschke K (1981) Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. *Plant Physiol* 68: 1170–1174
- Sharkey TD, Imai K, Farquhar GD and Cowan IR (1982) A direct confirmation of the standard method of estimating intercellular partial pressure of CO_2 . *Plant Physiol* 69: 657–659
- Sharkey TD, Vassey TL, Vanderveer PJ and Vierstra RD (1991) Carbon metabolism enzymes and photosynthesis in transgenic tobacco (*Nicotiana tabacum* L.) having excess phytochrome. *Planta* 185: 287–296
- Siebke K and Weis E (1995a) Assimilation images of leaves of *Glechoma hederacea*: Analysis of non-synchronous stomata related oscillations. *Planta*, 196:155–165
- Siebke K and Weis E (1995b) Imaging of chlorophyll-*a*-fluorescence in leaves: Topography of photosynthetic oscillations in leaves of *Glechoma hederacea*. *Photosynth Res* 45:225–237
- Solomon AK (1974) Apparent viscosity of human red blood cell membranes. *Biochim Biophys Acta* 373: 145–149
- Sultemeier D and Rinast KA (1996) The CO_2 permeability of the plasma membrane of *Chlamydomonas reinhardtii*: mass-spectrometric ^{18}O -exchange measurements from $^{13}\text{C}^{18}\text{O}_2$ in suspensions of carbonic anhydrase-loaded plasma-membrane vesicles. *Planta* 200:358–368
- Syvertsen JP, Lloyd J, McConchie C, Kriedemann PE and Farquhar GD (1995) On the site of biophysical constraints to CO_2 diffusion through the mesophyll of hypostomatous leaves. *Plant Cell Env* 18: 149–157
- Tenney SM and Remmers JE (1963) Comparative quantitative morphology of the mammalian lung: Diffusing area. *Nature*, 197: 54–56
- Terashima I (1992) Anatomy of non-uniform leaf photosynthesis. *Photosynth Res* 31: 195–212
- Terashima I, Wong SC Osmond CB and Farquhar GD (1988) Characterisation of non-uniform photosynthesis induced by abscisic acid in leaves having different mesophyll anatomies. *Plant Cell Physiol* 29:385–394
- Turrell FM (1936) The area of the internal exposed surface of dicotyledon leaves. *Amer J Bot* 23: 255–263
- Utsunomiya E and Muto S (1993) Carbonic anhydrase in the plasma membranes from leaves of C_3 and C_4 plants. *Physiol Plant* 88: 413–19
- Villarr R, Held AA and Merino J (1994) Comparison of methods to estimate dark respiration in the light in leaves of two woody species. *Plant Physiol* 105:167–172
- Vitousek PM, Field CB and Matson PA (1990) Variation in foliar $\delta^{13}\text{C}$ in Hawaiian *Metrosideros polymorpha*: a case of internal resistance? *Oecologia* 84: 362–370
- Vogel JC (1993) Variability of carbon isotope fractionation during photosynthesis. In: Ehleringer JR, Hall AE and Farquhar GD (eds) Stable Isotopes and Plant Carbon-Water Relations, pp 29–46. Academic Press, San Diego

- von Caemmerer S and Evans JR (1991) Determination of the average partial pressure of CO₂ in chloroplasts from leaves of several C₃ plants. *Aust J Plant Physiol* 18: 287–305
- von Caemmerer S and Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153: 376–387
- von Caemmerer S, Evans JR, Hudson GS and Andrews TJ (1994) The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. *Planta*, 195:88–97
- von Caemmerer S, Ludwig M, Millgate A, Farquhar GD, Price D, Badger M and Furbank RT (1997) Carbon isotope discrimination during C₄ photosynthesis: insights from transgenic plants. *Aust J Plant Physiol* 24: 487–494
- Watanabe N, Evans JR and Chow WS (1994) Changes in the photosynthetic properties of Australian wheat cultivars over the last century. *Aust J Plant Physiol* 21: 169–183
- Williams TG, Flanagan LB and Coleman JR (1996) Photosynthetic gas exchange and discrimination against ¹³CO₂ and C¹⁸O¹⁶O in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase. *Plant Physiol* 112:319–326
- Willmer C and Flicker M (1996) Stomata. Chapman and Hall, London
- Wong SC, Cowan IR and Farquhar GD (1979) Stomatal conductance correlates with photosynthetic capacity. *Nature* 282:424–426
- Wong SC, Cowan IR and Farquhar GD (1985a) Leaf conductance in relation to rate of CO₂ assimilation. I. Influence of nitrogen nutrition, phosphorus nutrition, photon flux density, and ambient partial pressure of CO₂ during ontogeny. *Plant Physiol* 78: 821–825
- Wong SC, Cowan IR and Farquhar GD (1985b) Leaf conductance in relation to rate of CO₂ assimilation. II. Effects of short-term exposures to different photon flux densities. *Plant Physiol* 78: 826–829
- Yoshie F (1986) Intercellular CO₂ concentration and water-use efficiency of temperate plants with different life-forms and from different microhabitats. *Oecologia* 68: 370–374
- Zeiger E, Farquhar GD and Cowan IR (1987) Stomatal function. Stanford University Press, Stanford

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Chapter 15

Carbonic Anhydrase and Its Role in Photosynthesis

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Summary

Carbonic anhydrase, an enzyme which catalyzes the reversible hydration of CO_2 , is a major protein component of most photosynthetic microorganisms and higher plant tissues. Once thought to be represented in plants by a single enzyme type, it is now apparent that DNA sequences and/or the encoded proteins for the evolutionarily distinct α , β , and γ forms of carbonic anhydrase are present in cyanobacteria, green algae, and higher plants. While exhibiting a wide range in structure, localization, and regulation of expression, some progress has been made in the establishment of roles for these various enzyme forms. It would appear that the primary role of many of the α and β isoforms is the establishment of inorganic carbon species equilibration. As a result of this activity, enzymes or transport systems which require either CO_2 or HCO_3^- are not limited by the slow, uncatalyzed rate of $\text{CO}_2/\text{HCO}_3^-$ interconversion. In contrast, little is known about patterns of expression or role(s) for γ isoforms. Recent studies on carbonic anhydrase described in this chapter include the isolation and characterization of new isoforms, the generation and phenotypic description of carbonic anhydrase mutants, as well as elucidation of mechanisms responsible for regulation of expression.

I. Introduction

Carbonic anhydrase (CA; EC 4.2.2.1) is a zinc-containing enzyme which catalyzes the interconversion of CO_2 and HCO_3^- . In this reversible hydration/dehydration reaction, described as $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$, the final equilibrium concentrations of HCO_3^- and CO_2 are established primarily by the pH of the aqueous environment. For example, when inorganic carbon species are at equilibrium and a pH value of 8.0, over 80% of the inorganic carbon (C_i) in solution is in the form of HCO_3^- . Conversely, as the pH declines the proportion of C_i in solution in the form of CO_2 increases, such that at pH 6.0, approximately 75 % of the total C_i is CO_2 . Why the need for carbonic anhydrase in biological systems if the interconversion of CO_2 and HCO_3^- will proceed spontaneously? The answer is readily apparent if one examines the uncatalyzed rate of C_i species equilibration. In the absence of the enzyme, the establishment of CO_2 and HCO_3^- equilibrium is very slow, exhibiting a $t_{1/2}$ of approximately 15 s at 25 °C and pH 8.0. As such, enzymatic reactions or active transport processes that utilize a specific C_i species preferentially could soon experience declines in activity if the rate of substrate use exceeds the rate of uncatalyzed C_i equilibration. Reductions in activity would become more extreme as the $\text{HCO}_3^-/\text{CO}_2$ ratio moves further away from equilibrium. Where CA is present, the rapidity of the enzyme catalyzed reaction effectively eliminates the possibility of C_i equilibration limiting a biological process. As CA appears to be found in all organisms yet examined, the ubiquitous nature of this enzyme would seem to indicate an important role in many physiological events. Indeed, in animal systems CA has a pivotal position in such diverse processes as respiration, kidney function, and bone formation (Tashian, 1989). The abundance of CA in many photosynthetic organisms, the molecules involved in the catalyzed reaction (CO_2 , HCO_3^- and H^+), and its primary spatial and temporal association with photosynthetic tissue, have all suggested to many researchers that CA should also play a key role in photosynthesis. This speculation generated much research activity in the past however only recently has more direct evidence for a specific role of CA in some photosynthetic organisms been

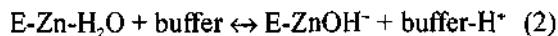
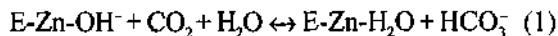
shown, and indeed, much more work needs to be done. In the following sections I will attempt to highlight some of the recent progress which has been made on characterization of the enzyme and determination of role(s) of CA in higher plants, microalgae, and cyanobacteria. In addition, I will also suggest some important areas for future research. I have not attempted to review all the literature in this area as it is voluminous and there are many older reviews on plant and algal carbonic anhydrases that provide a historical perspective (Poincelot, 1979; Reed and Graham, 1981; Graham et al., 1984; Aizawa and Miyachi, 1986; Tsuzuki and Miyachi, 1989). For complementary information, readers are also directed to two comprehensive reviews on CA in plants and other photosynthetic organisms by Sultemeyer et al. (1993) and Badger and Price (1994).

II. Enzyme Types, Structures And Kinetics

Carbonic anhydrases from a variety of sources, although catalyzing the same reaction, have been recently sub-divided on the basis of primary amino acid sequence into three major groups (Hewett-Emmett and Tashian, 1996). These groups are (a) α -CAs, typified by the well studied vertebrate-type enzyme (Tashian, 1989); (b) β -CAs, the group first characterized as a higher plant, chloroplast-localized enzyme (Burnell et al., 1990a; Fawcett et al., 1990; Roeske and Ogren, 1990; Majeau and Coleman, 1991, 1992); and (c) γ -CAs, a recently identified group first characterized in the *Archaeabacteria* (Alber and Ferry, 1994). The highly divergent sequences of these three groups certainly suggests multiple evolutionary origins but what is more intriguing is that many single organisms appear to express CAs from more than one group. For example, the cyanobacterium *Synechococcus* PCC 7942 expresses both α - and β -CAs and also contains a protein with significant amino acid sequence homology to the γ group of CAs (Fukuzawa et al., 1992; Yu et al., 1992; Price et al., 1993; Soltes-Rak et al., 1997). The higher plant *Arabidopsis* also contains DNA sequences significantly similar to α - and γ -CAs as well as the expressed β -CA isoforms found in the chloroplast and cytosol (Raines et al., 1992; Fett and Coleman, 1994; Newman et al., 1994). Although regulation of expression and localization studies for the 'non-plant-like,' α - and γ -CAs in higher plants has not been done, characterization of these enzymes

types in cyanobacteria and eukaryotic algae suggest that higher plants will also be found to use all three enzyme groups for specific functions in various tissues, cells and/or organelles.

Although all three CA groups are zinc-metallo-enzymes, with one Zn atom localized in the active site of each monomer, there are considerable differences in the structures of the active sites. For the **α -type** enzyme isolated from humans and animals, many crystal structures have shown that three essential His residues co-ordinate the position of the Zn atom in the active site at the bottom of a conical cavity (Tashian, 1989; Silverman, 1991). The sequence similarity between the periplasm-localized CAH1 and CAH2 proteins isolated from the eukaryotic green alga *Chlamydomonas reinhardtii* (Fujiwara et al., 1990) and the similarly localized ECA proteins in the cyanobacteria (Soltes-Rak et al., 1997) suggest that these **α -type** CAs will also have homologous active sites. The reaction mechanism within the active site of mammalian **α -type** enzymes involves a zinc-bound water molecule which can ionize to a Zn-bound hydroxide. In this basic form of the enzyme the CO_2 hydration reaction is catalyzed whereas the dehydration of HCO_3^- requires the protonated enzyme form. The actual catalytic mechanism is a two step process (Silverman, 1991):



Equation (1) describes the interaction of the catalytically Zn-bound hydroxide with CO_2 , the formation of HCO_3^- and the replacement of the OH^- with a H_2O bound to the Zn. Equation (2) describes what is the rate-limiting step in the reaction, the regeneration of the Zn-bound hydroxide by the protolysis of the Zn-bound H_2O and the transfer of the liberated H^+ to the surrounding medium (identified as a buffer).

A. **α -Carbonic Anhydrase Enzymes**

In terms of structure, the best studied examples of an **α -CA** from a photosynthetic organism are the homologous periplasmic proteins encoded by *cah1* and *cah2* in the eukaryotic alga *Chlamydomonas*. Translated as a 42–44 kDa precursor protein on 80S ribosomes, this extracellular CA is proteolytically processed to yield 35 and 4 kDa monomers (Coleman

and Grossman, 1984; Kamo et al., 1990). The holoenzyme is approximately 76 kDa and is tetramer of two 35 kDa monomers linked by a disulfide bond, and two 4 kDa monomers each linked to the larger subunit by a disulfide bond (Kamo et al., 1990). Following synthesis, the protein is glycosylated, assembled and exported in the periplasmic space. Amino acid sequence comparisons with vertebrate **α -CAs** indicates about 20% overall sequence similarity, however extensive conservation of zinc-binding His residues and other important active site amino acids certainly suggests a common catalytic mechanism. Recently, an extracellular **α -CA** has been identified in the cyanobacteria *Synechococcus* and *Anabaena* (Soltes-Rak et al., 1997). Again strict conservation of almost all active site residues suggest a common catalytic mechanism however the oligomeric structure of this enzyme has yet to be determined.

B. **β -Carbonic Anhydrase Enzymes**

Although no crystal structures are yet available for the **β -type** CAs, site-directed mutagenesis studies and extended X-ray fine structure analysis indicate that two Cys and one His residue are involved in coordination of the Zn atom in the active site (Provart et al., 1993; Bracey et al., 1994). Specific site-directed changes of H220N, C160S, or C223S in the gene encoding chloroplastic localized CA isolated from pea (*Pisum sativum*) (Provart et al., 1993) and the corresponding changes H210Q, C150A, or C213A in a similar cDNA encoding spinach (*Spinacea oleracea*) chloroplast CA (Bracey et al., 1994) all resulted in an inactive enzyme which was incapable of binding Zn. Additional site-directed changes in the pea enzyme of H209N and E276A produced enzymes with compromised catalytic activity which could be restored by the addition of imidazole buffers capable of entering the active site. Similar lesions and imidazole buffer complementation in the **α -type** mammalian CAII enzymes involving H64 suggest that this residue is involved in the intramolecular transfer of protons necessary for regeneration of the catalytically active Zn-hydroxide. The pea and spinach enzyme data suggest that a similar strategy may be employed by the **β -type**, plant enzymes. Kinetic analysis of pea (Johansson and Forsman, 1993) and spinach (Rowlett et al., 1994) show clearly that although structurally very different, **β -type** CAs isolated from higher plants are as efficient as the well

described **α -type** mammalian CAII enzyme. For example, in a recent, detailed kinetic study of the chloroplastic localized pea CA, a turnover number/subunit (k_{cat}) at pH 9.0 of 4×10^5 s $^{-1}$ and a k_{cat}/K_m value of $1.8 \times 10 M^{-1} s^{-1}$ were obtained. Representative values for the human CAII are 1×10^6 s $^{-1}$ and $1.2 \times 10^8 M^{-1} s^{-1}$ (Johansson and Forsman, 1993). Kinetic analysis of the spinach enzyme have provided similar data and also show that a H $^{+}$ transfer step is the rate determining factor in the catalytic mechanism, although it was not possible to conclude if an intramolecular H $^{+}$ transfer system was operational (Rowlett et al., 1994). Taken together, the structural and kinetic data indicate that the **β -type** enzyme active site residues and organization are different from the **α -type** enzymes but that the catalytic mechanisms are similar.

The oligomeric structure of the **β -type** enzyme in the absence of crystal structures is still unresolved and the literature contains a number of reports describing native enzymes of differing molecular mass. The most recent estimates however, indicate a structure of eight monomeric units per oligomer for CAs isolated from leaves of dicots. (Guliev et al., 1992; Rumeau et al., 1996; Bjorkbacka et al., 1997). Cysteine residues have also been implicated in interaction of subunits, and a model of the chick pea leaf enzyme based on electron microscopy suggests a P 422 symmetry for the octomer (Guliev et al., 1992; Bjorkbacka et al., 1997) Reported monomeric sizes of leaf enzymes range from 24 kDa (*Pisum*) to 31 and 35 kDa (*Flavaria*) indicating some heterogeneity which may reflect differences between cytosolic and chloroplastic leaf isoforms and/or variation in transit peptide processing sites following uptake of the nascent polypeptide into the chloroplast (Johansson and Forsman, 1992; Forsman and Pilon, 1995; Ludwig and Burnell, 1995). Few studies of monocot leaf CAs have been performed, however older data suggest that the oligomeric form may be a dimer (Reed and Graham, 1981). Recently, isolation of cDNAs encoding chloroplast-localized CAs from barley (Bracey and Bartlett, 1995) and rice (Suzuki and Burnell, 1995) have shown that the monomeric sizes and overall aa sequences are similar to CAs from leaves of dicots although a 10 aa C terminal sequence conserved among dicot CAs is missing in monocots. Sufficient aa sequence variation does exist, however, such that antibodies directed against spinach CA (a dicot) fail to cross-react with CAs from C₃ and C₄ monocots (Okabe et al., 1984; Burnell, 1990)

whereas antisera derived against maize leaf CAs exhibited some cross-reactivity with both C₃ and C₄ monocots and dicots (Burnell, 1990). A recent paper by Burnell and Ludwig (1997) describes the isolation and characterization of two cDNAs encoding mesophyll cell CA isoforms. Although the cDNAs have open reading frames of 71 and 53 kDa, immunological evidence shows that the two 180 kDa oligomeric native structures are composed of 27 kDa or 45–47 kDa monomers, suggesting some form of translational or post-translational processing. Certainly more detailed analysis of the oligomeric structure of monocot CAs is necessary before definitive statements on differences with dicots oligomeric structures can be made.

C. **γ -Carbonic Anhydrase Enzymes**

The only characterized example of a **γ CA** is from the methanogenic archaeabacterium *Methanosarcina thermophila* (Alber and Ferry, 1994) although similar deduced amino acid sequences have been found in photosynthetic organisms such as the CcmM protein in *Synechococcus* which is involved in the CO₂ concentrating mechanism (Price et al., 1993), and other deduced amino acid sequences from cDNAs obtained from *Arabidopsis* (Newman et al., 1994). With no aa sequence similarity to the other CA groups, the holoenzyme in *Methanosarcina* appears to be homotrimer of the 37 kDa monomer. Interestingly, crystal structure analysis has shown the coordination of the Zn atoms (three per holoenzyme) is a function of His residues from two adjacent monomers resulting in the three active sites at the monomer interfaces (Kisker et al., 1996). The geometry of the His residues required for Zn binding is similar to that seen in **α -CAs** suggesting a similar catalytic mechanism.

III. Localization, Regulation of Expression and Role

A. **C₄** Plants

It is now generally accepted that the principal enzyme in the photosynthetic tissue of C₄ plants is a **β -CA** localized in the cytosol of mesophyll cells (Hatch and Burnell, 1990). Little if any CA activity is found in bundle-sheath cells. To date the best data sets were obtained by separation of the two cell types and

estimating levels of mesophyll cell contamination of bundle sheath preparation using mesophyll enzyme markers such as PEPCase and pyruvate, P_i dikinase (Burnell and Hatch, 1988). These studies show that CA activities of bundle sheath cells rarely exceed the level of contaminating mesophyll enzyme activities, suggesting little if any expression of CA outside of the mesophyll cell environment. Although most of the CA in the mesophyll tissue appears to be soluble and cytoplasmic, there is at least one study indicating that a portion of the CA, as determined by activity and western blot analysis, is associated with the cytoplasmic side of purified plasma membranes from *Zea mays* (Utsunomiya and Muto, 1993). It has been suggested that two CA cDNAs recently isolated from *Zea* encode the plasma membrane associated form and a soluble cytosolic enzyme (Burnell and Ludwig, 1997). Questions on CA localization in C_4 plants could benefit from careful cytological studies involving *in situ* hybridization of both protein antibody and RNA probes.

Models of C_4 photosynthesis and hypothesized roles for CA predict the observed pattern of localization. Within the mesophyll cells, CA catalyzes the hydration of CO_2 that diffuses across the plasma membrane. The resulting HCO_3^- is then fixed by PEPCase, thus initiating the C_4 pathway. Quantitative analysis of CA activity in *Zea* also support this role. By taking into account estimated mesophyll intracellular CO_2 concentrations of 4 μM and CA $K_m CO_2$ and V_{max} values of 2 mM and 34,000 $\mu\text{mol CO}_2 \text{ hydrated. min}^{-1} \cdot \text{mg Chl}^{-1}$, respectively, Burnell and Hatch (1990) determined that there was just sufficient CA activity *in vivo* to support observed rates of C_4 photosynthesis. What is particularly noteworthy of this study is that it addresses the supposed excess of CA activity long commented on by many researchers. By estimating *in vivo* activities in terms of mol CO_2 hydration capacity instead of the arbitrary and physiologically irrelevant Wilbur-Anderson units (determined at saturating levels of CO_2), the measured CA levels are now seen to be necessary component of the C_4 pathway. In an additional point on the role of CA in the C_4 mesophyll cell, calculations of CO_2 conductance across the cell wall to the cytoplasm indicate that the flux appear to be approximately two-fold higher than in C_3 plants (Evans and von Caemmerer, 1996). Although a number of possible factors, including thinner cell walls, and shorter diffusion paths may contribute to more rapid CO_2 flux, as reported in *Zea mays* it is possible that the

significant amounts of CA activity associated with the mesophyll plasmalemma may facilitate CO_2 diffusion into the mesophyll cell (Utsunomiya and Muto, 1993).

Inhibition of CA activity in C_4 plants following vacuum infiltration of leaf pieces with the sulfonamide inhibitor ethoxzolamide has also shown that CA plays a prominent role in photosynthesis. At low levels of external C_i , inhibition of photosynthetic O_2 evolution approached 80 to 90% when leaf pieces of *Zea* and *Amaranthus* were treated with the CA inhibitor (Badger and Pfanz, 1995). The degree of inhibition was reduced as external C_i concentrations were increased. A somewhat puzzling observation in these studies however was that even when treated with ethoxzolamide, rates of photosynthesis still exceeded the uncatalyzed rate of CO_2 production in the external medium. A number of explanations were provided. It was possible that inhibition of all cytosolic CA activity was not achieved, or that the high external HCO_3^- concentration may access the mesophyll cells directly through the apoplasm and plasmodesmata. It was also proposed that an additional CA (perhaps the reported plasma membrane-localized enzyme) was less sensitive to ethoxzolamide inhibition and was still able to facilitate CO_2 uptake and intracellular hydration. Whatever the explanation, the effective inhibition of photosynthesis by the reduction of CA activity, particularly at low external C_i concentrations does support the importance of CA in the C_4 pathway.

That CA activity is a requirement for initiation of the C_4 pathway is also supported by data on regulation of expression. In *Zea*, exposure to light and increased N availability result in enhanced levels of CA and PEPCase activity (Burnell et al., 1990b). These changes in activity resulted from co-ordinated increased transcript abundance and not from light activation of the enzymes or other post-translational events. Experiments with various N sources and the glutamine synthetase inhibitor, methionine sulfoximine (MSX), have shown that N stimulation of CA and PEPCase expression appears to be mediated by changes in Gln levels (Sugiharto et al., 1992). This amino acid and/or its downstream metabolites seem to act as a positive effector resulting in a significant increase in CA and PEPCase transcript levels (Sugiharto et al., 1992). The similarity in patterns of regulation of expression of CA and PEPCase by light and N suggest that common mechanisms of gene activation are present. It would be interesting to determine if similar regulatory *cis* elements are

present in genomic promoter sequences of both CA and C₄ PEPCase isoforms.

A similar strategy of juxtaposing empirical data and modeling was employed to show that a bundle-sheath cell localized CA would be an impediment to the efficient Rubisco catalyzed fixation of CO₂ generated by the decarboxylation of C₄ acids. All decarboxylases in the bundle sheath cells generate CO₂ as a product (Jenkins et al., 1987). As such, and as a result of the slow rate of uncatalyzed hydration, the intracellular level of CO₂ in this tissue is estimated to be at least 70 μM, significantly above equilibrium levels (Jenkins et al., 1989). As CO₂ is the active species for Rubisco, the carboxylation reaction is favored and little if any oxygenase activity occurs. If CA were present, the catalyzed conversion of CO₂ to HCO₃⁻ would rapidly proceed to chemical equilibrium with the concomitant reduction in the rate of carboxylation. In addition, the formation of HCO₃⁻ would result in an increased C_i leak rate from the bundle sheath cells down a concentration gradient through the plasmodesmata and into the mesophyll cells. Modeling the impact of CA on bundle sheath cell CO₂ concentrations indicates that maintenance of CO₂ concentrations in excess of 100 μM cannot occur if the hydration rate exceeds approximately 50 times the uncatalyzed rate (Burnell and Hatch, 1988). This level of CA activity represents less than 5% of the average total CA activity in C₄ plant leaves. Measured CA levels appear to be even lower (Burnell and Hatch, 1988).

The use of C₄ transgenics would provide some interesting data on the role of CA in these plants. Recently, significant advances have been made in this area. Antisense technology has been used to assess the impact of reduced Rubisco activity in the C₄ plant *Flaveria bidentis* (Furbank et al., 1996). Using a similar strategy, mesophyll cell-specific antisense expression directed against the cytoplasm-localized CA should result in a severe inhibition of photosynthetic performance, particularly in a low external CO₂, high light environment. Additionally, the over-expression of CA activity in bundle sheath tissue should also result in a inhibition of photosynthesis by enhancing the C_i leak rate from these cells. This has now been shown experimentally. Using the same host and transformation strategy, tobacco CA over-expression in leaves of *Flaveria* resulted in plants with up to a 25% reduction in assimilation and a significant increase in C isotope discrimination relative to controls (Ludwig et al., 1998). The

reduction in assimilation was enhanced with increasing O₂ concentration, suggesting increased rates of photorespiration. All of these data are consistent with increased leakage of CO₂ from bundle sheath cells, and that this leakage is the result of over-expression of CA in this tissue.

B. CAM Plants

Our understanding of the role that CA plays in the functioning of CAM photosynthesis is very limited. With the exception of one study by Tsuzuki et al. (1982) on activities and localization of CA in a variety of CAM species, little has been done on this group of plants. This earlier paper showed that total CA activity for a variety of CAM species, such as *Ananas comosus*, *Sedum praealtum*, *Hoya carnosa* and *Mesembryanthemum crystallinum* were similar to many C₃ and C₄ plants but that localization varied. Plants which utilize PEP carboxykinase for decarboxylation contained only extrachloroplastic CA that partitioned with PEPCase during separation of intracellular compartments. In contrast, plants utilizing malic enzyme for decarboxylation contained chloroplast-localized CA activity. An additional observation was that facultative CAM plant *Mesembryanthemum* exhibited no significant differences in either CA activity or localization when performing photosynthesis in either C₃ or CAM mode. Given what we now suspect about the role of CA in the mesophyll cell cytoplasm of C₄ plants, it is surprising that it is absent from this compartment in any one group of CAM plants. It may be that higher nocturnal CO₂ conductances in combination with lower levels of PEPCase in some CAM plants result in a requirement for less cytosolic CA than other CAM species. The apparent chloroplastic localization of CA activity in some CAM plants, such as *Sedum*, also differs from the C₄ model. In these CAM plants, the chloroplastic CA and the resulting enhancement of C_i equilibration may not impair decarboxylase-mediated CO₂ production as there are no additional cell layers for plasmodesmal loss of HCO₃⁻. In fact, loss of C_i by CO₂ leakage may be reduced by rapid equilibration within the single cell of the CAM photosynthetic unit. The presence of chloroplastic CA may also be indicative of the amount of direct CO₂ diffusion, Rubisco-mediated fixation that takes place in CAM plants prior to malic acid synthesis and following deacidification. Direct CO₂ fixation by Rubisco can be an important component of the

total carbon assimilated during the diurnal cycle. It is interesting to note that a study by Maxwell et al. (1997) showed that the CAM plant *Kalanchoe daigremontia* exhibited extremely low levels of internal CO₂ conductance which reduced the efficiency of assimilation during phase IV photosynthesis in which atmospheric CO₂ is fixed directly by Rubisco. It was hypothesized that these low levels of CO₂ conductance were a consequence of the succulent nature of the thick leaves, with reduced air spaces and a densely packed mesophyll. The limited amount of CA that can be found in some CAM plant chloroplasts may be required to partially offset the restricted movement of CO₂ to the site of Rubisco carboxylation. It is apparent, however, that considerably more research is needed with CAM plants. Although an earlier study reported no changes in activity, it would seem that the well-characterized, NaCl induced C₃-CAM switching of *Mesembryanthemum* would be an ideal system for the molecular analysis of CA expression.

C. C₃ Plants

CA is an abundant polypeptide in the leaves of C₃ plants and may constitute upwards of 2% of the total soluble leaf protein. Although the bulk of the CA activity is associated with the chloroplast fraction, recent measurements have shown that at least 10 to 15% of the total activity is cytoplasmic (Rumeau et al., 1996). Even this higher than anticipated percentage of activity under-represents the significance of cytoplasmic CA unless one takes into account compartment volumes. As described in the paper by Rumeau et al. (1996) on subcellular distribution of CA in *Solanum tuberosum* leaves, assuming subcellular volumes of 9.5% and 3.4% for mesophyll cell stroma and cytosol, respectively, CA concentration in the chloroplast was only twofold higher than in the cytosol.

The CA isoform located in the chloroplast is synthesized as a larger precursor polypeptide in the cytosol and is then processed to the mature monomeric size during or following transport across the chloroplast envelope. The transit peptide of the nascent CAs that have been studied are all very long, in excess of 100 aa and are composed of two parts. The N-terminal region of approximately 70 aa has all the characteristics normally seen in chloroplast transit peptides. The C-terminal region of approximately 35 aa contains many acidic residues and is very unlike

typical C terminal regions of other transit peptides. Careful in vitro chloroplast import studies with partial, full length, or chimeric *Pisum sativum* CA constructs have shown that it is only the N-terminal region that is required for correct transport, processing and localization in the chloroplast stroma (Forsman and Pilon, 1995). This study also provides some evidence for two step processing of the nascent CA during or following transport into the chloroplast. No specific role for the C-terminal region of the transit peptide has been shown.

First evidence for a cytosolic CA in C₃ plants was presented by Kachru and Anderson (1974) who reported on the partial purification of cytosolic and chloroplastic forms of CA in *Pisum*. Purification of the *Solanum* cytosolic isoform has shown that it is structurally and biochemically similar, and immunologically related to the chloroplastic form, although its monomeric mass is somewhat larger (30 kDa vs. 27 kDa), respectively (Rumeau et al., 1996). Cytosolic and chloroplastic isoforms are also present in *Arabidopsis* and cDNAs encoding each enzyme have been characterized (Fett and Coleman, 1994). Comparison of the deduced aa sequences revealed approximately 85% identity, however as expected, the cDNA encoding the cytosolic enzyme was missing a 78 aa N-terminus transit peptide sequence found on the chloroplastic isoform cDNA. Molecular masses of the two processed monomers are similar to that observed in *Solanum*, with the cytosolic isoform somewhat larger than the chloroplastic enzyme. Whereas two distinct nuclear genes encode the CA isoforms in *Arabidopsis*, in *Pisum*, data suggest that the two CA isoforms result from differential transcription of a single gene and/or subsequent processing of its mRNA (Majeau and Coleman, unpublished).

Immunogold localization studies with antisera directed against the chloroplast CA isoform show that CA is randomly distributed through-out the cytosol of both *Solanum* and *Pisum* (Rumeau et al., 1996; Anderson et al., 1996). In the chloroplast, these same studies show the bulk of the label to be found in the stroma but a significant fraction was also found associated with granal and stromal thylakoids. Little if any label was found in association with the chloroplast envelope. These electron micrographs showing the presence of chloroplastic β-CA associated with thylakoid membranes has provided some support for a hypothesized thylakoidal CA (Stemler, 1997 and references within). It has

been suggested that CA (perhaps a distinct isoform) is associated with higher plant, algal and cyanobacterial thylakoids, specifically Photosystem II, and is required for efficient electron transport. Although a single protein component has never been isolated, extensively washed thylakoids and Photosystem II-enriched membrane fragments have been shown to catalyze the reversible hydration of CO_2 , suggesting that this CA activity is the product of an intrinsic membrane protein. In the absence of any identified, novel CA proteins, it is possible that the hydration activity of washed membrane fragments simply represents contamination by the abundant, soluble enzyme. Until a distinct CA isoform has been biochemically and genetically characterized, the presence and role of a thylakoidal CA remains an unresolved issue. Perhaps a cryptic isoform, either a novel α - or γ -type enzyme resides in the thylakoids and has yet to be characterized.

Another possibility for the cyto-immunological identification of CA in close proximity to thylakoids is that a portion of the CA may be a component of a complex with other Calvin cycle enzymes that form a loose association with photosynthetic lamellae. A number of studies have shown that enzymes such as Rubisco, phosphoribulokinase (PRK), glyceraldehyde phosphate dehydrogenase (GAPDH), **ferredoxin-NADP⁺** reductase (FNR), and ribose-5-P-isomerase (RPI) as well as others appear to form heteromeric complexes capable of CO_2 fixation (Süss et al., 1993 and references within). It has been hypothesized that such thylakoid-associated enzyme complexes would facilitate channeling of intermediates, and an association with NADPH and ATP generating sites (the thylakoids) would greatly improve the efficiency of substrate utilization. Recently, biochemical evidence has been provided to show that CA is associated with a Calvin cycle heteromeric enzyme complex in the chloroplasts of tobacco (Jebanathirajah and Coleman, 1997). Following non-denaturing FPLC separation of stromal extracts, CA, Rubisco and other Calvin cycle enzymes were found to co-purify in the same fractions. Trypsin treatment followed by SDS-PAGE analysis of these purified complexes suggested that CA was localized on the periphery or in a less tight association than some of the intrinsic heteromeric complex proteins such as PRK and FNR (Jebanathirajah and Coleman, 1997). The presence of CA in an association with Rubisco certainly strengthens the argument that CA is involved in the direct

provision of CO_2 to the C_3 Rubisco.

A description of location within the C_3 plant cell, numbers of isoforms, and possible association with other proteins leads logically to a discussion on the role of CA in C_3 higher plants. Although some progress has been made in this area, definitive evidence for any one role is still lacking. In all, three specific roles for the chloroplast-localized β -CA have been suggested. The first involves CA facilitating diffusion of CO_2 across the chloroplast envelope and maximizing the flux of C_i through the alkaline stroma to the site of Rubisco-mediated carboxylation. As CO_2 appears to be the only C_i species capable of crossing the chloroplast envelope, its diffusion from the more acidic cytoplasmic environment would be enhanced by the catalyzed hydration of CO_2 upon entry into the chloroplast. The rapid equilibration of the C_i species to that point established by the pH of the stroma also ensures a maximal rate of C_i flux through the stroma to the site of carboxylation. The rate of movement through the stroma is enhanced because both C_i species are components of the gradient established by Rubisco-catalyzed CO_2 consumption (Cowan, 1986). The second major proposed role for CA is that which occurs at the site of carboxylation where catalyzed dehydration of the abundant HCO_3^- pool in the alkaline stroma is presumed to maintain the supply of CO_2 for Rubisco. The association of a portion of the chloroplastic CA in a Rubisco-containing Calvin cycle complex is in support of this hypothesis. Certainly these two major roles for CA are not mutually exclusive and the spatial arrangement of CA in the chloroplast presumably complements both functions. The third hypothesized role for CA catalyzed $\text{CO}_2/\text{HCO}_3^-$ exchange in the stroma is that it is an important component in moderating rapid, localized pH changes induced by fluctuations in light intensity. The production or utilization of H^+ during dehydration/hydration reactions could act as a biological buffering system, however, there is no experimental evidence to support or refute this hypothesis.

The confirmation of the existence of a relatively abundant cytoplasmic CA has also prompted some speculation as to a role for this enzyme. As has been suggested for the C_4 plant mesophyll CA, the primary function of a cytoplasmic C_3 CA may be to provide HCO_3^- for PEPCase. PEPCase in C_3 plant mesophyll tissue plays an anaplerotic role in the biosynthesis of C_4 acids, and for maximal activity may require CA to speed hydration of CO_2 . In active guard cells, the

enhanced role of guard cell PEPCase would also require significant CA activity for HCO_3^- formation, however no guard cell-specific CA activity or localization measurements have been performed.

Recent attempts to identify specific roles for CA in C_3 plants have been based on methods to manipulate CA activity followed by an examination of plant growth or photosynthesis. Methods used to modulate CA activity have included growth of plants under Zn or N deficient conditions, use of sulfonamide inhibitors of CA with intact plants or isolated chloroplasts, and most recently, molecular technology employing transgenics expressing CA antisense constructs. Modulation of growth conditions by reducing the availability of N or Zn will reduce CA activity however the pleiotropic effects of these treatments and the high percentage of residual CA activity make the data from such studies difficult to interpret. Vacuum infiltration of the CA inhibitor ethoxyzolamide into C_3 plant leaf pieces has been used recently to improve the access of these inhibitors to the target tissue (Badger and Pfanz, 1995). In this study, CA inhibited *Spinacea oleracea*, *Nicotiana rustica*, and *Hordeum vulgare* leaf pieces exhibited 30 to 50% inhibition of photosynthesis at low external C_i concentrations. As levels of C_i increased, inhibition of photosynthesis was diminished. These data suggest that CA plays a relatively prominent role in photosynthetic carbon fixation. This is certainly different from observations with transgenic *Nicotiana* in which CA levels have been reduced by antisense expression. In two independent studies, growth and photosynthesis at air concentrations of CO_2 and high or low light levels were not markedly affected by levels of CA as low as 1% to 2% of wild type plants (Majeau et al., 1994; Price et al., 1994). Interestingly, $^{13}\text{CO}_2$ discrimination values for on-line gas samples and for leaf dry matter of CA antisense plants were quite different from wild type plants. ^{13}C tissue concentrations were consistently higher in low CA plants (Price et al., 1994; Williams et al., 1996). In combination with gas exchange analysis, these data indicated that chloroplastic CO_2 concentrations were approximately 15 to 20 $\mu\text{mol} \cdot \text{mol}^{-1}$ lower in plants with low CA levels. The relatively small decrease in intracellular CO_2 would not result in a significant or easily observed decline in photosynthesis. Some initial data (Majeau et al., 1994) did suggest that low CA plants were compensating by increasing stomatal conductance to improve CO_2 entry into the leaf however this finding was not confirmed in a more

detailed gas exchange study of these plants (Williams et al., 1996). To further confound this issue, however, there is at least one published report of *Arabidopsis* antisense CA plants being unable to grow in the absence of sucrose or high levels of external CO_2 suggesting that in some plants a reduction in CA activity can have a profound influence (Kim and Bartlett, 1996).

The isolation of null isoform-specific CA mutants would be an ideal strategy for further study of the role of CA in C_3 plants. Antisense or co-suppressed plants will always have some residual activity which may be sufficient for growth and photosynthesis under most conditions. In addition, the antisense constructs used to date are not isoform or tissue specific, again making the interpretation of any recognized phenotype somewhat difficult. One possibility would be to screen mutagenized populations of *Arabidopsis* to look for a null allele, however, electrophoretic or chromatographic separation and activity determination of the two isoforms would be necessary. Simple activity analysis of crude leaf preparations will only identify a mutant deficient in both isoforms, an extremely unlikely event.

Regulation of carbonic anhydrase expression in C_3 plants has been studied in a number of species, primarily in terms of activity modulation under various environmental conditions. Certainly the availability of Zn influences activity levels but it is only under severe Zn deprivation that CA levels are reduced and as such it is not likely to be a true regulating agent in vivo. Both N and CO_2 availability influence CA expression in concert with Rubisco expression. In most C_3 plants as leaf nitrogen values increase, both Rubisco and CA levels in plants (with the exception of *Triticum*) increase proportionally (Makino et al., 1992). This increase in carboxylation capacity, however, is not completely realized as enhanced in vivo rates of CO_2 fixation. As well as electron transport limitations on the production of RuBP, resistance to CO_2 flux from outside the leaf through to the substomatal cavity, and CO_2 transfer resistance between the intercellular airspaces and the carboxylation sites limits the availability of CO_2 (Evans and von Caemmerer, 1996). Even as CA levels increase (in concert with Rubisco), the partial pressure of CO_2 at the carboxylation sites is lowered by the increased abundance of Rubisco. These data suggest that although increased CA levels occur, they alone are unable to maintain CO_2 concentrations

within the chloroplast. It is possible that an increased CA/Rubisco ratio would be more effective in maintaining chloroplast CO_2 levels, however it is more likely that the intercellular air space transfer resistance and/or stomatal conductance play a more prominent role in limiting CO_2 flux, and thus in vivo Rubisco activity. Reduced levels of N or elevated (above ambient) levels of CO_2 result in declines in both Rubisco and CA activities, protein and transcript abundance. Conversely, the transfer of plants to elevated N levels or from elevated to ambient CO_2 concentrations enhances Rubisco and CA expression. The response to CO_2 can be quite rapid with an over three-fold increase in both chloroplast CA (*ca*) and Rubisco small sub-unit (*rbcS*) transcript abundance occurring in *Pisum* within three to six hours after transfer from 1000 to 350 $\mu\text{mol} \cdot \text{mol}^{-1}$ CO_2 (Majeau and Coleman, 1996). CA expression is even greater at below ambient levels of CO_2 , conditions under which Rubisco levels decline, with the result that a considerable enhancement of the CA/Rubisco ratio occurs (Majeau and Coleman, 1996). Modulation of CA expression (and Rubisco) in response to elevated levels of CO_2 is presumably mediated by changes in carbohydrate levels and/or metabolism. Actual mechanisms or signaling agents have not yet been identified although considerable evidence supports the idea that specific hexokinase isoform activity is involved in carbohydrate induced down-regulation of transcription (Jang et al., 1997). The capacity for enhanced CA activity at less than ambient levels of CO_2 , conditions which seem to repress *rbcS* transcript levels, suggests that CA expression may respond differentially to very low levels of carbohydrates or additional signals, perhaps CO_2 itself. In addition to N and CO_2 , in *Pisum*, both chloroplastic CA and Rubisco expression at the levels of transcript and protein abundance are coordinated during leaf ontogeny and in response to light (Majeau and Coleman, 1994). This coordination of CA and Rubisco activity has also been described in cultivars of rice which exhibit different rates of leaf photosynthesis (Sasaki et al., 1996).

D. Cyanobacteria and Microalgae

Studies on regulation of CA expression and role in photosynthesis of aquatic microorganisms are numerous and have been extensively reviewed in previous years (Aizawa and Miyachi, 1986; Tsuzuki and Miyachi, 1989; Coleman, 1991; Sultemeyer et

al., 1993; Badger and Price, 1994). In this section I will provide a general overview of the area and focus on recent research efforts and existing gaps in our knowledge.

The aquatic environment impacts significantly in a number of ways on the supply of CO_2 to the site of carboxylation. The rate of CO_2 diffusion in water is slower than in air by a factor of 10^4 . pH values above 7 result in HCO_3^- becoming the predominant C_i species with the concentration of CO_2 in air equilibrated water remaining at 10 to 12 μM . In addition, the slow, uncatalyzed rate of dehydration of the external HCO_3^- pool (particularly at alkaline pHs) restricts its use in the provision of the species for CO_2 -requiring reactions. As a result, many (if not all) aquatic photosynthetic microorganisms have developed CO_2 concentrating mechanisms (CCMs) (some induced by limiting C_i) which are capable of active transport of CO_2 and/or HCO_3^- and the formation of an intracellular C_i pool. High concentrations of CO_2 derived from the intracellular C_i pool provide sufficient substrate for Rubisco that the oxygenase reaction is minimized and little if any photorespiration occurs. The efficiency of the CCMs is revealed by the low affinity of the microalgal and cyanobacterial Rubisco which exhibit $k_m \text{CO}_2$ values ranging from 50 μM for *Chlamydomonas* to over 200 μM for *Synechococcus*. Apparently, the delivery of CO_2 to the site of carboxylation is sufficiently effective that there has been little need for selection of a high affinity Rubisco (as in terrestrial C_3 plants). Along with a reduced affinity for CO_2 of the algal and cyanobacterial Rubisco there has been a corresponding increase in Rubisco k_{cat} values with the result that, in concert with efficient CCM activity, many of these organisms can exhibit very high levels of productivity over a wide range of growth conditions.

What is the role of CA in the activity of cyanobacterial and microalgal CCMs and photosynthesis? It is well established that cyanobacteria and microalgae express higher levels of CA activity when transferred from replete to limiting concentrations of C_i , conditions which maximize the expression of CCMs. The cellular localization and role of CA within the CCMs and photosynthesis, however, can vary among groups of organisms. In the unicellular cyanobacterium *Synechococcus* PCC 7942, which has become the model organism for much research in this area, a β -type CA, the product of the *icfA* (*ccaA*) gene (Fukuzawa et al., 1992; Yu et

al., 1992) has been identified and appears to be associated with carboxysomes (Price et al., 1992). Carboxysomes are protein membrane encapsulated Rubisco aggregates found in cyanobacteria (and some other photosynthetic prokaryotes) and are the primary sites of CO_2 fixation. Both carboxysome number and CA levels increase following growth at limiting concentrations of C_i (Price et al., 1992; McKay et al., 1993). Mutants deficient in either carboxysome formation or *icfA* expression, although still able to transport and generate an intracellular pool of C_i , are unable to utilize this pool efficiently and require high external levels of CO_2 for growth (Fukuzawa et al., 1992; Yu et al., 1992; Price et al., 1993). The *icfA* gene product (and carboxysomes) are presumed to be an integral part of the cyanobacterial CCM. CA is thought to speed the dehydration of HCO_3^- that diffuses into the carboxysome such that the CO_2 generated is in close proximity to the active Rubisco. The close association of *icfA* gene product and Rubisco minimizes the leakage of CO_2 out of the cyanobacterial cell however some loss does occur.

Additional CAs or CA-like proteins/activity are also found in cyanobacteria. One intriguing but still cryptic protein is also a suspected component of the cyanobacterial carboxysome. Although not known to have CA activity, the *ccmM* gene product of *Synechococcus* PCC 7942 is a 58 kDa polypeptide with considerable sequence similarity in the N-terminal region to the trimeric γCA isolated from the archaeabacterium *Methanoscincina thermophila*. The *ccmM* gene product also displays some limited sequence similarity to the small sub-unit of Rubisco including key residues required for binding of the small sub-unit to the large sub-unit of Rubisco (Price et al., 1993). Insertional inactivation of the *ccmM* gene results in cell lines which require high CO_2 concentrations for growth, although the potential disruption of carboxysome structure by such an inactivation strategy is also known to generate this phenotype (Price et al., 1993). Assuming a carboxysomal protein shell location for this protein, it is possible that it facilitates movement of HCO_3^- into the carboxysome. The phenotype of cyanobacterial mutants generated by site-specific mutation constructs in which any catalytic capacity of the *ccmM* encoded protein is inactivated but coherent transcription/translation of it and the surrounding operon still occurs may yet answer questions on the role of this protein.

Extracellular (presumably periplasmic) α -and

β -type CAs have also been identified in cyanobacteria (Soltes-Rak et al., 1997; G. Espie, unpublished). Again, their role (if any) in the CCM remains cryptic. Although expression appears to be modulated by external CO_2 levels, insertional inactivation of the genes encoding these proteins results in only minor phenotypic differences when the cell lines are compared with wild type cells grown at low, ambient or elevated CO_2 concentrations (Soltes-Rak et al., 1997). It is possible that the primary role of these extracellular CAs may be as sensors rather than catalytic elements, and as such they would be the initial component of a signal transduction pathway that allow the cyanobacteria to rapidly respond to changes in extracellular C_i . There is some evidence to suggest that cyanobacteria and microalgae can directly sense and respond to changing C_i levels independently of photosynthetic carbon metabolism. Proteins, such as CA which directly interact with CO_2 or HCO_3^- molecules, could play a role in such a signalling system. An unidentified CA-like moiety is also assumed to be an integral component of the cyanobacterial CCM. The activity of a ethoxy-zolamide-sensitive, presumably membrane-associated protein results in the conversion of CO_2 to HCO_3^- prior to or during transport across the cytoplasmic membrane. The isolation and characterization of a putative vectorial CA, if it exists, would be a major step in our understanding of inorganic transport systems in aquatic organisms.

For the eukaryotic microalgae, it is primarily studies of CCMs of the green algae *Chlamydomonas* and *Chlorella* that have advanced our understanding of the roles that CA can play in this process. Indeed, the first well characterized CA from a photosynthetic organism and the first protein associated with the acclimation of algae to limiting CO_2 concentrations was the periplasmic localized α -type CA of *Chlamydomonas reinhardtii*. Both transcript and protein levels encoded by *cah1* increase several fold following transfer of cells from surfeit to limiting levels of CO_2 , and this pattern of expression accompanies the induction of the high affinity C_i transport systems (Bailly and Coleman, 1988; Fukuzawa et al., 1990). An additional extracellular CA, the product of *cah2*, is expressed at much lower levels and in a reciprocal fashion to *cah1*, with transcript and protein present only at high levels of external CO_2 (Fujiwara et al., 1990; Rawat and Moroney, 1991). Although not absolutely required for either CO_2 or HCO_3^- transport, the presumed role

of the extracellular CAs is to maintain the external $\text{CO}_2/\text{HCO}_3^-$ equilibrium and thus ensure the availability of either C_i species for the respective transporters.

The study of intracellular CAs in microalgae has been a complicated issue. There were many earlier reports of various soluble and insoluble CA isoforms exhibiting differing responses to CA inhibitors and CO_2 levels, and localized in both the cytoplasm and chloroplast (Sultemeyer et al., 1993 and references within; Husic and Marcus, 1994; Amoroso et al., 1996), however, definitive protein or molecular evidence for many of these isoforms was lacking. Certainly some variation in data from the various research groups could be the result of numbers of different organisms and isolation procedures used, but it is only recently that substantive and compelling evidence for two specific intracellular isoforms has been presented. A novel α -type CA (*cah3* gene product) has been isolated and characterized, and in the initial study the CA appeared to be localized in the *Chlamydomonas* chloroplast (Karlsson et al., 1995). These data suggested perhaps an association with Rubisco in the pyrenoid (a starch-sheathed proteinaceous complex), where the CA would catalyze the supply of CO_2 from the intracellular HCO_3^- pool for fixation. The postulated association of Rubisco and CA in the pyrenoid would be analogous to that which is thought to occur in the cyanobacterial carboxysome, where CA and Rubisco are also co-localized. In a later study, it was shown that *Chlamydomonas* mutants exhibiting a high CO_2 requiring phenotype were genetically complemented with the *cah3* gene, thus helping to define a role for this CA in *Chlamydomonas* (Funke et al., 1997). In a recent study, it has now been shown that the *cah3* gene product is in fact found in the chloroplast, but localized to the thylakoid lumen of the *Chlamydomonas* chloroplast, and is required for growth at ambient levels of CO_2 (Karlsson et al., 1998). Expression appears to be regulated by C_i levels, with CA activity increasing at low levels of extracellular C_i . It has been hypothesized that within the lumen, CA would speed the dehydration of HCO_3^- transported into the lumen during light-induced proton uptake, and thus generate CO_2 for Rubisco catalyzed fixation. CA-containing thylakoids which traverse the Rubisco-rich pyrenoid may provide the co-localization needed for the efficient coupling of the intracellular HCO_3^- pool and the requisite formation of CO_2 .

In addition to the α -type chloroplast CA, a new β -type CA localized in the mitochondria of *Chlamydomonas* has been characterized (Eriksson et al., 1996). Two highly homologous nuclear genes (*ca1* and *ca2*) encode two identical 20.7 kDa proteins which are both expressed following transfer of the cells to limiting CO_2 concentrations. The postulated role for this enzyme is to assist in stabilizing mitochondrial pH. Following transfer of the cells to low CO_2 concentrations and prior to the full induction of the algal CCM, there is an increased flux of photorespiratory intermediates produced by Rubisco operating in a CO_2 limited environment. Mitochondrial decarboxylation of glycine may produce sufficient amounts of NH_3 that alkalization of the mitochondrial matrix could occur. CA activity would ameliorate this problem by speeding the hydration of photorespiratory CO_2 and the concomitant production of H^+ (Eriksson et al., 1996). It is interesting to note that another study has described changes in mitochondria location in *Chlamydomonas* cells acclimating to limiting CO_2 concentrations and have identified by immunogold labeling a mitochondria-localized polypeptide synthesized in response to low CO_2 . (Geraghty and Spalding, 1996).

An intriguing and unknown element in the C_i regulation of CA (and the CCMs) expression in microalgae and cyanobacteria is the nature of the inducing signal. It had long been supposed that various carbon metabolite levels, presumably photorespiratory in origin, increased when cells were transferred from surfeit to limiting C_i concentrations and that this organic carbon flux triggered the induction of CA along with CCM expression (Coleman, 1991 and references within). The reported absolute requirement for light and photosynthesis, as well as the O_2 sensitivity of the induction process all supported this argument. There were also, however, reports showing that induction of *Chlamydomonas cah1* transcript and protein could also occur when cells were transferred to limiting CO_2 concentrations in the dark, and in *Chlorella*, CA activity could be induced at low CO_2 in the presence of the electron transport inhibitor DCMU or very low light (Bailly and Coleman, 1988; Shiraiwa and Miyachi, 1983). Recently, these earlier reports have been complemented by additional studies that show that *cah1* expression in *Chlamydomonas* can be circadianly entrained (Fujiwara et al., 1996), and can be induced in the dark following transfer from high to limiting CO_2 concentrations in both synchronous (Rawat and

Moroney, 1995) and asynchronous cultures (Villarejo et al., 1996). Studies with *Chlorella ellipsoidea* have also shown that induction of the CCM occurs in response to external CO_2 concentrations, not intracellular CO_2 levels, and that light speeds but is not required for CCM induction (Matsuda and Colman, 1995). Data obtained from a novel set of experiments in which the *ca1/ca2* promoter region of the mitochondria-localized CA was ligated to the arylsulfatase reporter gene and used to transform *Chlamydomonas* support these observations (Villand et al., 1997). As expected, arylsulfatase expression was induced by limiting CO_2 concentrations (as is wild type *ca1/ca2* expression) but was not repressed by exposure to low O_2 concentrations and limiting CO_2 levels. These conditions should limit the production of photorespiratory metabolites, and yet CA expression still occurred. Such an experimental system is ideal for more detailed promoter analysis and characterization of both *cis* and *trans* elements required for CO_2 sensing and CA expression. In addition, using this reporter system in combination with *Chlamydomonas* mutagenesis it should be possible to determine the entire signal transduction pathway for acclimation to external CO_2 in microalgae. The elucidation of algal mechanisms of CO_2 sensing and response may also lead to a better understanding of CO_2 responsive elements in both higher plants and cyanobacteria.

References

- Aizawa K and Miyachi S (1986) Carbonic anhydrase and CO_2 concentrating mechanisms in microalgae and cyanobacteria. *FEMS Microbiol Rev* 39: 215–233
- Alber BE and Ferry JG (1994) A carbonic anhydrase from the archaeon *Methanosc礼na thermophila*. *Proc Natl Acad Sci USA* 91: 6909–6913
- Amoroso G, Weber C, Sultemeyer D and Fock H (1996) Intracellular carbonic anhydrase activities in *Dunaliella tertiolecta* (Butcher) and *Chlamydomonas reinhardtii* (Dangeard) in relation to inorganic carbon concentration during growth: Further evidence for the existence of two distinct carbonic anhydrases associated with the chloroplast. *Planta* 199: 177–184
- Anderson LE, Gibbons JT and Wang X (1996) Distribution often enzymes of carbon metabolism in pea (*Pisum sativum*) chloroplasts. *Int J Plant Sci* 157: 525–538
- Badger MR and Pfanz H (1995) Effect of carbonic anhydrase inhibition on photosynthesis by leafpieces of C_3 and C_4 plants. *Aust J Plant Physiol* 22: 45–49
- Badger MR and Price GD (1994) The role of carbonic anhydrase in photosynthesis. *Ann Rev Plant Physiol Plant Molec Biol* 45: 369–392
- Bailly J and Coleman JR (1988) Effect of CO_2 concentration on protein biosynthesis and carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiol* 87: 833–840
- Bjorkbacka H, Johansson I-M, Skarstad E and Forsman C (1997) The sulfhydryl groups of Cys 269 and Cys 272 are critical for the oligomeric state of chloroplast carbonic anhydrase from *Pisum sativum*. *Biochemistry* 26: 4287–4294
- Bracey MH and Bartlett SG (1995) Sequence of a cDNA encoding carbonic anhydrase from barley. *Plant Physiol* 108: 433–434
- Bracey MH, Christiansen J, Tovar P, Cramer SP and Bartlett SG (1994) Spinach carbonic anhydrase: Investigation of the zinc-binding ligands by site-directed mutagenesis, elemental analysis, and EXAFS. *Biochemistry* 33: 13126–13131
- Burnell JN (1990) Immunological study of carbonic anhydrase in C_3 and C_4 plants using antibodies to maize cytosolic and spinach chloroplastic carbonic anhydrase. *Plant Cell Physiol* 31:423–427
- Burnell JN and Hatch MD (1988) Low bundle sheath carbonic anhydrase is apparently essential for effective C_4 pathway operation. *Plant Physiol* 86:1251–1256
- Burnell JN and Ludwig M (1997) Characterisation of two cDNAs encoding carbonic anhydrase in maize leaves. *Aust J Plant Physiol* 24: 451–458
- Burnell JN, Gibbs MJ and Mason JG (1990a) Spinach chloroplastic carbonic anhydrase: Nucleotide sequence analysis of cDNA. *Plant Physiol* 92: 37–40
- Burnell JN, Suzuki I and Sugiyama T (1990b) Light induction and the effect of nitrogen status upon the activity of carbonic anhydrase in maize leaves. *Plant Physiol* 94: 384–387
- Coleman JR (1991) The molecular and biochemical analyses of CO_2 concentrating mechanisms in cyanobacteria and green algae. *Plant Cell Environ* 14: 861–867
- Coleman JR and Grossman AR (1984) Biosynthesis of carbonic anhydrase in *Chlamydomonas reinhardtii* during adaptation to low CO_2 . *Proc Natl Acad Sci USA* 81: 6049–6053
- Cowan IR (1986) Economics of carbon fixation in higher plants. In: Givnish TJ (ed) On the Economy of Plant Form and Function, pp 130–170. Cambridge University Press, London
- Eriksson M, Karlsson J, Ramazanov Z, Gardestrom P and Samuelsson G (1996) Discovery of an algal mitochondrial carbonic anhydrase: Molecular cloning and characterization of a low- CO_2 -induced polypeptide in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 93: 12031–12034
- Evans JR and von Caemmerer S (1996) Carbon dioxide diffusion inside leaves. *Plant Physiol* 110: 339–346
- Fawcett TW, Browse JA, Volokita M and Bartlett SG (1990) Spinach carbonic anhydrase primary structure deduced from the sequence of a cDNA clone. *J Biol Chem* 265: 5414–5417
- Fett JP and Coleman JR (1994) Characterization and expression of two cDNAs encoding carbonic anhydrase in *Arabidopsis thaliana*. *Plant Physiol* 105: 707–713
- Forsman C and Pilon M (1995) Chloroplast import and sequential maturation of pea carbonic anhydrase: The roles of various parts of the transit peptide. *FEBS Lett* 358: 39–42
- Fujiwara S, Fukuzawa H, Tachiki A and Miyachi S (1990) Structure and differential expression of two genes encoding carbonic anhydrase in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 87: 9779–9783
- Fujiwara S, Ishida N and Tsuzuki M (1996) Circadian expression of the carbonic anhydrase gene *Cah1*, in *Chlamydomonas*

- reinhardtii*. Plant Molec Biol 32: 745–749
- Fukuzawa H, Fujiwara S, Yamamoto Y, Dionisio-Sese ML and Miyachi S (1990) cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*: Regulation by environmental CO₂ concentration. Proc Natl Acad Sci USA 87: 8383–8387
- Fukuzawa H, Suzuki E, Komukai Y and Miyachi S (1992) A gene homologous to chloroplast carbonic anhydrase (*icfA*) is essential to photosynthetic carbon fixation in the cyanobacterium *Synechococcus* PCC7924. Proc Natl Acad Sci USA 89: 4437–4441
- Funke RP, Kovar JL and Weeks DP (1997) Intracellular carbonic anhydrase is essential to photosynthesis in *Chlamydomonas reinhardtii* at atmospheric levels of CO₂. Demonstration via genomic complementation of the high CO₂-requiring mutant *ca-1*. Plant Physiol 114: 237–244
- Furbank RT, Chitty JA, von Caemmerer S and Jenkins CLD (1996) Antisense RNA inhibition of *RbcS* gene expression reduces Rubisco level and photosynthesis in the C₄ plant *Flaveria bidentis*. Plant Physiol 111: 725–734
- Geraghty AM and Spalding MH (1996) Molecular and structural changes in *Chlamydomonas* under limiting CO₂. A possible mitochondrial role in adaptation. Plant Physiol 111: 1339–1347
- Graham D, Reed ML, Patterson BD, Hockley DG and Dwyer MR (1984) Chemical properties, distribution and physiology of plant and algal carbonic anhydrases. Ann NY Acad Sci 429: 222–237
- Guliev NM, Bairamov SM and Aliev DA (1992) Functional organization of carbonic anhydrase in higher plants. Soviet Plant Physiol 39: 537–544
- Hatch MD and Burnell JN (1990) Carbonic anhydrase activity in leaves and its role in the first step of C₄ photosynthesis. Plant Physiol 93: 380–383
- Hewett-Emmett D and Tashian RE (1996) Functional diversity, conservation and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. Mol Phylogenet Evol 5: 50–77
- Husic HD and Marcus CA (1994) Identification of intracellular carbonic anhydrase in *Chlamydomonas reinhardtii* with a carbonic anhydrase directed photoaffinity label. Plant Physiol 105: 133–139
- Jang J-C, Leon P, Zhou L and Sheen J (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell 9: 5–19
- Jebanathirajah JA and Coleman JR (1998) Association of carbonic anhydrase with a Calvin cycle enzyme complex in *Nicotiana tabacum*. Planta 204: 117–182
- Jenkins CLD, Burnell JN and Hatch MD (1987) Form of inorganic carbon involved as a product and as an inhibitor of C₄ acid decarboxylases operating in C₄ photosynthesis. Plant Physiol 85: 952–957
- Jenkins CLD, Furbank RT and Hatch MD (1989) Mechanism of C₄ photosynthesis. A model describing the inorganic carbon pool in bundle sheath cells. Plant Physiol 91: 1372–1381
- Johansson I-M and Forsman C (1992) Processing of the chloroplast transit peptide of pea carbonic anhydrase in chloroplasts and in *E. coli*. Identification of two cleavage sites. FEBS Lett 314: 232–236
- Johansson I-M and Forsman C (1993) Kinetic studies of pea carbonic anhydrase. Eur J Biochem 218: 439–446
- Kachru RB and Anderson L (1974) Chloroplast and cytoplasmic enzymes. V. Pea-leaf carbonic anhydrase. Planta 118: 235–240
- Kamo T, Shimogawara K, Fukuzawa H, Muto S and Miyachi S (1990) Subunit composition of carbonic anhydrase from *Chlamydomonas reinhardtii*. Eur J Biochem 192: 557–562
- Karlsson J, Hiltonen T, Husic DH, Ramazanov Z and Samuelsson G (1995) Intracellular carbonic anhydrase of *Chlamydomonas reinhardtii*. Plant Physiol 109: 533–539
- Karlsson J, Clarke AK, Huggins SY, Park Y-L, Husic HD, Moroney JV and Samuelsson G (1998) A novel α-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂. EMBO J 17: 1208–1216
- Kim HJ and Bartlett SG (1996) Transgenic *Arabidopsis* plants expressing carbonic anhydrase in the antisense orientation can not grow on MS media without sucrose. Plant Physiol (Suppl) 111: 96
- Kisker C, Schindelin H, Alber BE, Ferry JG and Rees DC (1996) A left-handed helix revealed by the crystal structure of a carbonic anhydrase from the archaeon *Methanosarcina thermophila*. EMBO J 15: 2323–2330
- Ludwig M and Burnell JN (1995) Molecular comparison of carbonic anhydrase from *Flaveria* species demonstrating different photosynthetic pathways. Plant Molec Biol 29: 353–365
- Ludwig M, von Caemmerer S, Price DE, Badger MR and Furbank RT (1998) Expression of tobacco carbonic anhydrase in the C₄ dicot *Flaveria bidentis* leads to increased leakiness of the bundle sheath and a defective CO₂-concentrating mechanism. Plant Physiol 117: 1071–1081
- Makino A, Sakashita H, Hidema J, Mae T, Ojiima K and Osmond B (1992) Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase in wheat leaves to nitrogen nutrition and their possible relationships to CO₂-transfer resistance. Plant Physiol 100: 1737–1743
- Majeau N and Coleman JR (1991) Isolation and characterization of a cDNA coding for pea chloroplastic carbonic anhydrase. Plant Physiol 95: 264–268
- Majeau N and Coleman JR (1992) Nucleotide sequence of a complementary DNA encoding tobacco chloroplastic carbonic anhydrase. Plant Physiol 100: 1077–1078
- Majeau N and Coleman JR (1994) Correlation of carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase expression in pea. Plant Physiol 104: 1393–1399
- Majeau N and Coleman JR (1996) Effect of CO₂ concentration on carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase expression in pea. Plant Physiol 112: 569–574
- Majeau N, Arnoldo MA and Coleman JR (1994) Modification of carbonic anhydrase activity by antisense and over-expression constructs in transgenic tobacco. Plant Molec Biol 25: 377–385
- Matsuda Y and Colman B (1995) Induction of CO₂ and bicarbonate transport in the green alga *Chlorella ellipsoidea*. Plant Physiol 108: 253–260
- Maxwell K, von Caemmerer S and Evans JR (1997) Is the low internal conductance to CO₂ diffusion a consequence of succulence in plants with crassulacean acid metabolism. Aust J Plant Physiol 24: 777–786
- McKay MR, Gibbs SP and Espie GS (1993) Effect of dissolved inorganic carbon on the expression of carboxysomes,

- localization of Rubisco and the mode of inorganic carbon transport in cells of the cyanobacterium *Synechococcus* UTEX 625. Arch Microbiol 159: 21–29
- Newman T, de Bruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikhel N, Somerville S, Thomashow M, Retzel E and Somerville C (1994) Genes galore: A summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol 106: 1241–1255
- Poincelot RP (1979) Carbonic anhydrase. In: Gibbs M and Latzko E (eds) Encyclopedia of Plant Physiology, Vol 6 (2), pp 230–238. Springer-Verlag, Berlin
- Price GD, Coleman JR and Badger MR (1992) Association of carbonic anhydrase activity with carboxysomes isolated from the cyanobacterium *Synechococcus* PCC7942. Plant Physiol 100: 784–793
- Price GD, Howitt SM, Harrison K and Badger MR (1993) Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. Strain PCC7942 involved in carboxysome assembly and function. J Bacteriol 175: 2871–2879
- Price GD, von Caemmerer S, Evans JR, Yu J-W, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A and Badger MR (1994) Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco has a minor effect on photosynthetic CO_2 assimilation. Planta 193: 331–340
- Provart NJ, Majeau N and Coleman JR (1993) Characterization of pea chloroplast carbonic anhydrase. Expression in *E. coli* and site-directed mutagenesis. Plant Molec Biol 22: 937–942
- Raines CA, Horsnell PR, Holder C and Lloyd JC (1992) *Arabidopsis thaliana* carbonic anhydrase: cDNA sequence and effect of CO_2 on mRNA levels. Plant Molec Biol 20: 1143–1148
- Rawat M and Moroney JV (1991) Partial characterization of a new isozyme of carbonic anhydrase isolated from *Chlamydomonas reinhardtii*. J Biol Chem 266: 9719–9723
- Rawat M and Moroney JV (1995) The regulation of carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase activase by light and CO_2 in *Chlamydomonas reinhardtii*. Plant Physiol 109: 937–944
- Reed ML and Graham D (1981) Carbonic anhydrase in plants: Distribution, properties and possible physiological roles. Progress Phytochem 7: 47–94
- Roeske CA and Ogren WL (1990) Nucleotide sequence of pea cDNA encoding chloroplast carbonic anhydrase. Nucl Acid Res 18: 3413
- Rowlett RS, Chance MR, Wirt MD, Sidelinger DE, Royal JR, Woodroffe M, Wang Y-HA, Saha R and Lam MG (1994) Kinetic and structural characterization of spinach carbonic anhydrase. Biochemistry 33: 13967–13976
- Rumeau D, Ciune S, Fina L, Gault N, Nicole M and Peltier G (1996) Subcellular distribution of carbonic anhydrase in *Solanum tuberosum* L. leaves. Planta 199: 79–88
- Sasaki H, Samejima M and Ishii R (1996) Analysis of $\delta^{13}\text{C}$ measurement on the mechanism of cultivar difference in leaf photosynthesis of rice (*Oryza sativa* L.). Plant Cell Physiol 37: 1161–1166
- Shiraiwa Y and Miyachi S (1983) Factors controlling induction of carbonic anhydrase and efficiency of photosynthesis in *Chlorella vulgaris* 11h cells. Plant Cell Physiol 24: 919–923
- Silverman DN (1991) The catalytic mechanism of carbonic anhydrase. Can J Bot 69: 1070–1078
- Soltes-Rak E, Mulligan M and Coleman JR (1997) Identification and characterization of a gene encoding a vertebrate-type carbonic anhydrase in cyanobacteria. J Bacteriol 179: 769–774
- Stemler AJ (1997) The case for chloroplast thylakoid carbonic anhydrase. Physiol Plant 99: 348–353
- Sugiharto B, Suzuki I, Burnell JN and Sugiyama T (1992) Glutamine induces the N-dependent accumulation of mRNAs encoding phosphoenolpyruvate carboxylase and carbonic anhydrase in detached maize leaf tissue. Plant Physiol 100: 2066–2070
- Sultemeyer D, Schmidt C and Fock HP (1993) Carbonic anhydrases in higher plants and aquatic microorganisms. Physiol Plant 88: 179–190
- Süss K-H, Arkona C, Manteuffel R and Adler K (1993) Calvin cycle multienzyme complexes are bound to chloroplast thylakoid membranes of higher plants in situ. Proc Natl Acad Sci USA 90: 5514–5518
- Suzuki S and Burnell JN (1995) Nucleotide sequence of a cDNA encoding rice chloroplastic carbonic anhydrase. Plant Physiol 107: 299–300
- Tashian RE (1989) The carbonic anhydrases: Widening perspectives on their evolution, expression and function. Bioessays 10: 186–192
- Tsuzuki M, Miyachi S, Winter K and Edwards GE (1982) Localization of carbonic anhydrase in crassulacean acid metabolism plants. Plant Sci Lett 24: 211–218
- Tsuzuki M and Miyachi S (1989) The function of carbonic anhydrase in aquatic photosynthesis. Aquatic Bot 34: 85–104
- Utsunomiya E and Muto S (1993) Carbonic anhydrase in the plasma membrane from leaves of C_3 and C_4 plants. Physiol Plant 88: 413–419
- Villand P, Eriksson M and Samuelsson G (1997) Carbon dioxide and light regulation of promoters controlling the expression of mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. Biochem J 327: 51–57
- Villarejo A, Reina GG and Ramazanov Z (1996) Regulation of the low CO_2 inducible polypeptides in *Chlamydomonas reinhardtii*. Planta 199: 481–485
- Williams TG, Flanagan LB and Coleman JR (1996) Photosynthetic gas exchange and discrimination against $^{13}\text{CO}_2$ and $\text{C}^{18}\text{O}^{16}\text{O}$ in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase. Plant Physiol 112: 319–326
- Yu J-W, Price GD and Badger MR (1992) Isolation of a putative carboxysomal carbonic anhydrase gene from the cyanobacterium *Synechococcus* PCC7942. Plant Physiol 100: 794–800

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Chapter 16

CO₂ Acquisition, Concentration and Fixation in Cyanobacteria and Algae

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Summary

Aquatic photosynthetic organisms face a number of unique problems with regard to the supply of CO_2 for photosynthesis. These stem largely from the physical chemistry of the water phase in which they live, where the diffusion of C_i species is slow and C_i can exist as both CO_2 and HCO_3^- , depending on the pH of the medium. Given the constraints, a number of solutions have evolved to optimize photosynthetic CO_2 fixation in algae and cyanobacteria. The two chief strategies that are apparent are the development of CO_2 -concentrating mechanisms based on the active uptake of both CO_2 and HCO_3^- and the evolution of more efficient forms of Rubisco which are able to fix CO_2 at limiting levels of CO_2 . This chapter examines aspects of co-evolution of Rubisco and CO_2 -concentrating mechanisms in both algae and cyanobacteria. Particular emphasis is placed on what is known about the mechanism of the operation of carbon concentrating mechanisms (CCMs) in cyanobacteria and green microalgae. In cyanobacteria, multiple active C_i transporters drive the CCM on the plasma membrane. These are energized by photosynthetic ATP and NADPH production, with the NAD(P)H dehydrogenase complexes playing a critical role. A pool of HCO_3^- is accumulated within the cell and this is used by the Rubisco-containing carboxysome to generate CO_2 within this localized micro-environment. Carboxysomal carbonic anhydrase is crucial to this CO_2 generation process. For green microalgae, active C_i transport occurs at both the plasma membrane and the chloroplast envelope. A HCO_3^- pool is accumulated in the chloroplast stroma, with the aid of photosynthetic energy, and this pool is used to elevate CO_2 around Rubisco. Rubisco is primarily localized to the pyrenoid. A crucial part of this conversion appears to be a thylakoid lumen carbonic anhydrase, which may use the luminal protons to drive this process. The CCM is inducible in nature in both cyanobacteria and algae, increasing its affinity for external C_i when cells are grown at limiting C_i conditions. In both green and non-green algae, the presence of significant CCM activity is correlated with the presence of pyrenoids and single chloroplasts within cells. This chapter examines the possible diversity of CCM operation among the green and non-green algae, highlighting possible variation in CCM operations compared to the models developed for green microalgae. Considerable work remains to be done to identify specific variation of CCM mechanisms in non-green algae.

I. Supply of CO_2 in an Aquatic Environment

Algae and cyanobacteria undertake photosynthesis in an aquatic environment where they face a number of unique problems regarding the efficient operation of photosynthesis. Among these, the acquisition of CO_2 from the external medium and its supply as substrate for the primary CO_2 -fixing enzyme Rubisco (EC 4.1.1.39) has been of great significance. The evolution of terrestrial plants was a major step in altering the limitations imposed by these aquatic problems. Air filled leaves reduced the effective aqueous diffusion path to a few micrometers and the photosynthetic cells have rapid access to the CO_2 existing in the bulk atmosphere (Raven, 1970; Kerby and Raven, 1985; Raven et al., 1985). Since the

diffusion coefficient for CO_2 in air is some 10^4 higher than in water, this greatly reduces the problems of CO_2 supply to Rubisco.

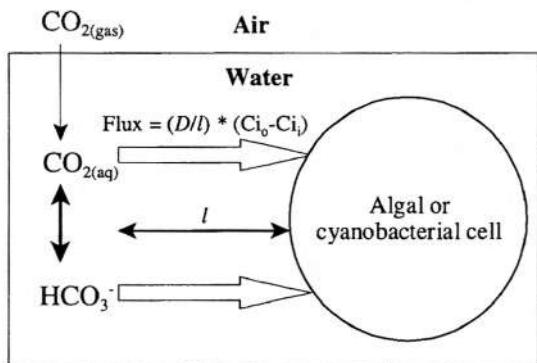
The nature of CO_2 supply problems and opportunities facing an aquatic photosynthetic cell stem largely from the physical chemistry relating to inorganic carbon (C_i) species in solution. Figure 1 depicts aspects of this supply problem. There is relatively slow equilibration of CO_2 between the air and the water, which can result in a depletion of the water [C_i] during conditions of active photosynthesis (Talling, 1985; Adams, 1985). In solution, all species of C_i , including CO_2 , HCO_3^- and CO_3^{2-} diffuse relatively slowly, so if unstirred layer thicknesses are significant then draw-down can occur between the bulk water and the cell surface. For small cells such as microalgae and cyanobacteria, the diffusion path through the unstirred layer (l) is very small (less than a few μm) so unstirred layer thicknesses are not a limitation. However, this is not so for macrophyte algae which have much larger multicellular dimensions (Kerby and Raven, 1985).

Perhaps the most significant feature of the aquatic

Abbreviations: CA – carbonic anhydrase; CCM – CO_2 -concentrating mechanism; C_i – inorganic carbon ($\text{CO}_2 + \text{HCO}_3^-$); DCMU – dichlorodiphenylmethoxy urea; PEP – phosphoenolpyruvate; PGA – 3-phosphoglyceric acid; RuBP – ribulose 1,5-bisphosphate; Rubisco – ribulose bisphosphate carboxylase/oxygenase; S_{rel} – relative specificity factor for Rubisco relating the carboxylase to oxygenase reaction kinetics $(V_c * K_o) / (K_o * V_o)$.

Ci Supply to the Aquatic Photosynthetic Cell

Inorganic carbon in the aquatic environment



Where,

 $\text{Flux} = \text{mol.m}^{-2}.\text{s}^{-1}$ $D = \text{diffusivity (m}^2\text{s}^{-1}\text{)}$ $Ci_0 = [\text{Ci}] \text{ in bulk phase (mol m}^{-3}\text{)}$ $Ci_i = [\text{Ci}] \text{ inside cell (mol m}^{-3}\text{)}$ $l = \text{thickness of unstirred layer (m)}$

Fig. 1. Inorganic carbon supply (C_i) to the aquatic photosynthetic cell.

system are that all species of C_i (CO₂, HCO₃⁻, and CO₃²⁻) are interconverted by a series of hydration, dehydration and protonation reactions as described in Fig. 2. The significant features of this chemistry are that firstly the ratio of CO₂ to HCO₃⁻ in solution is highly dependent on the pH of the aquatic environment and secondly, that the hydration of CO₂ to HCO₃⁻ is very slow compared to the use of CO₂ by photosynthesis (Badger and Price, 1994). The consequences of this chemistry are two-fold. Firstly, HCO₃⁻ becomes the dominant C_i species in solution above pH 6.3. This is particularly so in marine environments where the pH is above 8, HCO₃⁻/CO₂ is > 50 and the [HCO₃⁻] is around 1 mM compared to 10 μM for [CO₂]. Secondly, in environments where photosynthesis is relatively rapid per unit volume (either in the bulk phase or unstirred layers) both the slow conversion of HCO₃⁻ to CO₂ and solubilization of CO₂ from the air can cause a significant depletion of CO₂ below its air equilibrium value. Accompanying rises in pH in relatively unbuffered water bodies (Adams, 1985; Spence and Maberly, 1985; Talling, 1985) exacerbates this.

As will be described later, both algae and cyanobacteria have developed mechanisms to enable both CO₂ and HCO₃⁻ to be used to support photosynthesis. For some cells, this has been achieved by direct transport of HCO₃⁻ across otherwise

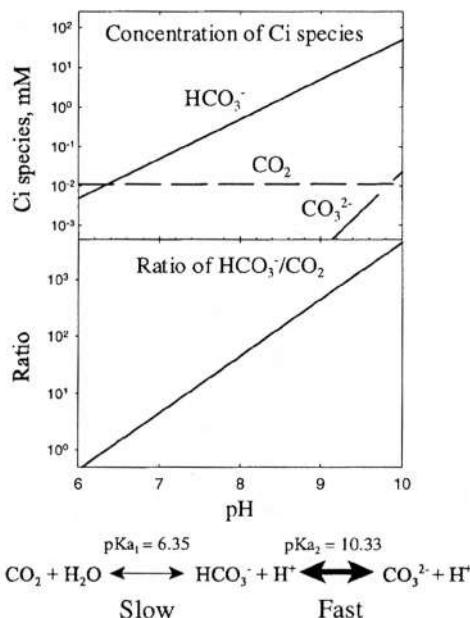


Fig. 2. The effect of pH on the distribution of inorganic carbon species in the aquatic environment. The CO₂ in the water is presumed to be in equilibrium with the air and shown as constant across the pH range.

impermeable lipid cell membranes while others are able to use variable amounts of CO₂ either by passive diffusion into the cell or active uptake. For the uptake of CO₂, the frequent prevalence of extracellular CA in algae, in particular, allows these cells to make rapid use of the external HCO₃⁻ pool and overcome its chemically slow dehydration to CO₂ (see Badger and Price, 1994).

II. Efficient CO₂ Capture Mechanisms Evolved Following Changes in Atmospheric CO₂ and O₂

The process of photosynthetic CO₂ fixation has evolved over time in response to a number of environmental constraints and photosynthetic organisms have presumably developed to optimize their efficiency under a range of environmental conditions. In particular, the extent to which CO₂, the primary substrate for Rubisco, operates as a limiting factor has changed dramatically over the time span of evolution. In view of this, it is not surprising that

there has been diverse adaptation by organisms in the mechanisms that have developed to alleviate the limitation which CO_2 availability imposes on photosynthesis.

The primary CO_2 fixing enzyme in all photosynthetic organisms, Rubisco, appears to have arisen once in the evolution of life, appearing with the chemolithotrophs, which arose some 3.5×10^9 years ago (Broda, 1975). In these environments, the CO_2 level was high and the O_2 was very low. Carbon dioxide was unlikely to have presented a substrate limitation to Rubisco and it is likely to have operated close to substrate saturation in these organisms. In addition, the deleterious effects of the oxygenase activity of Rubisco would not have been significant and photorespiration was unlikely to have been a problem.

Eventually, however, the operation of CO_2 consuming processes, particularly Rubisco carboxylation, led to a decline in the level of atmospheric CO_2 , particularly in the carboniferous era some 350 million years ago (Berner, 1990, 1993). Furthermore, with the advent of cyanobacteria and oxygenic photosynthesis, the O_2 levels began to rise. The result of these changes was that the CO_2/O_2 ratio fell dramatically and the atmospheric and aquatic environments became progressively less favorable for carboxylation by Rubisco. Primitive Rubiscos had poor kinetic properties with respect to their affinity for CO_2 and relatively high oxygenase potentials (Badger and Andrews, 1987; Badger et al., 1998). Eventually, net CO_2 fixation would have been threatened if changes had not occurred to either the properties of the Rubisco or the manner in which CO_2 was supplied to Rubisco from the external environment.

Not unexpectedly then, photosynthetic organisms have adapted to the changing CO_2 conditions of the environment in two ways. Firstly, Rubisco itself has shown considerable ability to evolve by increasing its affinity for CO_2 ($K_m\text{CO}_2$) and decreasing the relative activity of the oxygenase reaction, as evidenced by a higher S_{rel} value (see section below). Secondly, organisms have developed mechanisms to insulate Rubisco from the external environment where the CO_2 levels are actively elevated (Badger, 1987).

If a broad range of aerobic photosynthetic organisms is considered, including cyanobacteria, algae and higher plants, then it appears that the majority have developed some form of CO_2 -concentrating mechanism (CCM) which aids the

performance of Rubisco. In higher plants, the most obvious example is the development of C_4 photosynthesis, but functionally equivalent mechanisms have also developed in algae and cyanobacteria. Although there are significant differences between the particular details of each CCM, comparisons show that there are similar functional elements of CCMs across all systems. Table 1 and Fig. 3 present a comparison of these functional CCM components in C_4 higher plants, algae and cyanobacteria. The common components between cyanobacteria and algae include: active Ci transport systems, energized by photosynthesis; an internal pool of Ci which is the source of CO_2 for Rubisco; a compartmentalized Rubisco, in either a carboxysome or pyrenoid, together with carbonic anhydrase that generates CO_2 from HCO_3^- ; a barrier to the efflux of CO_2 .

In all CCMs, the balance between the rate at which CO_2 is produced in the localized Rubisco compartment and the rate at which it leaks back to the external environment or is fixed by Rubisco determines the extent to which CO_2 is elevated around Rubisco. Detailed aspects of the operation of the CCMs in cyanobacteria and algal species will be explored more fully later in this chapter.

III. Co-evolution of CCMs and Rubisco

In recent years, evolutionary and biochemical studies of Rubisco have given some new and exciting insights into the events which have led to the distribution of different Rubisco lineages among the algae, cyanobacteria and higher plants. The current evolutionary tree of algal and cyanobacterial Rubiscos is summarized in Fig. 4. It is evident that Rubisco shows an evolutionary lineage that is quite different to the light harvesting machinery of the thylakoids (Delwiche and Palmer, 1996; Watson and Tabita, 1997).

Within algae, cyanobacteria and higher plants, there are at least 3 distinct types of Rubisco enzymes. Form 1 L_8S_8 Rubisco forms dominate algal and higher plant chloroplasts, but there are distinct differences among these enzymes. Most of the cyanobacteria, chlorophyte algae and higher plants possess what has been termed the green Form 1a type enzymes with evolutionary lineage from the cyanobacterial Form 1 enzyme. Interestingly, it has been recently recognized that some cyanobacteria (e.g. *Synechococcus WH7803*) possess a green Form

Table 1. A comparison of the components of CO₂ concentrating mechanisms present in C₄ higher plants, algae and cyanobacteria. The generic CCM components are described in the model shown in Fig. 3.

CCM component	Photosynthetic species		
	C ₄ plants	Algae	Cyanobacteria
1. An active CO ₂ capture system	PEP carboxylase in the cytosol of the specialized mesophyll cells	Active CO ₂ and HCO ₃ ⁻ transport on both chloroplast and cell membranes	Active CO ₂ and HCO ₃ ⁻ transport systems on the cell membrane
2. Photosynthetic energy supply	ATP provided by chloroplasts for PEP regeneration	Photosynthetically derived ATP serves to support active transport	Photosynthetically derived ATP and perhaps NADPH energize transport
3. An intermediate pool of captured CO ₂	C ₄ acids, malate and aspartate	HCO ₃ ⁻ in the chloroplast stroma	HCO ₃ ⁻ in the cytoplasm
4. A mechanism to release CO ₂ from the intermediate CO ₂ pool	Specific C ₄ acid decarboxylating enzymes in the bundle sheath cells	Carbonic anhydrase specifically associated with the pyrenoid and the thylakoids	Carbonic anhydrase specifically in the carboxysome
5. A compartment to concentrate CO ₂ around Rubisco	The bundle sheath compartment with specialized bundle sheath cells and chloroplasts	The pyrenoid compartment in the chloroplast	The carboxysome compartment in the cytoplasm
6. A means to reduce CO ₂ leakage from the site of CO ₂ elevation	The bundle sheath structure, including suberized layers, as well as an absence of CA in this compartment	Some physical property of the pyrenoid, combined with an absence of CA from the stroma	Some physical property of the carboxysome, combined with an absence of CA from the cytoplasm
7. Modification of the kinetic properties of Rubisco	An intermediate affinity for CO ₂ , high S _{rel} and increased V _{max}	Depends on whether it is a green or non-green alga. See Fig. 4.	A low affinity for CO ₂ , low S _{rel} and a very high V _{max}

1b enzyme with evolutionary linkages to the enzyme from β/γ proteobacteria such as *Chromatium vinosum* (Watson and Tabita, 1996). The non-green algae, containing Chrysophytes, Rhodophytes and Phaeophytes, are distinctly different from the Chlorophyte algae. They possess a Red Form 1 Type Rubisco, with evolutionary linkages to the α/β purple proteobacteria such as *Alcaligenes eutrophus*. Dinoflagellates such as *Amphidinium carterae* are much more divergent than other non-green algae, possessing an L₈ Form II Rubisco enzyme, related to proteobacteria such as *Rhodospirillum rubrum*.

The existence of such distinctly different forms of Rubisco enzymes within the algae has implications when considering the evolution of strategies to acquire CO₂ from the surrounding medium. Figure 4 also summarizes the measured kinetic properties for Rubisco enzymes across the algal species spectrum

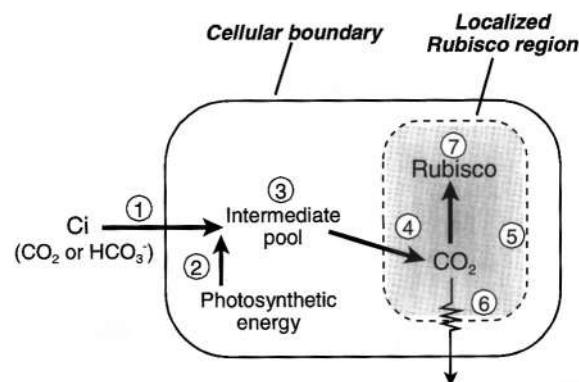
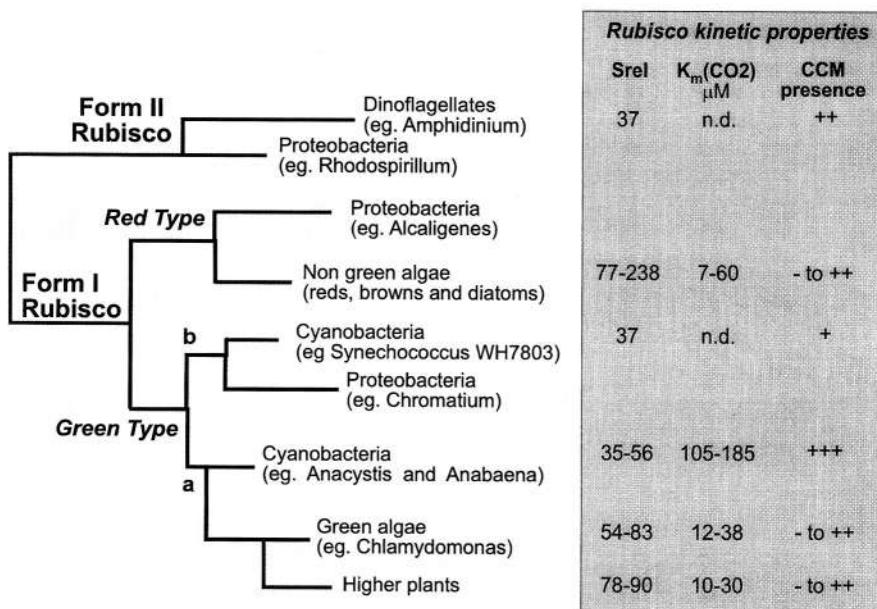


Fig. 3. A generalized model of components of a photosynthetic CO₂ concentrating mechanism. Components 1 to 7 are labeled as the generic component steps of a CCM. See Table 1 for a comparison of their particular nature of the components in C₄ higher plants, algae and cyanobacteria.

Evolution of Rubisco in Algae and Cyanobacteria



Form I = L8S8 ; Form II = L2

Fig. 4. A phylogenetic tree showing the evolutionary relationships between Rubisco enzymes found in photosynthetic organisms, together with their kinetic properties. The phylogenetic tree is adapted from Delwiche and Palmer (1996), and the kinetic parameters and CCM characteristics are taken from data summarized by Badger et al. (1998).

as well as for their ancestral relatives (for a more detailed review see Badger et al., 1998). Some salient points can be summarized. Firstly, despite the ancestral lineage of the Rubisco, there has been evolution of its kinetic properties to meet some of the constraints of its photosynthetic environment. Some examples of this can be seen in the following. For Dinoflagellates, the kinetic properties of the Form II enzyme (as measured by S_{rel}) are considerably improved over its proteobacterial ancestors. Such evolution is obviously necessary to allow the enzyme to function in an atmosphere of 21% O_2 and reduced CO_2 . However, a CCM is an important part of Dinoflagellate photosynthesis suggesting that this Rubisco cannot operate effectively at air levels of CO_2 and O_2 . The Form I enzyme from red algae such as *Cyanidium* and *Galdieria* (Uemura et al., 1997) have much higher S_{rel} values and lower $K_m(\text{CO}_2)$ when compared to other red algal counterparts. This appears to be an adaptation to growth in high temperature acid hot-springs environments where there is definite pressure to evolve a Rubisco with a

higher affinity for CO_2 . In the green alga *Coccomyxa* (Palmqvist et al., 1995), a lower $K_m(\text{CO}_2)$ and higher S_{rel} are seen compared to other Chlorophytes with CCMs, such as *Chlamydomonas*. This is clearly a response to adapt to an environment with a passive supply of CO_2 in these algae that lack a CCM.

The different Rubisco forms in the algae also have intrinsic properties that lead to different kinetic constraints, which may have to be dealt with by each group. Studies of both Rubisco kinetic properties and photosynthetic oxygen exchange (Badger et al., 1998) have shown that there are considerable differences in the potential oxygenase reactions exhibited by the Red and Green Form I types. In general, Red Types have less potential for oxygenase activity. This is largely due to a much-reduced affinity of the oxygenase reaction for O_2 and may in some cases be due to a change in the ratio between carboxylase and oxygenase V_{max} . This is summarized in Fig. 5 for four different Form I enzymes from algal, cyanobacterial and higher plant sources. Thus, following the evolution of the Chlorophyte Rubisco

**Carboxylase and Oxygenase activities at air
in algal and cyanobacterial Rubiscos**

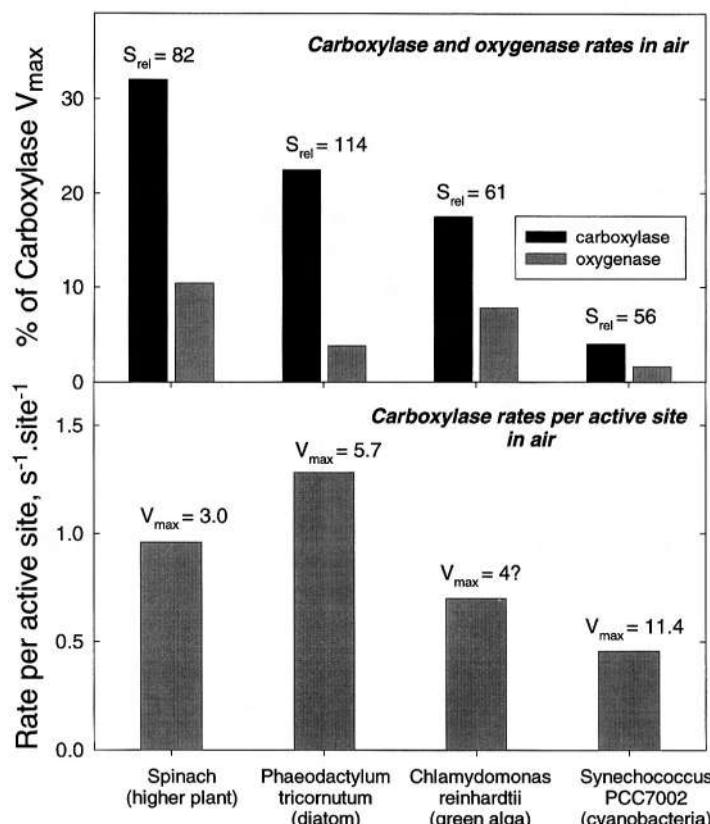


Fig. 5. Carboxylase and oxygenase activities for various algal, cyanobacterial and higher plant Rubiscos. The rates for each reaction were calculated assuming air levels of oxygen (21%) and CO₂ (350 µbar), and competitive kinetics between the two reactions. Kinetic parameters used for the calculations were taken from data summarized by Badger et al. (1998). The V_{max} for Chlamydomonas was assumed to be 4, as no data exists for any purified green algal Rubisco.

into higher plants, although it has increased the affinity for CO₂, it has also increased the affinity of the oxygenase reaction. This has led to a situation in which the higher plant chloroplast has to deal with potentially high rates of oxygenase reaction. This has been accommodated by the development of the photorespiratory cycle to recycle glycolate (Ogren, 1984). For non-green algae, the reduced oxygenase reaction (see Fig. 5) may mean that strategies to deal with glycolate synthesis are much less important. Interestingly, although the S_{rel} value of cyanobacterial enzyme is low, it shows a poor affinity for oxygen, which means that at atmospheric levels of oxygen it displays low oxygenase activity. However, the K_m(CO₂) is also quite high in the Type 1a cyanobacterial Rubisco enzyme, with the consequence

that cyanobacteria cannot function effectively without the aid of a CCM.

An interesting correlation with changes in K_m(CO₂) in Rubisco enzymes is that the V_{max} of the enzyme per active site increases with higher K_m(CO₂) (Fig. 5). Thus, algae and cyanobacteria employing a CCM are able to achieve a relatively high rate of photosynthesis per unit Rubisco. A cyanobacterium with a CCM may achieve Rubisco carboxylase turnover rates of 11 s⁻¹.site⁻¹, while spinach in air would be less than 1. This may contribute to improved nitrogen use efficiency for these organisms which often grow in nutrient limited waters. Interestingly, the Red Type enzyme from *Phaeodactylum*, because of its high S_{rel} and higher V_{max}, would actually appear to be better than the spinach enzyme at current air levels of

substrate CO_2 and O_2 .

There has been considerable interest recently in the fact that non-green algae (such as reds and diatoms) may possess a Rubisco kinetically superior to the higher plant green Form I enzyme (Read and Tabita, 1994; Uemura et al., 1997). Interestingly, all non-green algal Rubiscos with high S_{rel} values probably come from algae with some level of CCM. This includes *Phaeodactylum*, *Porphyridium*, and *Cylindrotheca* (see Badger et al., 1998). It appears that for these enzymes, the oxygenase reaction is not very relevant for the functional performance of the chloroplast, but the lower affinity ($K_m(\text{CO}_2)$ 20–40 μM) for external CO_2 probably is. Thus a CCM must still be used to allow Rubisco to fix CO_2 efficiently. Further studies are needed to clarify the role of a CCM in non-green algae.

IV. Operation of CCMs in Cyanobacteria

A number of reviews dealing with cyanobacterial CCMs have appeared in recent years (Badger, 1987; Miller et al., 1990; Coleman, 1991; Kaplan et al., 1991; Price and Badger, 1991; Badger and Price, 1992; Kaplan et al., 1994), and readers are directed here for a detailed analysis of current research perspectives. The purpose of the description that follows is to summarize the current state of our knowledge and to point out the areas that are unresolved.

A generalized description of the operation and components of the CCM in cyanobacteria is shown in Fig. 6. Inorganic carbon is transported by a number of C_i transporters on the cell membrane and enters the cell as HCO_3^- . This transport is energized by photosynthetic electron transport and probably involves both cyclic and linear electron transport. The concentrated cytosolic HCO_3^- pool enters the carboxysome where it is converted to CO_2 through the action of carboxysomal CA. CO_2 is elevated in the carboxysome environment through some property of the carboxysome that restricts CO_2 efflux. The analogy of this CCM to other CCM-systems is shown in Fig. 3 and Table 1.

A. Inorganic Carbon Transport

There are multiple C_i transport systems that appear to be associated with the plasma membrane of cyanobacteria. These transport systems have the

The Cyanobacterial CCM

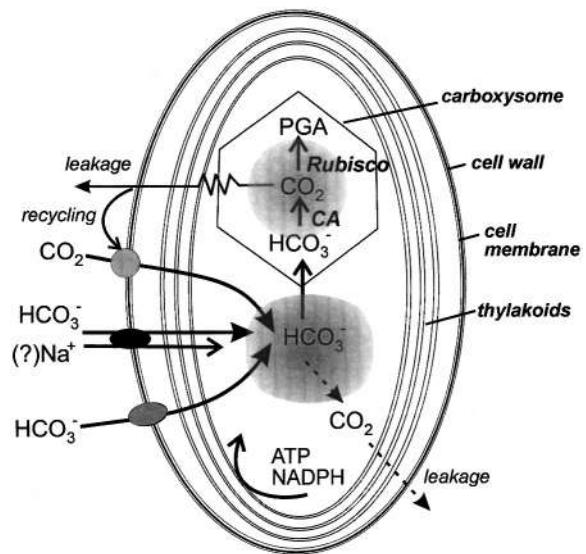


Fig. 6. A model showing the currently recognized components of a cyanobacterial cell that are important in the CO_2 concentrating mechanism. Features shown include: CO_2 and HCO_3^- transporters on the cell membrane; production of ATP and NADPH by the thylakoids; accumulation of a HCO_3^- pool within the cell; and the carboxysomes located within the central region of the cell, containing Rubisco and carbonic anhydrase (CA).

ability to actively accumulate both CO_2 and HCO_3^- from the external medium (see reviews Miller et al., 1990; Badger and Price, 1992; Kaplan et al., 1994). However, despite what appears to be multiple transport systems, there is strong evidence to suggest that all transport processes deliver HCO_3^- to the cytosol of the cell. This leads to an elevation of the internal HCO_3^- pool up to 1,000-fold over that in the external environment (Badger and Price, 1992). Although both CO_2 and HCO_3^- can be actively transported, most cyanobacteria grow in environments where the pH is in excess of 7.5, and under these conditions (see Fig. 2), it is likely that HCO_3^- uptake is the dominant transport process contributing to C_i uptake.

Until recently, physical evidence for the existence of multiple transporters or any understanding of how they functioned has not existed and speculation has relied on a number of physiological experimental approaches. Multiple transporters were predicted several years ago, largely based on experiments examining the requirement for Na^+ for C_i transport and the effects of various CO_2 and Na^+ analogues on

C_i transport (see Miller et al., 1990). Evidence has been presented for what appears to be both Na^+ -dependent and Na^+ -independent HCO_3^- transport systems. The role of Na^+ in C_i uptake is unknown, but it is likely to be involved in pH regulation via Na^+/H^+ antiport mechanisms. However, a direct involvement of Na^+ in a $\text{Na}^+/\text{HCO}_3^-$ symport system (Espie and Kandasamy, 1994) cannot be completely discounted. A separate CO_2 transport system is also apparent and this is inhibited by compounds such as COS and NaS (Miller et al., 1991). Interestingly, physiological analyses have also shown that there are a number of commonalities between the CO_2 and HCO_3^- -transport systems. This includes inhibition by the carbonic anhydrase inhibitor ethoxzolamide (Price and Badger, 1989a; Badger et al., 1994; Tyrrell et al., 1996) and a common requirement for a competent NADH dehydrogenase complex for what appears to be energization (Ogawa, 1990, 1991b, 1992). A final understanding of just how many transport components exist, how they operate and in what ways they are connected remains to be resolved. However, this understanding will almost certainly arise from molecular genetic approaches that are currently yielding new and exciting information about genes and proteins involved in C_i transport systems.

Genetic evidence for multiple C_i transporters has recently emerged, with the finding that the *cmpABCD* operon from *Synechococcus* PCC7942 codes for what appears to be a HCO_3^- transporter (Fig. 7). This transporter has characteristics which suggest that it is involved in high-affinity HCO_3^- uptake, it is induced

under severe C_i limitation and appears to be Na^+ -independent (Okamura et al., 1997; T. Omata and G. D. Price, personal communication). This work clearly indicates that there is at least one specific HCO_3^- transporter, and this appears to be highly analogous to a diverse sub-family of ABC (ATP Binding Cassette) transporters found in prokaryotes (see Price et al., 1998). The *cmpA* gene codes for the precursor of the 42 kD plasma membrane protein which was previously found to be induced under C_i limitation (Omata and Ogawa, 1985, 1986) and highlight stress (Reddy et al., 1989). Analysis of *cmpABCD* mutants also confirms that this transport complex is only one of at least two HCO_3^- uptake systems (T. Omata and G. D. Price, personal communication). There is a significant Na^+ -dependent transport system, which appears more important at high- C_i growth conditions, and there is a distinct CO_2 transport system that remains unaffected in the *cmpABCD* mutants. The existence of other HCO_3^- transport systems still remains a distinct possibility.

A potential structure for the *cmpABCD* HCO_3^- transporter is shown in Fig. 7. This is drawn by analogy with other ABC transporters which have been characterized (Higgins, 1992; Omata et al., 1993). *CmpA* codes for a periplasmically exposed HCO_3^- binding protein (42 kDa); *cmpB* is an intrinsic membrane protein and most probably forms a dimer within the membrane; *cmpC* is a large extrinsic membrane protein with an ATP binding site; *cmpD* is a smaller related protein also with an ATP binding site. The closest homologue of *cmpC* is part of the

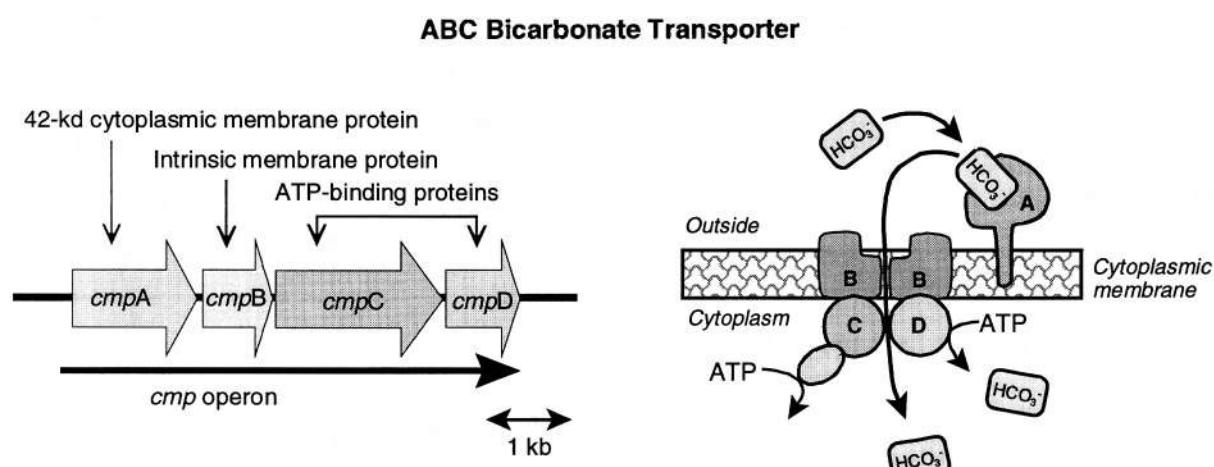


Fig. 7. The operon and protein structure of the ABC-bicarbonate transporter characterized from *Synechococcus* PCC6803. The figure is modified from Omata et al. (1997).

nitrate transporter, *nrtC*, which has been shown to be involved in regulation of activity in the presence of ammonia (Kobayashi et al., 1997). *cmpC* may also be involved in regulation of this ABC-type HCO_3^- transporter. *CmpA* exists in stoichiometric excess to other components of the transporter (Omata and Ogawa, 1986), which is often a feature of binding proteins in ABC transporters (Higgins, 1992).

B. Energization of C_i Transport

It appears that the operation of C_i transport systems, both CO_2 and HCO_3^- are highly dependent on the presence of light and photosynthetic electron transport. Experiments clearly show that there is little C_i transport activity in the dark or in the presence of DCMU, and hence the involvement of respiratory energy sources is relatively insignificant in being able to energize the transport (Badger, 1987; Miller et al., 1990; Kaplan et al., 1991). Action spectrum studies have suggested that PS I driven cyclic electron transport was primarily responsible for the supply of energy, but some PS II activity is apparently required for activation of C_i uptake (Ogawa et al., 1985; Kaplan et al., 1987). The existence of an ATP-using **ABC-HCO₃⁻-transporter** would suggest that this transport process could be dependent on PS I driven cyclic ATP production, with activation in the light being regulated in some specific manner, perhaps being dependent on photosynthetic reductant. However, there is also evidence to suggest that whole chain electron transport can also support C_i transport (Miller et al., 1991; Li and Canvin, 1997).

Analysis of mutants has also added to the picture of light-driven energization. The NAD(P)H dehydrogenase complex has been found to be essential for the functioning of both CO_2 and HCO_3^- transport processes. Genetic elimination of particular subunits of this complex (*ndhB* K and L) (Ogawa, 1991a, 1992; Marco et al., 1993) had dramatic effects on eliminating both CO_2 and HCO_3^- transport. These studies also led to the discovery that the NAD(P)H complex was intimately involved in mediating the reduction of the thylakoid plastoquinone pool using NAD(P)H reductant (Mi et al., 1992, 1994, 1995), thus catalyzing one form of cyclic electron transport around PS II. The obvious interpretation arising from these studies was that a thylakoid located NAD(P)H complex was involved in mediating the cyclic photophosphorylation which produced ATP to drive C_i uptake by ATP-dependent C_i transporters

such as the then-unknown ATP-dependent ABC HCO_3^- transporter.

Further studies of the NAD(P)H dehydrogenase mutants and gene expression have added complexity the role of this complex in C_i transport. Inactivation of a number of the subunits of this complex (*ndhF* and D in particular) apparently having more subtle effects on C_i transport (Ohkawa et al., 1998; Price et al., 1998) appearing to have specific effects on reducing high-affinity CO_2 transport (Sültmeyer et al., 1997a; Ohkawa et al., 1998; Price et al., 1998). With the publishing of the complete genome for *Synechocystis* PCC6803 (Kaneko and Tabata, 1997; Nakamura et al., 1998), it has become apparent that both *ndhD* (6 homologues) and F (3 homologues) are members of a multigene family, while the other *ndh* genes are single copy. Furthermore, it is apparent that only one member of the *ndhD* and *ndhF* gene families may be involved in mediating the functioning of high-affinity CO_2 transport. This has been clearly shown for *Synechococcus* PCC7002 (Price et al., 1998) and *Synechocystis* PCC6803 (Ohkawa et al., 1998) and in *Synechocystis*, the *ndhD3* gene shows specific upregulation of expression during growth at low CO_2 (Ohkawa et al., 1998).

The evidence is beginning to suggest that there may in fact be multiple NAD(P)H dehydrogenase complexes in the cell, with perhaps both thylakoid and plasma membrane locations (see Price et al., 1998). The complexes may have common core components, such as the *ndhB*, K and L, but there may be variability of some of the polypeptide components, such as the *ndhD* and F. This throws into question just which NAD(P)H complex is involved in energizing C_i transport. The original *ndhB*, K and L mutants would have eliminated both plasma membrane and thylakoid complexes. So perhaps there is a role for plasma membrane NAD(P)H complexes which remains to be more fully described (Price et al., 1998).

Another gene product that appears to play a role in energizing C_i uptake, at least in *Synechococcus* PCC7002, is the *PsaE* protein associated with PS I (Sültmeyer et al., 1997b). This protein appears to be involved in mediating an alternative type of PS I cyclic electron flow (Yu et al., 1993), and inactivation of *psaE* leads to a loss of high-affinity HCO_3^- transport, which is developed at low- C_i growth conditions.

C. Cyanobacterial Carbonic Anhydrases

The model shown in Fig. 6 implies specific localization of carbonic anhydrase in the cyanobacterial cell. For this model to function there is a carbonic anhydrase associated with the carboxysome, and a specific absence from the cytosolic compartment. The *Synechocystis* PCC6803 genome sequence indicates that there are two potential carbonic anhydrase genes. One of them has close homology with what has been shown to be a carboxysomal CA (*icfA*) (Fukuzawa et al., 1992; Price et al., 1992b; Yu et al., 1992). The other has closest homology to the *Chlamydomonas* mitochondrial β CA, but its function seems cryptic. Inactivation of this gene in *Synechocystis* produces no discernible phenotype relating to the operation of the CCM (T. Ogawa and G. S. Espie personal communication). Adding to this evidence is the observation that expression of a human CA protein in the cytosol of *Synechococcus* PCC7942 leads to the loss of C_i accumulation and a **high-C_i** requiring phenotype (Price and Badger, 1989b). Another animal-like α CA protein and gene has been discovered in *Synechococcus* PCC7942 and *Anabaena* PCC7120 (Soltész-Rak et al., 1997). This CA appears to be periplasmically located and is expressed more highly at elevated CO₂. Inactivation of the gene in *Synechococcus* PCC7942 did not impair the operation of the CCM. Thus at this stage, a carboxysomal CA protein is the only CA known to be specifically involved in the operation of the CCM.

D. Role of the Carboxysome

The carboxysomes, located within the cyanobacterial cell, have emerged to have a central role in the CCM and evidence for their functional roles has been reviewed by a number of authors over recent years (Price and Badger, 1991; Badger and Price, 1992; Kaplan et al., 1994; Price et al., 1998). These are the compartments where Rubisco and CA are specifically localized and CO₂ is elevated around the active site of Rubisco, similar in functional outcome to the C₄ bundle sheath (see Table 1). A number of mutants have been isolated and created with impaired carboxysome structure, and in all cases cells with missing or aberrant carboxysomes have a **high-CO₂-requiring** phenotype (see above reviews).

A number of models have been developed to theoretically describe how carboxysomes might achieve their functional roles (Reinhold et al., 1989;

The role of the carboxysome in the CCM

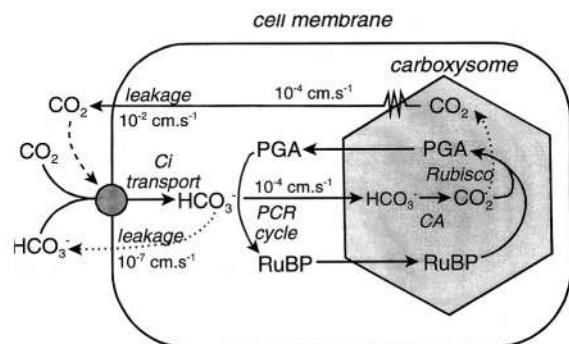


Fig. 8. The role of the carboxysome in the cyanobacterial CO₂ concentrating mechanism. The model assumes that the major resistance to CO₂ leakage from the cell is associated with the carboxysome, and not the cell membrane. Assumed conductances for both HCO₃⁻ and CO₂ across the cell membrane and the carboxysome shell are shown. RuBP and PGA cycle into and out of the carboxysome and their conductances are assumed to be similar to HCO₃⁻. Rubisco and carbonic anhydrase are specifically localized to the carboxysome.

Reinhold et al., 1991). Figure 8 summarizes one possible scenario. The key feature of this model is that the carboxysome protein shell provides a restriction to CO₂ efflux, which is around 2–3 orders of magnitude higher than that found for normal lipid bilayers such as the thylakoids and cell membrane. In addition, it must have an increased permeability for anions such as HCO₃⁻, PGA and RuBP. A conductance of around 10⁻⁴ cm s⁻¹ is sufficient to restrict CO₂ efflux and allow CO₂ to be elevated, while still allowing the exchange of HCO₃⁻, PGA and RuBP at rates sufficient to support CO₂ fixation. This is because the gradients for CO₂ are around 500 μM, while for HCO₃⁻ and the sugar phosphates it will be in the 10–30 mM range. The plasma membrane is assumed to be relatively permeable to CO₂ but relatively impermeable to HCO₃⁻. This model assumes that Rubisco and CA are homogeneously distributed throughout the carboxysome. A more complex version of this model considers that CA molecules may be surrounded by a Rubisco shell, thus acting as a leak reduction mechanism (Reinhold et al., 1991). There is no particular evidence for this model as opposed to the simpler model shown in Fig. 8, except that one carboxysome mutant, with an extension to the C terminus of Rubisco, shows loose carboxysome structure and a **high-CO₂-requiring** phenotype, suggesting a role for specific Rubisco packing. Such

fine details of carboxysome structure/function remain to be resolved.

Several genes have been identified which are involved in the formation of functional carboxysomes (see Price et al., 1998, for a detailed review). Obviously the Rubisco genes and proteins are essential (*rbcL* and *rbcS*—Rubisco null mutants do not have carboxysomes—Pierce et al., 1989) and the carboxysomal CA (*icfA*). In addition to these a number of CCM genes and their protein products have also been identified which are involved in some aspect of the assembly of carboxysome structures. This includes *ccm A, J, K, L, M, N* and *O* (Price et al., 1998). Of these, only the *ccmM* appears to code for a carboxysome structural protein. This is a 58 kDa protein which at this stage is most likely a major component of the carboxysome shell. This protein possesses 3–4 internally repeated regions (depending on species) which show homology to the Rubisco small subunit. This has led to speculation that these regions may interact with Rubisco large subunit within the carboxysome in order to organize or stabilize the carboxysome structure (Price et al., 1998). The *ccmK* and *L* gene products appear to code for soluble proteins, which may be involved in assembly functions.

E. Induction of the CCM

Most cyanobacteria that have been examined show the ability to adjust the properties of their CCM, depending on C_i limitation during growth. As shown in Fig. 9, cyanobacteria cannot exist without a CCM at ambient C_i conditions, largely because of the low CO_2 affinity of their Rubisco. Cells without a CCM would require in excess of 50 mM C_i at pH 8 to saturate photosynthesis. Thus a certain level of CCM is constitutively expressed in high- C_i cells. However, when cells are grown at limiting C_i , which may be at air or less levels of CO_2 , the cells develop a much higher affinity for external C_i . These have been termed **low- C_i** cells. As summarized in Table 2 certain changes occur to components of the CCM to achieve this induction. The primary change occurs to the kinetic properties of the C_i transport systems, with cell increasing their affinities for CO_2 and HCO_3^- . This is also accompanied by changes in the supply of energy to drive C_i uptake, which may include changes to the NAD(P)H dehydrogenase complexes, as well as cyclic electron transport (see energization of C_i transport section). These changes mean that cells can

Plot of C_i response for low and high- C_i cells

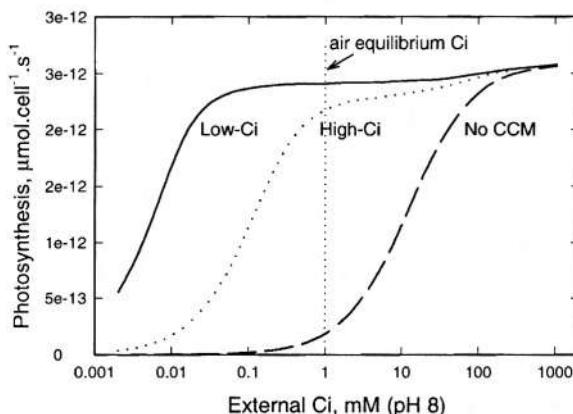


Fig. 9. A modeled response of photosynthesis in a cyanobacterial cell to external inorganic carbon. Three cell states are shown: No CCM, where active inorganic carbon transport is absent from the cell; High- C_i , where active C_i transport is present and has an affinity ($K_{0.5}$) for external C_i of 200 μM ; and Low- C_i , where the affinity of C_i transport for external C_i has increased through adaptation to growth at low- C_i to 20 μM . The model used for these calculations is described by Price and Badger (1989a).

accumulate a larger internal HCO_3^- pool at much lower external C_i levels. There are other smaller changes which also occur to the nature of the carboxysomes, including an increase in both Rubisco and CA per cell and production of a larger number of smaller carboxysomes in the cell (Price et al., 1992; McKay et al., 1993).

The manner in which these changes are achieved is not entirely clear. Certainly some de novo protein synthesis is involved. The CmpABCD HCO_3^- transport complex is upregulated at limiting C_i , increasing the affinity of HCO_3^- transport (Omata and Ogawa, 1986, 1987). In addition, such changes as the number and size of carboxysomes and levels of carboxysome carbonic anhydrase must also require protein synthesis changes. In general, when cells are transferred from high CO_2 to low CO_2 environments, adaptation to the new growth conditions takes 1–4 h and has been proposed to involve de novo protein synthesis (Omata and Ogawa, 1985; Kaplan et al., 1994). However, recently it has been shown that quite rapid induction of high-affinity HCO_3^- transport can occur in as little as 10 min after transfer to zero CO_2 , in *Synechococcus* PCC7002 and PCC7942, and that this can be blocked by addition of protein kinase but not protein phosphatase or protein synthesis inhibitors (Sütemeyer et al., 1998a,b).

Table 2. Adaptation of CCM components in cyanobacteria to growth at low CO₂. The CCM components are described in Fig. 3 and Table 1.

CCM Component	Adaptation to growth at low C _i
1. The C _i transport system	The activity of both CO ₂ and HCO ₃ ⁻ -transporters are changed, resulting in an increased affinity for both external CO ₂ and HCO ₃ ⁻ . The relative contributions of HCO ₃ ⁻ and CO ₂ -transport to photosynthesis varies between species.
2. Energization of C _i transport	There appears to be increased emphasis on a requirement for PS1 driven cyclic electron flow as well as the up regulation of specific NDH-dehydrogenase components, possibly located on the cytoplasmic membrane.
3. The cytosolic HCO ₃ ⁻ pool	Larger HCO ₃ ⁻ pools can be accumulated by low-C _i grown cells, at lower external C _i levels.
4. & 5. The carboxysome compartment	A number of carboxysome changes occur. There are increases in the number of smaller carboxysomes per cell, and increases in CA and Rubisco per cell.
6. Reduction of CO ₂ leakage	There appears to be little difference in the leakiness of low and high-C _i grown cells, but the increased activity of the C _i transport system may recycle CO ₂ leakage at low external C _i .

Thus, some part of the induction process appears to be modulated by phosphorylation of existing proteins associated with a HCO₃⁻ transport complex.

There is little evidence to suggest what components are involved in the sensing of the external C_i environment. Some suggestions have included signal transduction pathways linked to the sensing of internal photorespiratory intermediates, external and internal C_i. Some evidence, examining rapid induction with *Synechococcus* PCC7002, has suggested that the external HCO₃⁻ level may be critical to modulating the changes in phosphorylation (Sültmeyer et al., 1998a), but there may be other and multiple systems operating to control the induction of multiple CO₂ and HCO₃⁻ transporters as well as changes to carboxysomes. Cell signal transduction in relation to adaptation to C_i remains to be more fully explored.

V. Components and Adaptation of the CCM in Green Microalgae

A generalized description of the operation and components of the CCM in green microalgae is illustrated in Fig. 10. The salient features of this proposed model are that C_i enters the cell either by carrier as bicarbonate or CO₂ or by diffusion as CO₂, that C_i is transported into the chloroplast, accumulating in the stroma as bicarbonate, and that the accumulated bicarbonate enters the lumen of the pyrenoidal thylakoids where it is dehydrated by a thylakoid lumen CA (ctCA1; see Figs. 10, 11 and

The CCM in Chlorophyte Algae

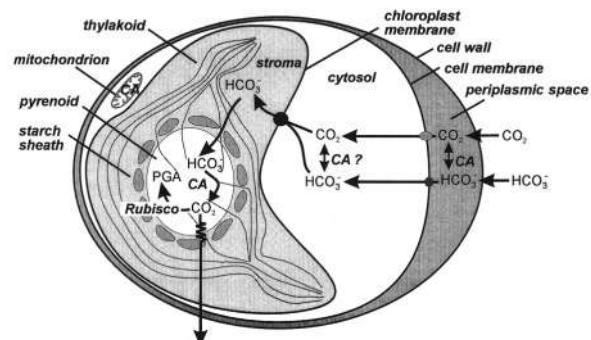


Fig. 10. A model of the components identified as being associated with the operation of the CCM in Chlorophyte algae such as *Chlamydomonas reinhardtii*. Components shown include: various specialized carbonic anhydrases located in the periplasmic space, the chloroplast, the mitochondria and the cytosol; C_i transporters located on both the cell membrane and the chloroplast envelope; transport of HCO₃⁻ into the chloroplast stroma; the pyrenoid in the chloroplast, containing Rubisco, thylakoids and possibly a specific carbonic anhydrase.

below) to provide substrate CO₂ to Rubisco in the pyrenoid. This model of the green algal CCM is based in part on that described above for cyanobacteria and reflects primarily characteristics drawn from study of the CCM of *Chlamydomonas reinhardtii*. Comparison of the characteristics of this CCM to those of other CCM-systems is shown in Fig. 3 and Table 1.

The chloroplast and pyrenoid

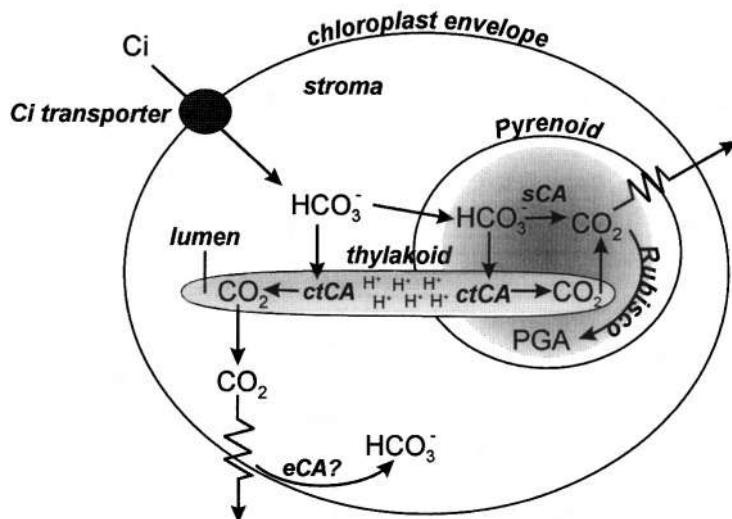


Fig. 11. A model for the involvement of the chloroplast and the pyrenoid in the algal CCM. Elements shown include: an envelope C_i transporter delivering HCO₃⁻ to the stroma; Rubisco localized to the pyrenoid; thylakoid carbonic anhydrase (ctCA) and soluble pyrenoid CA (sCA) involved in the conversion of HCO₃⁻ to CO₂; resistances to CO₂ leakage associated with either the pyrenoid or the chloroplast envelope; a possible envelope CA, recovering leaked CO₂.

A. Inorganic Carbon Transport

The requirement for active C_i transport in the green-microalgal CCM was clearly established by isolation and characterization of a *C. reinhardtii* mutant (*pmp1-1*) that lacks C_i transport and accumulation (Spalding et al., 1983b), but our understanding of C_i transport in microalgae remains somewhat unclear nonetheless, both in terms of location and substrate specificity. The use of carbon isotope disequilibrium studies to identify the C_i species taken up has established that the major flux of C_i into many green microalgae occurs through direct uptake of CO₂ across the plasmalemma via an active process (Sültmeyer et al., 1989; Gehl et al., 1990; Rotatore and Colman, 1991; Rotatore et al., 1992; Badger et al., 1994; Palmqvist et al., 1994b; Amoroso et al., 1998), although the data cannot distinguish between active CO₂ transport at the plasmalemma and active C_i transport at the chloroplast envelope following CO₂ diffusion into the cell. From these and similar studies, there also is good evidence for direct bicarbonate transport across the plasmalemma, but generally at a lower rate than CO₂ influx (Sültmeyer et al., 1989; Gehl et al., 1990; Thielmann et al., 1990;

Rotatore et al., 1991; Badger et al., 1994; Palmqvist et al., 1994b; Amoroso et al., 1998), except in *Dunaliella tertiolecta*, where bicarbonate appears to be the dominant inorganic carbon species taken up (Amoroso et al., 1998). Therefore, it appears that both of the predominant C_i species present in the aquatic environment (CO₂ and HCO₃⁻) can be used by microalgal cells, although most apparently prefer CO₂.

Chloroplast envelope C_i transport has been demonstrated in intact, isolated chloroplasts from *C. reinhardtii*, *D. tertiolecta*, and *Chlorella ellipsoidea*, and the light-dependent C_i accumulation activity of the first two was restricted to chloroplasts from air-adapted cells (Moroney et al., 1987; Sültmeyer et al., 1988; Goyal and Tolbert, 1989; Rotatore and Colman, 1990). Difficulties with obtaining high yields of intact, active chloroplasts generally have hindered extensive investigation of the C_i species transported, but recent work with isolated chloroplasts from *C. reinhardtii* and *D. tertiolecta* indicates that both algae have inducible, high-affinity bicarbonate and CO₂ transporters at the chloroplast envelope (Amoroso et al., 1998).

Therefore, transport of CO₂ and/or bicarbonate in

microalgae may occur at the plasmalemma and/or the inner chloroplast envelope, but the mechanism of transport is not understood for uptake of either C_i species across either membrane. There is evidence for the inhibition of C_i uptake by the H⁺-ATPase inhibitor vanadate both in whole cells and chloroplasts, suggesting that activity of a vanadate-sensitive H⁺-ATPase is essential for transport across both the plasmalemma and the chloroplast inner envelope (Palmqvist et al., 1988; Goyal and Tolbert, 1989a; Thielmann et al., 1990; Karlsson et al., 1994). The characteristics of the *C. reinhardtii pmp1-1* mutant, which completely lacks C_i transport, have raised the intriguing question as to how a single mutation apparently can eliminate virtually all C_i accumulation, even though there is good evidence demonstrating C_i transport across both the plasmalemma and the chloroplast envelope.

Based on the available evidence, green microalgae probably have one or two plasmalemma C_i transporters, one for bicarbonate and possibly another for CO₂. However, no plasmalemma transport proteins have been identified, nor have any proteins been identified as potential candidates for this function. The situation is similar for chloroplast envelope C_i carriers, except that cDNA clones of two *C. reinhardtii* genes (*Ccp1* and *Ccp2*; =LIP36-G1 and LIP36-G2; see Spalding, 1998 for nomenclature) encoding closely-related, **limiting-CO₂** inducible polypeptides showing sequence similarity with the mitochondrial carrier protein superfamily and apparently located in the chloroplast envelope have been identified recently (Ramazanov et al., 1993; Chen et al., 1997). This superfamily encompasses proteins representing a wide variety of transport substrate specificities and intracellular locations, including plastid and peroxisomal as well as mitochondrial carrier proteins (Chen et al., 1997). It is tempting to speculate that the Ccp1 and Ccp2 proteins might be involved in C_i transport across the chloroplast inner envelope, but much more information is needed before the function of these proteins is established. In *D. tertiolecta* as well, two low **CO₂-inducible**, chloroplast envelope proteins have been reported as potential components of a C_i transport mechanism (Thielmann et al., 1992). In addition, a *C. reinhardtii* plastid-encoded, chloroplast envelope protein (*ycf10* gene product) has been implicated in C_i uptake into chloroplasts (Rolland et al., 1997), although its role is unclear at present.

B. Energization of C_i Transport

The CCM requires an additional input of energy above that required for operation of the Calvin cycle, and it is clear from many early experiments that photosynthesis is the source of the additional energy (see Badger and Price, 1992). However, it is not certain how or in what form the additional energy is provided by photosynthesis. Internal C_i pools are reduced or eliminated in the presence of DCMU or darkness and in the presence of proton ionophores (Badger et al., 1980; Spalding et al., 1983a; Badger, 1987). Following from this it has been proposed that a substantial part of the additional energy is required as ATP to drive active transport of C_i. However, considering the essential role played in *C. reinhardtii* by ctCA1, its probable location in the thylakoid lumen and its suggested function in the CCM (Fig. 11 and see Section V.D.), it seems apparent that at least some energy associated with generation of the thylakoid ΔpH might be used to enhance the dehydration of bicarbonate in the thylakoid lumen.

Acclimation of **CO₂-enriched** *C. reinhardtii* and *Scenedesmus obliquus* to limiting CO₂ results in changes in the photochemical properties of the cells (Spalding et al., 1984; Palmqvist et al., 1986, 1990; Sundblad et al., 1990), indicating that the cells must adjust to the different energy demands of operating the CCM. Short term changes in the photochemical properties probably reflect the transient stress of decreased CO₂ availability prior to development of CCM activity, and long term photochemical changes may reflect an increased ratio of PS I to PS II to provide extra ATP to the CCM (Palmqvist et al., 1990).

C. The Role of Carbonic Anhydrases

In recent years it has become clear that three apparently independent evolutionary lines of CA (**α**, **β** and **γ**CA) can be found in various organisms (Hewett-Emmett and Tashian, 1996), and at least two of these lines, the **α** and **β** CAs, have been found in green microalgae (Fujiwara et al., 1990; Fukuzawa et al., 1990; Hiltonen et al., 1995; Eriksson et al., 1996; Fisher et al., 1996; Funke et al., 1997; Karlsson et al., 1998). The first microalgal CA identified was the abundant periplasmic CA (pCA1) induced by limiting CO₂ concentrations in *C. reinhardtii* (Coleman and Grossman, 1984; Coleman et al.,

1984), which has been studied rather extensively since its initial discovery. *C. reinhardtii* pCA1, encoded by the *Cah 1* gene, is expressed to very high levels only under limiting CO_2 conditions, but it still is rather unclear whether it plays any essential role in the *C. reinhardtii* CCM (Williams and Turpin, 1987; Sültemeyer et al., 1990). If it does, that role may be to dehydrate bicarbonate to supply CO_2 for active uptake, especially under alkaline conditions (Moroney et al., 1985), a role consistent with characteristics of periplasmic CAs reported from other green microalgae (Miyachi et al., 1983; Aizawa and Miyachi, 1984; Coleman et al., 1991; Goyal et al., 1992; Pesheva et al., 1994).

Intracellular CA isozymes have long been implicated in function of the microalgal CCM, but only recently has significant progress been made in unambiguously identifying any internal CA. So far, one chloroplast-localized CA, the thylakoid lumen α -CA (ctCA1, encoded by the *Cah3* gene) defective in mutants with lesions at the CA 1 locus (Spalding et al., 1983a; Karlsson et al., 1995; 1998; Funke et al., 1997), and two mitochondrial β -CAs (mtCA1 and mtCA2, =LIP-21; encoded by the *Mca1* and *Mca2* genes; = β -CA1 and β -CA2) (Eriksson et al., 1996; Geraghty and Spalding, 1996; see Spalding, 1998 for nomenclature) have been explicitly identified in *C. reinhardtii*. Other intracellular CA isozymes have been either postulated or tentatively detected in *C. reinhardtii* (Badger and Price, 1992, 1994; Sültemeyer et al. 1993; Amoroso et al., 1996, 1998; also see Figs. 10 and 11 and Section VI.A.), but hard evidence for their existence is lacking. It is not clear what the function of the inducible mitochondrial CAs might be, but it has been suggested that they might function in pH regulation (via hydration of photorespiratory CO_2) to prevent alkalization of the mitochondrial matrix during rapid production of photorespiratory ammonia from the glycine decarboxylase complex and to facilitate diffusion of CO_2 from the same source out of the mitochondrial matrix under high photorespiratory conditions (Eriksson et al., 1996).

There is strong genetic evidence from analysis of *C. reinhardtii* *ca1* mutants to indicate that bicarbonate is actively accumulated in the chloroplast and that a thylakoid lumen CA (ctCA1; *Cah3* gene product) is required for rapid dehydration of this accumulated bicarbonate to supply Rubisco with CO_2 at a physiological rate (Spalding et al., 1983a; Suzuki and Spalding, 1989; Sültemeyer et al., 1995; Funke

et al., 1997; Karlsson et al., 1998). The plastid location of ctCA1 indicates that active accumulation of bicarbonate occurs in the chloroplast, but it is not yet clear whether the accumulation occurs throughout the chloroplast or is restricted to some compartment within the plastid. It is reasonable to assume that the transported C_i enters and is accumulated in the stroma as bicarbonate, regardless of which species of C_i serves in the cytosol as transport substrate.

D. The Pyrenoid as the Site of CO_2 Elevation

It has long been suggested that, in microalgae, the acidic thylakoid lumen might participate in net dehydration of bicarbonate to supply CO_2 to Rubisco at concentrations higher than the equilibrium concentration of CO_2 in the (presumably) alkaline pyrenoid (Pronina and Borodin, 1993; Raven, 1997), as illustrated in Figure 11. The requirements for effective participation of such a system are that the thylakoid membrane transport (or be permeable to) bicarbonate and that CA activity be present in the thylakoid lumen but not in the compartment containing Rubisco. The localization of ctCA1 to the thylakoid lumen in *C. reinhardtii* (Karlsson et al., 1998) has brought renewed interest to the possibility that such a system might operate as part of the *C. reinhardtii* CCM. Since Rubisco is localized to the pyrenoid in *C. reinhardtii* (Lacoste-Royal and Gibbs, 1987; Kuchitsu et al., 1988b, 1991; McKay and Gibbs, 1989, 1991), it is tempting to speculate that ctCA1 is restricted to those thylakoids that traverse the pyrenoid and that the high bicarbonate concentration in the stroma has access to this same pyrenoidal thylakoid lumen, thus allowing for CO_2 release into the pyrenoid Rubisco pool at a concentration even higher than that expected at equilibrium in the alkaline stroma/pyrenoid.

It is clear that the site of CO_2 elevation in *C. reinhardtii* must at least overlap with the site where bicarbonate is actively accumulated and must include both the site where the ctCA1 is located and the site where Rubisco is located. Since active bicarbonate accumulation most likely occurs in the chloroplast stroma and Rubisco appears to be exclusively located within the chloroplast pyrenoid, it has been suggested that the microalgal pyrenoid might play a role in the CCM similar to the role apparently played by the carboxysome in cyanobacteria (Badger and Price, 1992, 1994). Depending on whether ctCA1 is located in all thylakoids or

restricted to those encompassed by the pyrenoid, the site of CO₂ elevation might be restricted to the pyrenoid or include the whole chloroplast. In this regard, it is intriguing to note that at least one alga that lacks a pyrenoid has also been found to lack a functional CCM (Palmqvist et al., 1994a, 1995).

In keeping with the hypothesis that the site of CO₂ release from actively accumulated bicarbonate might be restricted to the pyrenoid, it has been suggested that the starch sheath might serve as a diffusion barrier to minimize loss of CO₂ from the pyrenoid and thus from the site of Rubisco activity (Badger and Price, 1992, 1994). However, recent work with mutants of both *C. reinhardtii* and *Chlorella pyrenoidosa* lacking pyrenoid starch sheaths showed that the affinity of the mutants for CO₂ in photosynthesis was not significantly different from that of wild-type cells (Plumed et al., 1996; Villarejo et al., 1996a). Thus any role as a diffusion barrier played by the pyrenoid starch sheath must be minimal at best. Since the chloroplast envelopes probably do not represent a significant diffusion barrier to CO₂, the existence of any barrier to restrict diffusion of the elevated CO₂ away from its source, whether that be the pyrenoid or the whole chloroplast, is still in question.

E. Induction of the CCM and Related Adaptations to Limiting CO₂

Green microalgae can exist in at least two distinctly different physiological states depending on the CO₂ concentration (0.03% vs 1–5%) during growth (see Spalding, 1989), which reflects a whole suite of adaptive responses to limiting CO₂ (Table 3). Acclimation to limiting CO₂ in *C. reinhardtii* results

in, along with induction of a functional CCM, the induced expression of specific genes, including *Cah1*, *Ccp1* and *Ccp2*, and *Mca1* and *Mca2*, encoding the major periplasmic CA (pCA1), the putative chloroplast carrier proteins (Ccp1 and Ccp2) and the mitochondrial CAs (mtCA1 and mtCA2), respectively (Coleman and Grossman, 1984; Bailly and Coleman, 1988; Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Fujiwara et al., 1990; Fukuzawa et al., 1990; Geraghty et al., 1990; Spalding et al., 1991; Ramazanov et al., 1993; Eriksson et al., 1996; Geraghty and Spalding, 1996; Chen et al., 1997). In addition to the identified genes, two soluble polypeptides of 45–50kDa appear to be up-regulated (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989), and substantial increases also occur in the activity of photorespiratory enzymes (Marek and Spalding, 1991; Ramazanov and Cardenas, 1994). Two **limiting-CO₂-induced** polypeptides also have been reported in *D. tertiolecta* chloroplast envelopes (Thielmann et al., 1992).

Six *C. reinhardtii* cDNA clones of other limiting-CO₂ inducible genes were reported by Burow et al. (1996), one of which corresponded to the *Cah1* gene. The sequence of one, *Lci1*, showed no homology to any known genes, but its deduced amino acid sequence included four putative transmembrane spanning helices, suggesting it might encode a transmembrane protein. Another of the clones (*Att1*) was subsequently identified as encoding alanine: α -ketoglutarate aminotransferase, although it is not clear what role this aminotransferase might play in acclimation of *C. reinhardtii* to limiting CO₂ (Chen et al., 1996).

During acclimation of *C. reinhardtii* to limiting CO₂, expression of *Cah1*, *Mca1*, *Mca2*, *Ccp1* and

Table 3. Adaptation of carbon concentrating mechanism (CCM) components in green algae to growth at low CO₂. The CCM components are described in Fig. 3 and Table 1.

CCM Component	Adaptation to Growth at Low C _i
1. The C _i transport system	C _i transport, either as CO ₂ or HCO ₃ [−] , increases from little or no activity prior to adaptation both at cell and chloroplast membranes. The relative contributions of CO ₂ and HCO ₃ [−] -transport are unclear at both locations.
2. Energization of C _i transport	Although changes occur in the photochemical properties of the cells during adaptation, it is not yet clear how these are related to the increased energy demands associated with operation of the CCM.
3. The stromal HCO ₃ [−] pool	No significant HCO ₃ [−] pool can be detected prior to adaptation.
4. The thylakoid lumen CA	The significant changes appear to occur with regard to the thylakoid lumen CA.
5 & 6. The pyrenoid compartment	It is unclear whether any changes occur in the pyrenoid itself. The pyrenoid starch sheath increases, but it is not clear whether this is of any functional significance for the CCM.

Ccp2 all are regulated at the level of mRNA abundance and appear to be closely correlated (Bailly and Coleman, 1988; Dionisio-Sese et al., 1990; Fujiwara et al., 1990; Geraghty et al., 1990; Spalding et al., 1991; Eriksson et al., 1996, 1998; Geraghty and Spalding, 1996; Chen et al., 1997), suggesting that their expression may be coordinately regulated by a single mechanism, e.g., a single *trans*-acting factor. Along with the genes induced in limiting CO₂, some genes exhibit decreased expression in limiting CO₂, including stable down-regulation of the minor periplasmic CA (*Cah2*) (Fujiwara et al., 1990) and transient decrease in the synthesis of both subunits of Rubisco (the *RbcS1*, *RbcS2* and *rbcL* gene products) (Coleman and Grossman, 1984; Winder et al., 1992). Although *Cah2* expression is controlled at the level of mRNA abundance, as with the five induced genes discussed above (Fujiwara et al., 1990; Rawat and Moroney, 1991), the transient decrease in biosynthesis of both Rubisco subunits during acclimation to limiting CO₂ is controlled at the translational level (Winder et al., 1992).

Acclimation to limiting CO₂ also can involve substantial structural changes in microalgal cells, including an increased development of the pyrenoid starch sheath (Kuchitsu et al., 1988a, 1991; Ramazanov et al., 1994; Geraghty and Spalding, 1996), increased vacuolization (Geraghty and Spalding, 1996) and changes in mitochondrial distribution (Kramer and Findenegg, 1978; Geraghty and Spalding, 1996). Mitochondria of *C. reinhardtii* move during acclimation from a central position, within the cup of the chloroplast, to a peripheral position, between the chloroplast envelope and the plasmalemma (Geraghty and Spalding, 1996).

The mitochondrial relocation and the limiting-CO₂ induction of specific mitochondrial proteins (Eriksson et al., 1996; Geraghty and Spalding, 1996) argue that the mitochondria may play an important role in acclimation of green microalgae to limiting CO₂. It has been suggested that, because of their move to a peripheral location in air-adapted cells and the increased glycolate pathway flux in air-adapted cells, the mitochondria might be involved in scavenging a photorespiratory metabolite produced by the chloroplasts (Geraghty and Spalding, 1996).

The signal regulating acclimation of green microalgae to changes in CO₂ concentration is unknown (Coleman, 1991). Clearly, they must sense the change either directly as CO₂ (or C_i) concentration or by an indirect effect on a cellular process, such as carbohydrate metabolism. Several studies have

implicated a requirement for photosynthetic activity for induction in *C. reinhardtii* (Spalding and Ogren, 1982; Spencer et al., 1983; Dionisio et al., 1989a,b; Dionisio-Sese et al., 1990; Villarejo et al., 1996b). Based on evidence implicating photorespiration in induction, it has been suggested that a CO₂ deficiency might be signaled by a photorespiratory metabolite (Spalding and Ogren, 1982; Ramazanov and Cardenas, 1992; Villarejo et al., 1996b). However, this simplistic model fails to take into account reports indicating a more complicated signaling system, including induction by low CO₂ concentrations in the dark (Bailey and Coleman, 1988; Fett and Coleman, 1994; Rawat and Moroney, 1995; Villarejo et al., 1996b), enhanced induction by non-photosynthetic blue light (Dionisio et al., 1989a,b; Dionisio-Sese et al., 1990; Borodin et al., 1994) and partial repression by mixotrophic growth with acetate as a carbon source (Spalding and Ogren, 1982; Moroney et al., 1987; Coleman et al., 1991; Fett and Coleman, 1994; Ramazanov et al., 1994). Clearly the acclimation of *C. reinhardtii* to limiting CO₂ involves a signaling system more complex than a change in the concentration of a photorespiratory metabolite, but, at present, it is difficult to incorporate all the available information on *Cah1* and CCM induction into a cogent hypothesis for the signaling mechanism.

An important advance in understanding gene regulation mediated in *C. reinhardtii* by changes in CO₂ concentration was made with the identification and characterization of a mutant, *cia5*, which apparently does not acclimate to limiting CO₂ (Moroney et al., 1989). The *cia5* mutant lacks induction of C_i transport, induction of *Cah1*, *Mca1* and *Mca2*, *Ccp1* and *Ccp2*, *Lci1*, or any of the unidentified limiting-CO₂ induced polypeptides, up-regulation of phosphoglycolate phosphatase and glycolate dehydrogenase, and down-regulation of Rubisco biosynthesis (Moroney et al., 1989; Marek and Spalding, 1991; Spalding et al., 1991; Burow et al., 1996). The lack of any of these responses in *cia5* argues that one signal transduction pathway regulates a very diverse set of adaptations, including transcriptional (pre-translational) up-regulation of *Lci1*, *Cah1*, *Mca1*, *Mca2*, *Ccp1* and *Ccp2*, transcriptional (pre-translational) down-regulation of *Cah2*, transient, translational down-regulation of *rbcL* (chloroplast), *RbcS1* (nuclear) and *RbcS2* (nuclear) expression, long term up-regulation of glycolate dehydrogenase and transient up-regulation of phosphoglycolate phosphatase. Since *cia5* appears to lack any response to limiting CO₂, this mutant

probably represents a defect in a component of the signal transduction pathway that is required for induction of all of the observed limiting CO₂ adaptations.

VI. Diversity of CCM Function in Green and Non-Green Algae

A. Potential Diversity of CCMs in Algae

The existence of CCMs and their contribution to aquatic photosynthesis is well recognized (Aizawa and Miyachi, 1986; Badger, 1987; Spalding, 1989; Kaplan et al., 1991; Badger and Price, 1992). However, most of the studies of these systems have centered on model cyanobacterial and green microalgal systems. However, for algae in particular it is likely that considerable diversity in the operation of CCMs exists between genera belonging to both green and non-green algae. In this context, more detailed studies of algal species across all the algal groups are necessary before we can understand the range of solutions which have been found to circumvent Rubisco's limitations.

In considering the diversity of CCM function in both green and non-green alga it is useful to consider the possible functional elements which a eukaryotic cell may possess to enable an elevation of CO₂ within the chloroplast. In this context, consideration of Table 3 and Fig. 10 for *Chlamydomonas* is useful. These elements include:

1. One or more plasma membrane located C_i transport systems.
2. The presence of various forms of external periplasmic carbonic anhydrase.
3. One or more chloroplast membrane-located C_i transport systems which use photosynthetic energy to accumulate HCO₃⁻ within the chloroplast.
4. Pyrenoid or stromal Rubisco, which may be an integral part of CCM activity.
5. A pyrenoid carbonic anhydrase.
6. A thylakoid CA which might use luminal protons to generate CO₂ either in the pyrenoid or the stroma surrounding the thylakoids.

7. An envelope CA which could minimize CO₂ leakage by converting CO₂ back to HCO₃⁻.
8. The possible significance of single versus multi-chloroplast cells.
9. Variation in the kinetic properties of Rubisco.
10. Involvement of mitochondria, including mitochondrial CA.

Although the above list includes most known possible elements of an algal CCM, not all elements would be expected in all algae displaying CCM ability. It is possible to envisage a number of CCM arrangements, scaling from those that perhaps have no CCM at all, to algae where the maximum amount of CCM activity is engaged. However, all algal CCM models which have been thus far described (Spalding and Portis, 1985; Fridlyand, 1997; Raven, 1997; Badger et al., 1998; Spalding, 1998) have one property in common, that being the active accumulation of C_i, resulting in the accumulation of HCO₃⁻ in the chloroplast stroma. This HCO₃⁻ supply can be used in a number of ways to elevate CO₂, through the action of CA located in three possible places, the pyrenoid, the thylakoid lumen or the stroma (Badger et al., 1998)

B. Indications of CCM Diversity between Species

The assessment of the diversity of CCM functioning and activities across diverse microphyte and macrophyte algal genera depends on the availability of reliable indicators for measuring and quantifying the relative functioning of a CCM. A survey of the experimental evidence that has been accumulated for a range of algal species has recently been presented (Badger et al., 1998) and suggests that the following list of experimental indicators may be used to measure aspects of CCM activity. Table 4 shows a summary of both green and non-green algal species that have been examined for various CCM attributes and summarizes some general findings with regard to diversity. Aspects of this diversity are expanded in the following sections.

1. C_i Accumulation

Since the discovery of a CCM in both *Chlamydomonas reinhardtii* and *Anabaena variabilis* (Badger

Table 4. CCM characteristics and their variation across both green and non-green algal species. This table summarizes data presented by Badger et al. (1998). Readers are referred to this reference for a more detailed summary of particular features of various algal species listed in this table and the source of the data.

Green Algae	Non-Green Algae		
<i>Chlorophyta</i>	<i>Chrysophyta</i>	<i>Rhodophyta</i>	<i>Phaeophyta</i>
<i>Microalgae</i>	<i>Microalgae</i>	<i>Microalgae</i>	<i>Macroalgae</i>
<i>Chlamydomonas reinhardtii</i>	<i>Emiliania huxleyi</i>	<i>Porphyridium cruentum</i>	<i>Ascochyllum nodosum</i>
<i>Dunaliella</i> species	<i>Pleurochrysis carterae</i>	<i>Porphyridium purpureum</i>	<i>Fucus</i> species
<i>Chlorella</i> species	<i>Isochrysis galbana</i>	<i>Cyanidioschyzon merolae</i>	<i>Laminaria</i> species
<i>Scenedesmus obliquus</i>	<i>Phaeodactylum tricornutum</i>	<i>Gonniotrichopsis sublittoralis</i>	
<i>Euglena gracilis</i>	<i>Cylindrotheca fusiformis</i>	<i>Macroalgae</i>	<i>Pyrrrophyta</i>
<i>Stichococcus bacillaris</i>	<i>Cyclotella</i> sp.	<i>Palmaria palmata</i>	<i>Microalgae</i>
<i>Coccomyxa</i> sp.	<i>Nannochloropsis oculata</i>	<i>Chondrus crispus</i>	<i>Symbiodinium</i> sp.
<i>Macroalgae</i>	<i>Navicula pelliculosa</i>	<i>Gracilaria</i> species	<i>Amphidinium carterae</i>
<i>Ulva</i> sp.		<i>Porphyra leucosticta</i>	<i>Peridinium gatunense</i>

CCM Characteristics

- Up to 80 fold accumulation of inorganic carbon, however some species appear to have CCMs without significant C_i accumulation.
- $K_{0.5}(CO_2)$ of photosynthesis 5–30-fold less than $K_m(CO_2)$ Rubisco.
- Carbonic anhydrase inhibitors, AZA and EZA, reduce the affinity of photosynthesis for C_i .
- A general ability to use HCO_3^- for photosynthesis
- Species with CCM activity and HCO_3^- -usage generally show the ability to change their affinity for external C_i when grown at varying pH and C_i conditions.
- Variability in the presence of external CA. Can vary both within and between species, and is induced by growth conditions.
- Active transport of C_i into the cells may be associated with light stimulated CA activity.
- Considerable variation in chloroplast structure in cells. Most microalgae with CCM activity have single chloroplasts with pyrenoids. More advanced species tend to have multiple chloroplasts without pyrenoids.
- There is considerable variability in pyrenoid structure. This includes the presence and absence of thylakoid intrusions, starch sheaths and position within the chloroplast.
- Several species have been identified as lacking CCM activity. In all cases, this is associated with the lack of a chloroplast pyrenoid and perhaps multiple chloroplasts per cell.
- Macroalgae of all genera show greater diversity than microalgal species. There are many examples of single and multiple chloroplast cells, and the presence and absence of single or multiple pyrenoids and great variation in starch sheath and thylakoid arrangements.

et al., 1980; Kaplan et al., 1980) it has generally been recognized that there needs to be light-dependent active accumulation of an internal C_i pool for a CCM to function. The physical existence of this has been measured by both silicone-oil centrifugation techniques and by measuring uptake and evolution during dark/light/dark transients (Badger et al., 1980; Badger et al., 1985; Miller et al., 1991). While this has proved relatively easy with cyanobacteria, because of their large internal pools, evidence is much less reliably collected for algae which have smaller accumulation ratios (Burns and Beardall, 1987). In addition, these techniques do not work well with

macroalgae which are not amenable to some of these physical separation techniques. As has been pointed out by Raven (1997) and by Badger et al. (1998), the expected internal C_i pool may be very dependent on what particular CCM might operate. Certainly the involvement of the thylakoid proton-linked CA may reduce C_i pools, and actually lead to a depletion of total C_i but an accumulation of CO_2 . Thus, at this stage, while measurement of a C_i pool is useful in establishing that this is part of a particular CCM, the absence of significant accumulation does not mean the absence of a CCM. Indeed, it may be useful to establish in how many species C_i accumulation or

non-accumulation is a characteristic of a CCM.

Examining the available data (Badger et al., 1998), it is apparent that it is really only the green microalgae such as *Chlamydomonas*, *Dunaliella* and *Chlorella* which show what can be called significant levels of C_i accumulation in the cells. Non-green microalgae which have been measured have significantly lower levels of C_i accumulation, despite the fact that many of them can achieve similarly high affinities for external C_i, particularly at the alkaline pH of seawater. Examples of this include *Phaeodactylum*, *Isochrysis* and *Porphyridium*. *Chondrus crispus* is also an example of a red macroalga with an absence of apparent C_i accumulation but with a relatively high photosynthetic affinity for external C_i. Exceptions in the non-green algae appear to be the freshwater diatom, *Navicula pelliculosa* and the acid thermophile Rhodophyte *Cyanidioschyzon merolae*, which both show significant C_i accumulation. Significant C_i accumulation also appears to occur in the dinoflagellate *Peridinium gatunense*.

2. CO₂ Affinity of Photosynthesis Versus Rubisco

The driving force for the evolution of a CCM has been the low affinity of Rubisco for external CO₂. It is not surprising then that one of the most useful indicators for implying the existence of a CCM in any photosynthetic organism is to compare the affinity of photosynthesis for external CO₂ versus the affinity of its Rubisco. The major limitation of this approach is the ability to accurately measure the affinity of Rubisco extracted from various organisms that may be more or less recalcitrant to in vitro measurement techniques.

Using this comparative approach, it is relatively easy to conclude that *Chlamydomonas reinhardtii* with a K_m(CO₂-photosynthesis):K_m(CO₂-Rubisco) of about 1:30, clearly employs a CCM to improve Rubisco kinetics (see Badger et al., 1998). In contrast, *Coccomyxa* with a ratio of about 1:1 appears not to employ a CCM. A high photosynthetic affinity of intact cells for CO₂ compared to Rubisco exists in wide range of green and non-green algae, particularly microalgal species, achieving ratios of greater than 2:30 in many cases. The notable exceptions appear to be the microalgal species *Coccomyxa*, *Emiliania huxleyi* and *Gonniotrichopsis sublittoralis*, as well as the macroalgae *Palmaria palmata* and *Laminaria* species. Considering the dinoflagellates, which

possess a Form II Rubisco (see Fig. 4), one would also expect a relatively efficient CCM to achieve high affinities for external CO₂ despite a Rubisco which has a relatively low S_{rel} and probably a high K_m(CO₂).

3. Oxygen Inhibition of Photosynthesis and the CO₂ (C_i) Compensation Point

Based on the functioning of Rubisco in higher plants and the central role of its oxygenase reaction in the photorespiratory cycle, evidence for oxygen inhibition of photosynthesis in algae and the CO₂ compensation point has been used to infer some potential for CCM activity. The obvious assumption in this approach is that algal Rubisco enzymes are similar to those of higher plants and that when CO₂ is subsaturating, then atmospheric levels of oxygen would be expected to inhibit carboxylation and enhance the synthesis of phosphoglycolate. Interestingly, for most algal and cyanobacterial species, except some green algae, the effects of oxygen on photosynthesis are significantly less than that which would be expected in higher plants and could lead to the inference that CCM activity is present to suppress oxygenase activity, as occurs in C₄ higher plants (Kerby and Raven, 1985; Burns and Beardall, 1987; Beardall, 1989; Spalding, 1989; Raven et al., 1990; Johnston, 1991). With an increase in our knowledge of the kinetics of Rubisco enzymes from a variety of algal and cyanobacterial sources it is apparent that this approach may be of little use for most algal species, particularly non-green algae and cyanobacteria.

4. Carbonic Anhydrases

As CA is of central importance to all CCM models shown in Fig. 10, then examining the presence of both internal and external CAs and the effects of CA inhibitors has proven useful for implying the function of a CCM in algae.

External CA has been readily measured in a wide range of species and has been well correlated with the utilization of external HCO₃⁻. External CA has been shown to function in both micro and macroalgae in all algal Divisions (Burns and Beardall, 1987; Giordano and Maberly, 1989; Johnston, 1991; Badger and Price, 1992).

Internal CAs have been less well studied in non-green algae. A particular exception to this has been

chloroplast CA from *Porphyridium* (Mitsuhashi, personal communication) where recent evidence suggests that this may be a soluble protein localized in the matrix of the Rubisco containing pyrenoid, rather than being associated with the thylakoid as has been discovered for *Chlamydomonas* (Karlsson et al., 1998). The green alga *Coccomyxa* (Hiltonen et al., 1995), which lacks a pyrenoid, has also been shown to possess a soluble **β -type** CA which is presumed to be localized in the stroma. Thus we may expect the further discovery of multiple forms of internal CA with multiple locations and particular roles in a CCM. Of particular recent interest has been the description of a carbonic anhydrase gene from the oceanic diatom *Thalassiosira weissflogii* (Roberts et al., 1997) which shows little homology to other CA genes and proteins from either cyanobacteria or green algae.

5. Light-Stimulated CA Activity

A primary component of all CCMs is that light energizes the uptake of external inorganic carbon and places it in an internal environment containing CA. This constitutes a light-stimulated CA activity. Such light-stimulated CA activity is only measurable with a mass spectrometric approach, examining the exchange of ^{18}O from C_i species into ^{16}O labeled water. This has been used to show light-stimulated CA activity in both cyanobacteria (Badger and Price, 1989) and green algae (Palmqvist et al., 1994b) and non-green algae (Badger et al., 1998) which employ a CCM. It is apparent that there is considerable diversity in the appearance of light-stimulated CA among the non-green algae which remains to be explored and explained.

6. The Use of HCO_3^- as a Photosynthetic Substrate

Bicarbonate usage in photosynthesis has long been examined as a property of algae (Lucas, 1983). Usage of HCO_3^- by algae is obviously dependent on the participation of external CA and plasma membrane transporter(s) (Badger, 1987; Johnston, 1991; Badger and Price, 1992). In all algae so far examined, it would appear as though HCO_3^- usage is well correlated with the presence of a CCM (see Badger et al., 1998). The green algae *Stichococcus bacillaris* and *Chlorella saccharophila* and the Rhodophyte *Cyanidioschyzon merolae* are excep-

tions, largely using CO_2 but showing clear evidence of CCM activity.

7. Changes in the Affinity for External C_i , Depending on Growth Conditions

The induction of a high affinity for external C_i when cells are grown at limiting C_i is well recognized as being due to the induction of CCM activity in microalgae (Badger, 1987). Changes in the affinity for external C_i of 10 fold or more are well documented in both green and non-green algae (Badger et al., 1998). Such changes have been shown to involve changes in the affinity of C_i transport systems for external CO_2 and HCO_3^- as well as other changes involving increases in both internal and external CA activities (section V.D). Such inducible changes seem to be strong evidence of the participation of cellular infrastructure in the supply of CO_2 to Rubisco in the chloroplast and are easily measured by examining photosynthesis in intact cells. These inducible changes are not limited to microalgae and have also been observed in a number of macrophytes, including *Ulva* (Bjork et al., 1993), *Gracilaria* (Garcia Sanchez et al., 1994), *Porphyra* (Mercado et al., 1997) and *Fucus* (Johnston and Raven, 1990). While such changes indicate an active supply of CO_2 to Rubisco, particularly when coupled with HCO_3^- usage, they do not allow a distinction to be made as to whether the changes occur to processes involving the chloroplast or the cytosol/plasma membrane components of the cell. The lack of inducible changes in affinity for external C_i is certainly not evidence for the lack of a CCM. Inducible changes in a CCM probably only occur in species that experience periodic limitations to external C_i supply. Many algal species may employ a CCM with variable activity that is sufficient to meet their environmental needs. They may not have the very high affinities for external C_i exhibited by some microalgae but are nonetheless dependent on the engagement of some level of CCM.

8. Correlations between Pyrenoids, Plastid Number and CCMs

The role of the Rubisco containing pyrenoids in the algal CCM has been of considerable speculation since the identification of the cyanobacterial carboxysome as central to the cyanobacterial CCM (Badger and Price, 1992). But, speculation and correlation is all that it has remained. Certainly,

Rubisco has been found to be localized within a number of green and non-green algal pyrenoids (Lacoste-Royal and Gibbs, 1987; Kuchitsu et al., 1988b; McKay and Gibbs, 1989, 1991) and CA has been found in the pyrenoid of the red alga *Porphyridium* (Mitsuhashi, personal communication).

So far we have four examples where the absence of pyrenoids seems to be correlated with the absence or reduction in CCM activity. There are three algae, *Coccomyxa* (Palmqvist et al., 1994a, 1995), *Trentopohlia* (Smith and Griffiths, 1996a), *Gonniotrichopsis* (Badger et al., 1998) and one bryophyte, *Anthoceros crispulus* (Smith and Griffiths, 1996a,b) as examples where the absence of CCM activity appears to be correlated with an absence of pyrenoids in the chloroplast. In *Trentopohlia*, *Gonniotrichopsis* and *Anthoceros*, this is also correlated with multiplastid cells. Pyrenoids abound in all algal Divisions and the evolution of more advanced genera in all algal groups is associated with the loss of pyrenoids and a transition from large single plastids to smaller plastids and multiplastidic cells. Badger et al. (1998), have summarized data which show that there are plentiful examples of closely related algal genera showing variations in both the presence and absence of pyrenoids as well as the number of plastids.

Investigation of the pyrenoid literature (Griffiths, 1970; Dodge, 1973; Ettl, 1978, 1983; Garbary et al., 1980; Bold and Wynne, 1985; McKay and Gibbs, 1991) shows that there is a wide diversity in pyrenoid structure and morphology. The simplest pyrenoids consist mainly of a protein matrix containing Rubisco and with no chloroplast thylakoids entering this body. In more complicated pyrenoids, thylakoids enter the protein matrix in either a random or more organized arrangement. Mostly, pyrenoid bodies are contained within the general stromal compartment of the chloroplast but in some cases the pyrenoid is stalked, protruding outside the body of the chloroplast, often into the interior of the cell. In addition to this, starch and polysaccharide may be associated with pyrenoids, normally surrounding the pyrenoid as a sheath. There may also be one or more pyrenoids per chloroplast. There has been some speculation as to the role of various pyrenoid features, particularly as they may be related to CCM function. Studies of pyrenoid-located thylakoids have indicated that there may be a dominance of PS I in these lamellae and they may be involved in cyclic electron transport (McKay and Gibbs, 1991). It is tempting to speculate that cyclic

electron transport may be involved in the supply of protons which may aid in the conversion of HCO₃⁻ to CO₂, in the mechanism depicted in Fig. 11, but there is no evidence to support this notion. Pyrenoid structure has strong phylogenetic linkages in all algal groups and has been used by many as an aid in algal classification. The unique structure/function relationships of pyrenoids, particularly in their role in the chloroplast CCM, remain to be fully understood.

References

- Adams MS (1985) Inorganic carbon reserves of natural waters and the ecophysiological consequences of their photosynthetic depletion: (II) macrophytes. In: Lucas WJ and Berry JA (eds) Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms, pp 421–435. American Society of Plant Physiologists, Rockville
- Aizawa K and Miyachi S (1984) Carbonic anhydrase located on cell surface increases the affinity for inorganic carbon in photosynthesis of *Dunaliella tertiolecta*. FEBS Lett 173: 41–44
- Aizawa K and Miyachi S (1986) Carbonic-anhydrase and CO₂ concentrating mechanisms in microalgae and cyanobacteria (review). FEMS Micr Rev 39: 215–233
- Amoroso G, Weber C, Sültemeyer DF and Fock H (1996) Intracellular carbonic anhydrase activities in *Dunaliella tertiolecta* (Butcher) and *Chlamydomonas reinhardtii* (Dangeard) in relation to inorganic carbon concentration during growth: Further evidence for the existence of two distinct carbonic anhydrases associated with the chloroplasts. Planta 199:177–184
- Amoroso G, Sültemeyer D, Thyssen C and Fock HP (1998) Uptake of HCO₃⁻ and CO₂ in cells and chloroplasts from the microalgae *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta*. Plant Physiology 116: 193–201
- Badger MR (1987) The CO₂-concentrating mechanism in aquatic phototrophs. In: Hatch MD and Boardman NK (eds) The Biochemistry of Plants, Vol 10, pp 217–274. Academic Press, San Diego
- Badger MR and Andrews TJ (1987) Co-evolution of Rubisco and CO₂ concentrating mechanisms. In: Biggins J (ed) Progress in Photosynthesis Research, Vol III, pp 601–609. Martinus Nijhoff Publishers, Dordrecht
- Badger MR and Price GD (1989) Carbonic anhydrase activity associated with the cyanobacterium *Synechococcus PCC7942*. Plant Physiol 89: 51–60
- Badger MR and Price GD (1992) The CO₂ concentrating mechanism in cyanobacteria and microalgae. Physiol Plant 84: 606–615
- Badger MR and Price GD (1994) The role of carbonic anhydrase in photosynthesis. Ann Rev Plant Physiol Plant Mol Biol 45: 369–392
- Badger MR, Kaplan A and Berry JA (1980) Internal inorganic carbon pool of *Chlamydomonas reinhardtii*—evidence for a carbon dioxide concentrating mechanism. Plant Physiol 66: 407–113

- Badger MR, Bassett M and Comins HN (1985) A model for HCO_3^- accumulation and photosynthesis in the cyanobacterium *Synechococcus* sp. Theoretical predictions and experimental observations. *Plant Physiol* 77: 465–471
- Badger MR, Palmqvist K and Yu JW (1994) Measurement of CO_2 and HCO_3^- fluxes in cyanobacteria and microalgae during steady-state photosynthesis. *Physiol Plant* 90: 529–536
- Badger MR, Andrews TJ, Whitney SM, Ludwig M, Yellowlees DC, Leggat W and Price GD (1998) The diversity and Co-evolution of Rubisco, plastids, pyrenoids and chloroplast-based CCMs in the algae. *Can J Bot* 76: 1052–1071
- Bailly J and Coleman JR (1988) Effect of CO_2 concentration on protein biosynthesis and carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiol* 87: 833–840
- Beardall J (1989) Photosynthesis and photorespiration in marine phytoplankton. *Aquatic Botany* 43: 104–130
- Berner RA (1990) Atmospheric carbon dioxide levels over phanerozoic time. *Science* 249: 1382–1386
- Berner RA (1993) Paleozoic atmospheric CO_2 : Importance of solar radiation and plant evolution. *Science* 261: 68–70
- Björk M, Haglund K, Ramazanov Z and Pedersen M (1993) Inducible mechanisms for HCO_3^- utilization and repression of photorespiration in protoplasts and thalli of three species of *Ulva* (Chlorophyta). *J Phycol* 29: 166–173
- Bold HC and Wynne MJ (1985) Introduction to the Algae. Structure and reproduction. Prentice-Hall, Inc., Edgewood Cliffs
- Borodin V, Gardeström P and Samuelsson G (1994) The effect of light quality on the induction of efficient photosynthesis under low CO_2 conditions in *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa*. *Physiol Plant* 92: 254–260
- Broda E (1975) The Evolution of Bioenergetic Processes. Pergamon Press, Oxford
- Burns BD and Beardall J (1987) Utilization of inorganic carbon by marine microalgae. *J Exp Mar Biol Ecol* 107: 75–86
- Burow MD, Chen Z-Y, Mouton TM and Moroney JV (1996) Isolation of cDNA clones induced upon transfer of *Chlamydomonas reinhardtii* cells to low CO_2 . *Plant Mol Biol* 31:443–448
- Chen Z-Y, Burow MD, Mason CB and Moroney JV (1996) A low- CO_2 -inducible gene encoding an alanine: α -ketoglutarate aminotransferase in *Chlamydomonas reinhardtii*. *Plant Physiol* 112: 677–684
- Chen Z-Y, Lavigne LL, Mason CB and Moroney JV (1997) Cloning and overexpression of two cDNAs encoding the low- CO_2 -inducible chloroplast envelope protein LIP-36 from *Chlamydomonas reinhardtii*. *Plant Physiol* 114: 265–273
- Coleman JR (1991) The molecular and biochemical analyses of CO_2 -concentrating mechanisms in cyanobacteria and microalgae. *Plant Cell Environ* 14: 861–867
- Coleman JR and Grossman AR (1984) Biosynthesis of carbonic anhydrase in *Chlamydomonas reinhardtii* during adaptation to low CO_2 . *Proc Natl Acad Sci USA* 81: 6049–6053
- Coleman JR, Berry JA, Togasaki RK and Grossman AR (1984) Identification of extracellular carbonic anhydrase of *Chlamydomonas reinhardtii*. *Plant Physiol* 76: 472–477
- Coleman JR, Luinenburg I, Majea N and Provart N (1991) Sequence analysis and regulation of expression of a gene coding for carbonic anhydrase in *Chlamydomonas reinhardtii*. *Can J Bot* 69: 1097–1102
- Delwiche CF and Palmer JD (1996) Rampant horizontal transfer and duplication of Rubisco genes in eubacteria and plastids. *Mol Biol Evol* 13: 873–882
- Dionisio ML, Tsuzuki M and Miyachi S (1989a) Light requirement for carbonic anhydrase induction in *Chlamydomonas reinhardtii*. *Plant Cell Physiol* 30: 207–213
- Dionisio ML, Tsuzuki M and Miyachi S (1989b) Blue light induction of carbonic anhydrase activity in *Chlamydomonas reinhardtii*. *Plant Cell Physiol* 30: 215–219
- Dionisio-Sese ML, Fukuzawa H and Miyachi S (1990) Light-induced carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiol* 94: 1103–1110
- Dodge J (1973) The pyrenoid. In: Dodge J (ed) The Fine Structure of Algal Cells, pp 105–124. Academic Press, London
- Eriksson M, Karlsson J, Ramazanov Z, Gardeström P and Samuelsson G (1996) Discovery of an algal mitochondrial carbonic anhydrase: Molecular cloning and characterization of a low- CO_2 -induced polypeptide in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 93: 12031–12034
- Eriksson M, Villand P, Gardeström P and Samuelsson G (1998) Induction and regulation of expression of a low- CO_2 -induced mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiol* 116: 637–641
- Espie GS and Kandasamy RA (1994) Monensin inhibition of Na^+ -dependent HCO_3^- transport distinguishes it from Na^+ -independent HCO_3^- transport and provides evidence for Na^+ / HCO_3^- symport in the cyanobacterium *Synechococcus* UTEX 625. *Plant Physiol* 104: 1419–1428
- Ettl H (1978) Xanthophyceae. In: Ettl H, Gerloff J and Heyning H (eds) Süsswasserflora von Mitteleuropa. Gustav Fischer, Stuttgart
- Ettl H (1983) Chlorophyta I. In: Ettl H, Gerloff J and Heyning H (eds) Süsswasserflora von Mitteleuropa. pp i–xiv, 1–807. Gustaff Fischer Verlag, Stuttgart
- Fett JP and Coleman JR (1994) Regulation of periplasmic carbonic anhydrase expression in *Chlamydomonas reinhardtii* by acetate and pH. *Plant Physiol* 106: 103–108
- Fisher M, Gokhman I, Pick U and Zamir A (1996) A salt-resistant plasma membrane carbonic anhydrase is induced by salt in *Dunaliella salina*. *J Biol Chem* 271: 17718–17723
- Fridlyand LE (1997) Models of CO_2 concentrating mechanisms in microalgae taking into account cell and chloroplast structure. *Biosystems* 44: 41–57
- Fujiwara S, Fukuzawa H, Tachiki A and Miyachi S (1990) Structure and differential expression of two genes encoding carbonic anhydrase in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 87: 9779–9783
- Fukuzawa H, Fujiwara S, Yamamoto Y, Dionisio-Sese ML and Miyachi S (1990) cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*: Regulation by environmental CO_2 concentration. *Proc Natl Acad Sci USA* 87: 4383–4387
- Fukuzawa H, Suzuki E, Komukai Y and Miyachi S (1992) A gene homologous to chloroplast carbonic anhydrase (icfA) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC7942. *Proc Natl Acad Sci* 89: 4437–41
- Funke RP, Kovar JL and Weeks DP (1997) Intracellular carbonic anhydrase is essential to photosynthesis in *Chlamydomonas reinhardtii* at atmospheric levels of CO_2 . *Plant Physiol* 114: 237–244
- Garbary DJ, Hansen GI and Scagel RF (1980) A revised classification of the Bangiophyceae (Rhodophyta). Nova

- Hedwigia 33: 145–166
- Garcia Sanchez MJ, Fernandez JA and Niell X (1994) Effect of inorganic carbon supply on the photosynthetic physiology of *Gracilaria tenuistipitata*. *Planta* 194: 55–61
- Gehl KA, Colman B, and Sposato LM (1990) Mechanism of inorganic carbon uptake in *Chlorella saccharophila*: The lack of involvement of carbonic anhydrase. *J Exp Bot* 41: 1385–1391
- Geraghty AM and Spalding MH (1996) Molecular and structural changes in *Chlamydomonas* under limiting CO₂: A possible mitochondrial role in adaptation. *Plant Physiol* 111: 1339–1347
- Geraghty AM, Anderson JC and Spalding MH (1990) A 36 kilodalton **limiting-CO₂** induced polypeptide of *Chlamydomonas* is distinct from the 37 kilodalton periplasmic carbonic anhydrase. *Plant Physiol* 93: 116–121
- Giordano M and Maberly SC (1989) Distribution of carbonic anhydrase in British marine macroalgae. *Oecologia* 81: 534–539
- Goyal A and Tolbert NE (1989) Uptake of inorganic carbon by isolated chloroplasts from air-adapted *Dunaliella*. *Plant Physiol* 89: 1264–1269
- Goyal A, Shiraiwa Y, Husic HD and Tolbert NE (1992) External and internal carbonic anhydrases in *Dunaliella* species. *Marine Biol* 113: 349–355
- Griffiths DJ (1970) The pyrenoid. *Bot Reviews* 36: 29–58
- Hewett-Elliott D and Tashian RE (1996) Functional diversity, conservation, and convergence in the evolution of the α , β , and γ -carbonic anhydrase gene families. *Mol Phylogenet Evol* 5: 50–77
- Higgins CT (1992) ABC transporters: From microorganisms to man. *Ann Rev Cell Biol* 8: 67–113
- Hiltonen T, Karlsson J, Palmqvist K, Clarke AK and Samuelsson G (1995) Purification and characterisation of an intracellular carbonic anhydrase from the unicellular green alga *Coccomyxa*. *Planta* 195: 345–351
- Johnston AM (1991) The acquisition of inorganic carbon by marine macroalgae. *Can J Bot* 69: 1123–1132
- Johnston AM and Raven JA (1990) Effects of culture in high CO₂ on the photosynthetic physiology of *Fucus serratus*. *Br Phycol J* 25: 75–82
- Kaneko T and Tabata S (1997) Complete genome structure of the unicellular cyanobacterium *Synechocystis* PCC6803. *Plant Cell Physiol* 38: 1171–1176
- Kaplan A, Badger MR and Berry JA (1980) Photosynthesis and the intracellular inorganic carbon pool in the bluegreen alga *Anabaena variabilis*—response to external CO₂ concentration. *Planta* 149: 219–226
- Kaplan A, Zenvirth D, Marcus Y, Omata T and Ogawa T (1987) Energization and activation of inorganic carbon uptake by light in cyanobacteria. *Plant Physiol* 84: 210–213
- Kaplan A, Schwarz R, Lieman Hurwitz J and Reinhold L (1991) Physiological and molecular aspects of the inorganic carbon-concentrating mechanism in cyanobacteria. *Plant Physiol* 97: 851–855
- Kaplan A, Schwarz R, Lieman-Hurwitz J and Reinhold L (1994) Physiological and molecular studies on the response of cyanobacteria to changes in the ambient inorganic carbon concentration. In: Bryant D (ed) *The Molecular Biology of the Cyanobacteria*, pp 469–485. Kluwer Academic Publishers, Dordrecht
- Karlsson J, Ramazanov Z, Hiltonen T, Garderström P and Samuelsson G (1994) Effect of vanadate on photosynthesis and the ATP/ADP ratio in **low-CO₂-adapted** *Chlamydomonas reinhardtii* cells. *Planta* 192: 46–51
- Karlsson J, Hiltonen T, Husic HD, Ramazanov Z and Samuelsson G (1995) Intracellular carbonic anhydrase of *Chlamydomonas reinhardtii*. *Plant Physiol* 109: 533–539
- Karlsson J, Clarke AK, Chen ZY, Huggins SY, Park YI, Husic HD, Moroney JV and Samuelsson G (1998) A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂. *EMBO J* 17: 1208–1216
- Kerby NW and Raven JA (1985) Transport and fixation of inorganic carbon by marine algae. *Adv Bot Res* 11: 71–123
- Kobayashi M, Rodriguez R, Lara C and Omata T (1997) Involvement of the C-terminal domain of an ATP-binding subunit in the regulation of the ABC-type nitrate/nitrite transporter of the cyanobacterium *Synechococcus* sp. strain PCC7942. *J Biol Chem* 272: 27197–27201
- Kramer D and Findenegg GR (1978) Variations in the ultrastructure of *Scenedesmus obliquus* during adaptation to low CO₂ level. *Z Pflanzen* 89: 407–410
- Kuchitsu K, Tsuzuki M and Miyachi S (1988a) Changes in starch localization within the chloroplast induced by changes in CO₂ concentration during growth of *Chlamydomonas reinhardtii*: Independent regulation of pyrenoid starch and stromal starch. *Plant Cell Physiol* 29: 1269–1278
- Kuchitsu K, Tsuzuki M and Miyachi S (1988b) Characterization of the pyrenoid isolated from unicellular green alga *Chlamydomonas reinhardtii*: Paniculate form of RuBisCO protein. *Protoplasma* 144: 17–24
- Kuchitsu K, Tsuzuki M and Miyachi S (1991) Polypeptide composition and enzyme activities of the pyrenoid and its regulation by CO₂ concentration in unicellular green algae. *Can J Bot* 69: 1062–1069
- Lacoste-Royal G and Gibbs SP (1987) Immunocytochemical localization of ribulose-1,5-bisphosphate carboxylase in the pyrenoid and thylakoid region of the chloroplast of *Chlamydomonas reinhardtii*. *Plant Physiol* 83: 602–606
- Li QL and Canvin DT (1997) Inorganic carbon accumulation stimulates linear electron flow to artificial electron acceptors of photosystem i in air-grown cells of the cyanobacterium *Synechococcus utex* 625. *Plant Physiol* 114: 1273–1281
- Lucas WJ (1983) Photosynthetic assimilation of exogenous HCO₃[−] by aquatic plants. *Ann Rev Plant Physiol* 34: 71–104
- Manuel LJ and Moroney JV (1988) Inorganic carbon accumulation by *Chlamydomonas reinhardtii*. New proteins are made during adaptation to low CO₂. *Plant Physiol* 88: 491–496
- Marco E, Ohad N, Schwarz R, Lieman Hurwitz J, Gabay C and Kaplan A (1993) High CO₂ concentration alleviates the block in photosynthetic electron transport in an ndhb-inactivated mutant of *Synechococcus* sp. PCC 7942. *Plant Physiol* 101: 1047–53
- Marek LF and Spalding MH (1991) Changes in photorespiratory enzyme activity in response to limiting CO₂ in *Chlamydomonas reinhardtii*. *Plant Physiol* 97: 420–425
- McKay RML and Gibbs SP (1989) Immunocytochemical localization of ribulose 1,5-bisphosphate carboxylase oxygenase in light-limited and light saturated cells of *Chlorella pyrenoidosa*. *Protoplasma* 149: 31–37
- McKay RML and Gibbs SP (1991) Composition and function of

- pyrenoids: cytochemical and immunocytochemical approaches. *Can J Bot* 69: 1040–1052
- McKay RML, Gibbs SP and Espie GS (1993) Effect of dissolved inorganic carbon on the expression of carboxysomes, localization of Rubisco and the mode of inorganic carbon transport in cells of the cyanobacterium *Synechococcus* UTEX 625. *Arch Microbiol* 159: 21–29
- Mercado JM, Niell FX and Figueroa FL (1997) Regulation of the mechanism for HCO_3^- use by the inorganic carbon level in *Porphyra leucosticta* Thur in le Jolis (rhodophyta). *Planta* 201: 319–325
- Mi H, Endo T, Schreiber U, Ogawa T and Asada K (1992) Electron donation from cyclic and respiratory flows to the photosynthetic intersystem chain is mediated by pyridine nucleotide dehydrogenase in the cyanobacterium *Synechocystis* PCC 6803. *Plant Cell Physiol* 33: 1233–1237
- Mi H, Endo T, Schreiber U, Ogawa T and Asada K (1994) NAD(P)H dehydrogenase-dependent cyclic electron flow around photosystem I in the cyanobacterium *Synechocystis* PCC 6803: A study of dark-starved cells and spheroplasts. *Plant Cell Physiol* 35: 163–173
- Mi H, Endo T, Ogawa T and Asada K (1995) Thylakoid membrane-bound, NADPH-specific pyridine nucleotide dehydrogenase complex mediated cyclic electron transport in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol* 36: 661–668
- Miller AG, Espie GS and Canvin DT (1990) Physiological aspects of CO_2 and HCO_3^- -transport by cyanobacteria: A review. *Can J Bot* 68: 1291–1302
- Miller AG, Espie GS and Canvin DT (1991) Active CO_2 transport in cyanobacteria. *Can J Bot* 69: 925–935
- Miyachi S, Suzuki M and Avramova T (1983) Utilization modes of inorganic carbon for photosynthesis in various species of *Chlorella*. *Plant Cell Physiol* 24: 441–451
- Moroney JV, Husic HD and Tolbert NE (1985) Effects of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*. *Plant Physiol* 79: 177–183
- Moroney JV, Kitayama M, Togasaki RK and Tolbert NE (1987) Evidence for inorganic carbon transport by intact chloroplasts of *Chlamydomonas reinhardtii*. *Plant Physiol* 83: 460–463
- Moroney JV, Husic HD, Tolbert NE, Kitayama M, Manuel LJ and Togasaki RK (1989) Isolation and characterization of a mutant of *Chlamydomonas reinhardtii* deficient in the CO_2 concentrating mechanism. *Plant Physiol* 89: 897–903
- Nakamura Y, Kaneko T, Hirosewa M, Miyajima N and Tabata S (1998) Cyanobase, a www database containing the complete nucleotide sequence of the genome of *Synechocystis* sp. strain PCC6803. *Nuc Acids Res* 26: 63–67
- Ogawa T (1990) Mutants of *Synechocystis* PCC6803 defective in inorganic carbon transport. *Plant Physiol* 94: 760–765
- Ogawa T (1991a) Cloning and inactivation of a gene essential to inorganic carbon transport of *Synechocystis* PCC6803. *Plant Physiol* 96: 280–284
- Ogawa T (1991b) A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of *Synechocystis* PCC6803. *Proc Natl Acad Sci* 88: 4275–4279
- Ogawa T (1992) Identification and characterization of the *ictA/ndhL* gene product essential to inorganic carbon transport of *Synechocystis* PCC6803. *Plant Physiol* 99: 1604–1608
- Ogawa T, Miyano A and Inoue Y (1985) Photosystem-I-driven inorganic carbon transport in the cyanobacterium, *Anacystis nidulans*. *Biochim Biophys Acta* 808: 77–84
- Ogren WL (1984) Photorespiration—pathways, regulation, and modification. *Ann Rev Plant Physiol* 35: 415–442
- Ohkawa H, Sonoda M, Katoh H and Ogawa T (1998) The use of mutants in the analysis of the CCM in cyanobacteria. *Can J Bot* 76: 1035–1042
- Okamura M, Price GD, Badger MR, Ogawa T and Omata T (1997) The cmpABCD genes of the cyanobacterium *Synechococcus* sp. PCC7942 encode a HCO_3^- transporter. *Plant Cell Physiol* 38: supplement: 30
- Omata T and Ogawa T (1985) Changes in the polypeptide composition of the cytoplasmic membrane in the cyanobacterium *Anacystis nidulans* during adaptation to low CO_2 conditions. *Plant Cell Physiol* 26: 1075–1081
- Omata T and Ogawa T (1986) Biosynthesis of a 42-KD polypeptide in the cytoplasmic membrane of the cyanobacterium *Anacystis nidulans* strain-R2 during adaptation to low CO_2 concentration. *Plant Physiol* 80: 525–530
- Omata T and Ogawa T (1987) Immunochemical studies on the major proteins in cytoplasmic membranes of cyanobacteria. In: Biggins J (eds) Progress in Photosynthesis Research, Vol IV, pp 601–609. Martinus Nijhoff Publishers, Dordrecht
- Omata T, Andriesse X and Hirano A (1993) Identification and characterization of a gene cluster involved in nitrate transport in the cyanobacterium *Synechococcus* sp. PCC7942. *Mol Gen Genet* 236: 193–202
- Palmqvist K, Sundblad LG, Samuelsson G and Sundbom E (1986) A correlation between changes in luminescence decay kinetics and the appearance of a CO_2 -accumulating mechanism in *Scenedesmus obliquus*. *Photosynth Res* 10: 113–123
- Palmqvist K, Sjöberg S and Samuelsson G (1988) Induction of inorganic carbon accumulation in the unicellular green algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*. *Plant Physiol* 87: 437–442
- Palmqvist K, Sundblad L-G, Wingsle G and Samuelsson G (1990) Acclimation of photosynthetic light reactions during induction of inorganic carbon accumulation in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol* 94: 357–366
- Palmqvist K, Ogren E and Lernmark U (1994a) The CO_2 -concentrating mechanism is absent in the green alga *Coccomyxa*: a comparative study of photosynthetic CO_2 and light responses of *Coccomyxa*, *Chlamydomonas reinhardtii* and barley protoplasts. *Plant Cell Environ* 17: 65–72
- Palmqvist K, Yu J-W and Badger MR (1994b) Carbonic anhydrase activity and inorganic carbon fluxes in low- and high- C_i cells of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. *Physiol Plant* 90: 537–547
- Palmqvist K, Sültemeyer D, Baldet P, Andrews TJ and Badger MR (1995) Characterisation of inorganic carbon fluxes, carbonic anhydrase(s) and ribulose-1,5-biphosphate carboxylase-oxygenase in the green unicellular alga *Coccomyxa*—Comparisons with low- CO_2 cells of *Chlamydomonas reinhardtii*. *Planta* 197: 352–361
- Pesheva I, Kodama M, Dionisio-Sese ML and Miyachi S (1994) Changes in photosynthetic characteristics induced by transferring air-grown cells of *Chlorococcum littorale* to high- CO_2 conditions. *Plant Cell Physiol* 35: 379–387
- Pierce J, Carlson TJ and Williams JGK (1989) A cyanobacterial

- mutant requiring the expression of ribulose bisphosphate carboxylase from a photosynthetic anaerobe. Proc Natl Acad Sci 86: 5753–5757
- Plumed MdP, Villarejo A, Rios Adl, Garcia-Reina G and Ramazanov Z (1996) The CO₂-concentrating mechanism in a starchless mutant of the green unicellular alga *Chlorella pyrenoidosa*. Planta 200: 28–31
- Price GD and Badger MR (1989a) Ethoxzolamide inhibition of CO₂ uptake in the cyanobacterium *Synechococcus* PCC7942 without apparent inhibition of internal carbonic anhydrase activity. Plant Physiol 89: 37–43
- Price GD and Badger MR (1989b) Expression of human carbonic anhydrase in the cyanobacterium *Synechococcus* PCC7942 creates a high CO₂-requiring phenotype. Evidence for a central role for carboxysomes in the CO₂ concentrating mechanism. Plant Physiol 91: 505–513
- Price GD and Badger MR (1991) Evidence for the role of carboxysomes in the cyanobacterial CO₂-concentrating mechanism. Can J Bot 69: 963–973
- Price GD, Coleman JR and Badger MR (1992) Association of carbonic anhydrase activity with carboxysomes isolated from the cyanobacterium *Synechococcus* PCC7942. Plant Physiol 100: 784–793
- Price GD, Sültemeyer D, Klughammer B, Ludwig M and Badger MR (1998) The functioning of the CO₂ concentrating mechanism in several cyanobacterial strains: A review of general physiological characteristics, genes, proteins and recent advances. Can J Bot 76: 973–1002
- Pronina NA and Borodin VV (1993) CO₂ stress and CO₂ concentration mechanism: investigation by means of photosystem deficient and carbonic-anhydrase-deficient mutants of *Chlamydomonas reinhardtii*. Photosynthetica 28: 515–522
- Ramazanov Z and Cardenas J (1992) Involvement of photorespiration and glycolate pathway in carbonic anhydrase induction and inorganic carbon concentration in *Chlamydomonas reinhardtii*. Physiol Plant 84: 502–508
- Ramazanov Z and Cardenas J (1994) Photorespiratory ammonium assimilation in chloroplasts of *Chlamydomonas reinhardtii*. Physiol Plant 91: 495–502
- Ramazanov Z, Mason CB, Geraghty AM, Spalding MH and Moroney JV (1993) The low CO₂-inducible 36 kD protein is localized to the chloroplast envelope of *Chlamydomonas reinhardtii*. Plant Physiol 101: 1195–1199
- Ramazanov Z, Rawat M, Henk MC, Mason CB, Matthews SW and Moroney JV (1994) The induction of the CO₂-concentrating mechanism is correlated with the formation of the starch sheath around the pyrenoid of *Chlamydomonas reinhardtii*. Planta 195: 210–216
- Raven JA (1970) Exogenous inorganic carbon sources in plant photosynthesis. Biol Reviews 45: 167–221
- Raven JA (1997) CO₂-concentrating mechanisms—a direct role for thylakoid lumen acidification. Plant Cell Environ 20: 147–154
- Raven JA, Johnston AM and MacFarlane JJ (1990) Carbon metabolism. In: Cole KM and Sheath RG (eds) Biology of the Red Algae, pp 171–202. Cambridge University Press, Cambridge
- Raven JA, Osborne JA and Johnston AM (1985) Uptake of CO₂ by aquatic vegetation. Plant Cell Environ 8: 417–425
- Rawat M and Moroney JV (1991) Partial characterization of a new isozyme of carbonic anhydrase isolated from *Chlamydomonas reinhardtii*. J Biol Chem 266: 9719–9723
- Rawat M and Moroney JV (1995) The regulation of carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase activase by light and CO₂ in *Chlamydomonas reinhardtii*. Plant Physiol 109: 937–944
- Read BA and Tabita FR (1994) High substrate specificity factor ribulose bisphosphate carboxylase/oxygenase from eukaryotic marine algae and properties of recombinant cyanobacterial RubiSCO containing ‘algal’ residue modifications. Arch Biochem Biophys 312: 210–218
- Reddy KJ, Masamoto K, Sherman DM and Sherman LA (1989) DNA sequence and regulation of the gene (*cbpA*) encoding the 42-kilodalton cytoplasmic membrane carotenoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942. J Bacteriol 171: 3486–3493
- Reinhold L, Zviman M and Kaplan A (1989) A quantitative model for inorganic carbon fluxes and photosynthesis in cyanobacteria. Plant Physiol Biochem 27: 945–954
- Reinhold L, Kosloff R and Kaplan A (1991) A model for inorganic carbon fluxes and photosynthesis in cyanobacterial carboxysomes. Can J Bot 69: 984–988
- Roberts SB, Lane TW and Morel FMM (1997) Carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (bacillariophyceae). J Phycol 33: 845–850
- Rolland N, Dorne AJ, Amoroso G, Sültemeyer DF, Joyard J and Rochaix JD (1997) Disruption of the plastid *ycf10* open reading frame affects uptake of inorganic carbon in the chloroplast of Chlamydomonas. EMBO J 16: 6713–6726
- Rotatore C and Colman B (1990) Uptake of inorganic carbon by isolated chloroplasts of the unicellular green alga *Chlorella ellipsoidea*. Plant Physiol 93: 1597–1600
- Rotatore C and Colman B (1991) The acquisition and accumulation of inorganic carbon by the unicellular green alga *Chlorella ellipsoidea*. Plant Cell Environ 14: 377–382
- Rotatore C, Lew RR and Colman B (1992) Active uptake of CO₂ during photosynthesis in the green alga *Eremosphaera viridis* is mediated by a CO₂-ATPase. Planta 188: 539–545
- Smith EC and Griffiths H (1996a) The occurrence of the chloroplast pyrenoid is correlated with the activity of a CO₂-concentrating mechanism and carbon isotope discrimination in lichens and bryophytes. Planta 198: 6–16
- Smith EC and Griffiths H (1996b) A pyrenoid-based carbon-concentrating mechanism is present in terrestrial bryophytes of the class Anthocerotae. Planta 200: 203–212
- Soltes-Rak E, Mulligan ME and Coleman JR (1997) Identification and characterization of a gene encoding a vertebrate-type carbonic anhydrase in cyanobacteria. J Bacteriol 179: 769–774
- Spalding MH (1989) Photosynthesis and photorespiration in freshwater green algae. Aquatic Botany 34: 181–209
- Spalding MH (1990) Effect of photon flux density on inorganic carbon accumulation and net CO₂ exchange in a high-CO₂-requiring mutant of *Chlamydomonas reinhardtii*. Photosynth Res 24: 245–252
- Spalding MH (1998) CO₂ acquisition: Acclimation to changing carbon availability. In: Rochaix J-D, Goldschmidt-Clermont M and Merchant S (eds) The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas, pp 529–547. Kluwer

- Academic Publishers, Dordrecht
- Spalding MH and Jeffrey M (1989) Membrane-associated polypeptides induced in *Chlamydomonas* by limiting CO₂ concentrations. *Plant Physiol* 89: 133–137
- Spalding MH and Ogren WL (1982) Photosynthesis is required for induction of the CO₂-concentrating system in *Chlamydomonas reinhardtii*. *FEB S Lett* 145: 41–44
- Spalding MH and Portis AR (1985) A model of CO₂ assimilation in *Chlamydomonas reinhardtii*. *Planta* 73: 268–272
- Spalding MH, Spreitzer RJ and Ogren WL (1983a) Carbonic anhydrase deficient mutant of *Chlamydomonas* requires elevated carbon dioxide concentration for photoautotrophic growth. *Plant Physiol* 73: 268–272
- Spalding MH, Spreitzer RJ and Ogren WL (1983b) Reduced inorganic carbon transport in a CO₂-requiring mutant of *Chlamydomonas reinhardtii*. *Plant Physiol* 73: 273–276
- Spalding MH, Critchley C, Govindjee and Ogren WL (1984) Influence of carbon dioxide concentration during growth on fluorescence induction characteristics of the green alga *Chlamydomonas reinhardtii*. *Photosynth Res* 5: 169–176
- Spalding MH, Winder TL, Anderson JC, Geraghty AM and Marek LF (1991) Changes in protein and gene expression during induction of the CO₂-concentrating mechanism in wild-type and mutant *Chlamydomonas*. *Can J Bot* 69: 1008–1016
- Spence DH and Maberly SC (1985) Occurrence and ecological importance of HCO₃⁻ use among aquatic higher plants. In: Lucas WJ and Berry JA (eds) *Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms*, pp. 125–144. American Society of Plant Physiologists, Rockville
- Spencer KG, Kimpel DL, Fisher ML, Togasaki RK and Miyachi S (1983) Carbonic anhydrase induction in *Chlamydomonas reinhardtii* II. Requirements for carbonic anhydrase induction. *Plant Cell Physiol* 24: 301–304
- Sütemeyer DF, Klock G, Kreutzberg K and Fock HP (1988) Photosynthesis and apparent affinity for dissolved inorganic carbon by cells and chloroplasts of *Chlamydomonas reinhardtii* grown at high and low CO₂ concentrations. *Planta* 176: 256–260
- Sütemeyer DF, Miller AG, Espie GS, Fock HP and Canvin DT (1989) Active CO₂ transport by the green alga *Chlamydomonas reinhardtii*. *Plant Physiol* 89: 1213–1219
- Sütemeyer DF, Fock HP and Canvin DT (1990) Mass spectrometric measurement of intracellular carbonic anhydrase activity in high and low C_i cells of *Chlamydomonas*. *Plant Physiol* 94: 1250–1257
- Sütemeyer D, Schmidt, C and Fock HP (1993) Carbonic anhydrases in higher plants and aquatic microorganisms. *Physiol Plant* 88: 179–190
- Sütemeyer DF, Amoroso G and Fock H (1995) Induction of intracellular carbonic anhydrases during the adaptation to low inorganic carbon concentrations in wild-type and *ca-1* mutant cells of *Chlamydomonas reinhardtii*. *Planta* 196: 217–224
- Sütemeyer D, Klughammer B, Ludwig M, Badger MR and Price GD (1997a) Random insertional mutagenesis used in the generation of mutants of the marine cyanobacterium *Synechococcus* sp. strain PCC7002 with an impaired CO₂ concentrating mechanism. *Aust J Plant Physiol* 24: 317–327
- Sütemeyer D, Price GD, Bryant DA and Badger MR (1997b) PsaE- and ndhF-mediated electron transport affect bicarbonate transport rather than carbon dioxide uptake in the cyanobacterium *Synechococcus* sp PCC7002. *Planta* 201: 36–42
- Sütemeyer D, Klughammer B, Badger MR and Price GD (1998a) Fast induction of high-affinity HCO₃⁻ transport in cyanobacteria. *Plant Physiol* 116: 183–192
- Sütemeyer D, Klughammer B, Badger MR and Price GD (1998b) Protein phosphorylation and its possible involvement in the induction of the high-affinity CO₂ concentrating mechanism in cyanobacteria. *Can J Bot* 76: 954–961
- Sundblad LG, Samuelsson G, Wigge B and Gardeström P (1990) Luminescence decay kinetics in relation to quenching and stimulation of dark fluorescence from high and low CO₂ adapted cells of *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*. *Photosynth Res* 23: 269–282
- Suzuki K and Spalding MH (1989) Adaptation of *Chlamydomonas reinhardtii* high-CO₂-requiring mutants to limiting-CO₂. *Plant Physiol* 90: 1195–1200
- Talling JF (1985) Inorganic carbon reserves of natural waters and ecophysiological consequences of their photosynthetic depletion: microalgae. In: Lucas WJ and Berry JA (eds) *Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms*, pp 403–420. American Society of Plant Physiologists, Rockville
- Thielmann J, Tolbert NE, Goyal A and Senger H (1990) Two systems for concentrating CO₂ and bicarbonate during photosynthesis by *Scenedesmus*. *Plant Physiol* 92: 622–629
- Thielmann J, Goyal A and Tolbert NE (1992) Two polypeptides in the inner chloroplast envelope of *Dunaliella tertiolecta* induced by low CO₂. *Plant Physiol* 100: 2113–2115
- Tyrrell PN, Kandasamy RA, Crotty CM and Espie GS (1996) Ethoxysolamide differentially inhibits CO₂ uptake and Na⁺-independent and Na⁺-dependent HCO₃⁻ uptake in the cyanobacterium *Synechococcus* sp UTEX 625. *Plant Physiol* 112: 79–88
- Uemura K, Anwaruzzaman, Miyachi S and Yokota A (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from thermophilic red algae with a strong specificity for CO₂ fixation. *Biochem Biophys Res Comm* 233: 568–571
- Villarejo A, Martínez F, Plumed MdP and Ramazanov Z (1996a) The induction of the CO₂ concentrating mechanism in a starchless mutant of *Chlamydomonas reinhardtii*. *Physiol Plant* 98: 798–802
- Villarejo A, Reina GG and Ramazanov Z (1996b) Regulation of the low-CO₂-inducible polypeptides in *Chlamydomonas reinhardtii*. *Planta* 199: 481–485
- Watson GMF and Tabita FR (1996) Regulation, unique gene organization, and unusual primary structure of carbon fixation genes from a marine phycoerythrin-containing cyanobacterium. *Plant Mol Biol* 32: 1103–1115
- Watson GMF and Tabita FR (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* 146: 13–22
- Williams TG and Turpin DH (1987) The role of external carbonic anhydrase in inorganic carbon acquisition by *Chlamydomonas reinhardtii* at alkaline pH. *Plant Physiol* 83: 92–96
- Winder TL, Anderson JC and Spalding MH (1992) Translational regulation of the large and small subunits of ribulose bisphosphate carboxylase/oxygenase during induction of the CO₂-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiol* 98: 1409–1414

Yu J-W, Price GD, Song L and Badger MR (1992) Isolation of a putative carboxysomal carbonic anhydrase gene from the cyanobacterium *Synechococcus* PCC7942. Plant Physiol 100: 794–800

Yu L, Zhao JD, Muhlenhoff U, Bryant DA and Golbeck JH (1993) PsaE is required for in vivo cyclic electron flow around Photosystem I in the cyanobacterium *Synechococcus* sp. PCC7002. Plant Physiol 103: 171–180

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Chapter 17

Photosynthetic Fractionation of Carbon Isotopes

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Summary

During photosynthetic CO_2 fixation fractionation of stable carbon isotopes occurs and, consequently, plants are generally depleted in the heavier isotope ^{13}C . Carbon isotope discrimination (Δ) is a measure of this process and depends on fractionation during diffusion and during enzymatic carboxylation reactions. Discrimination during photosynthesis has a significant, though relatively small, effect on the isotopic composition of atmospheric CO_2 both at regional and global level; hence stable isotopes find relevant applications in the study of the global carbon cycle. In addition to variation in Δ among plants with different photosynthetic pathways, large variations are found within plant groups, resulting from genetic and environmental influences on the ratio of partial pressures of CO_2 at the sites of carboxylation and that in the free turbulent atmosphere. Experimental evidences confirming the theory of carbon isotope discrimination and known complications are discussed. Carbon isotope composition also varies among different metabolites, compartments and plant organs as a result of fractionation during secondary metabolism and variation in the ratio of diffusional and carboxylation limitations. Special emphases are given to measurements of Δ in different carbon pools such as bulk dry matter, cellulose, starch and sucrose, with different turnover rates and different integration of p_i/p_a and to the links with water-use efficiency. The application of carbon isotope discrimination to physiological and ecophysiological studies and to selection of genotypes with improved water-use efficiency and drought tolerance and the recent progress in this field are reviewed.

I. Introduction

In nature there are two stable isotopes of carbon, ^{12}C and ^{13}C . The lighter isotope ^{12}C is by far the most abundant with about 98.9% of the atoms, while the heavier isotope ^{13}C is present in variable proportion at around 1.1%. These two isotopes are not equally distributed in natural compounds because of isotope fractionation occurring during physical, chemical and biological processes involved in the carbon cycle. Normally plants are depleted in ^{13}C compared to atmospheric CO_2 because of carbon isotope fractionation occurring during photosynthetic CO_2 fixation.

The interest in stable isotope distribution in nature developed initially in the physical sciences and then became a focal point for geochemists. Indeed, the earliest contributions to botany with measurements of plant isotopic compositions were by geochemists interested in natural variations in isotope abundance

levels. Since the early systematic measurements of carbon isotope composition in plants (Wickman, 1952; Craig, 1953) there have been numerous efforts to understand the mechanisms determining the isotope fractionation during photosynthesis (Park and Epstein, 1960, 1961). Early systematic surveys of carbon isotope ratios in plants possessing the C_3 and the C_4 photosynthetic pathways were reported by Margaret Bender (1968, 1971) and Smith and Epstein (1971), shortly after the discovery of the C_4 photosynthetic pathway (Kortschak et al., 1965; Hatch and Slack, 1966). Then, carbon isotope abundance become a classical means to distinguish between different photosynthetic pathways and to study their geographical, taxonomic and ecological distributions. Subsequently, several mechanistic models were developed to explain the fractionation in C_3 and C_4 plants and to account for the variability in isotope composition observed among plants within each photosynthetic pathway. On the basis of these models, a large number of investigations have focused on the study of the relationship between carbon isotope fractionation and water-use efficiency, especially in C_3 species. The enormous success of these studies has allowed the application of stable isotopes in genetic studies and in breeding program for increased yield in dry environments.

The bases of carbon isotope fractionation in plants have been previously reviewed (O'Leary, 1981; Peterson and Fry, 1987; O'Leary, 1988; Farquhar et

Abbreviations: α – isotope effect; δ – carbon isotope composition relative to VPDB; Δ – carbon isotope discrimination; A – assimilation rate; b – fractionation during carboxylations; CAM – crassulacean acid metabolism; CCM – CO_2 concentrating mechanism; g_m – mesophyll conductance; g_s – stomatal conductance; p_c – chloroplastic CO_2 partial pressure; PEP – phosphoenolpyruvate; p_i/p_a – ratio of intercellular to atmospheric partial pressures of CO_2 ; PDB – Pee Dee Belemnite; R – isotope abundance ratio; RuBP – ribulose-1,5-bisphosphate; VPDB – Vienna Pee Dee Belemnite; W – photosynthetic water-use efficiency; WUE – water-use efficiency

al., 1989a). However, more recently a large number of studies has extended the knowledge of carbon isotope discrimination during photosynthesis.

In this chapter, the fundamental chemical, physical and biochemical principles of carbon isotope fractionation in plants are reviewed. The effects of fractionation processes occurring during photosynthesis and during carbon metabolism on plant isotope composition are discussed. Special attention is also given to recent developments in this area and to possible implications for physiological and ecological studies of photosynthesis and plant productivity. Perspectives of stable isotopes in the study of water-use efficiency and productivity and the application in breeding for increased yield and drought tolerance are also discussed.

II. Carbon Isotopes in Nature—The Global Carbon Cycle

Variation of carbon isotope composition occurs in the organic and inorganic carbon pools. Atmospheric carbon dioxide represents the major link between the inorganic and organic portions of the terrestrial global carbon cycle and between terrestrial and marine ecosystems (Siegenthaler and Sarmiento, 1993). It participates in the equilibrium exchange reactions between the ocean carbonates and serves as the major source of carbon for the entire biosphere, as a substrate for photosynthesis.

Apart from carbon in the earth's crust, dissolved inorganic carbon in the oceans is the largest pool in the global carbon cycle. It is mostly represented by HCO_3^- , but CO_3^{2-} and dissolved CO_2 are also present. The isotope ratio of total dissolved inorganic carbon and of marine carbonates are both close to 0‰ (Bauer et al., 1995) relative to the international standard PDB (Pee Dee Belemnite, see below), which is also a carbonate limestone of marine origin. Because of photosynthetic fractionation, the organic carbon pool in the marine environment is strongly depleted in ^{13}C compared to the inorganic carbon pool. Dissolved organic carbon is mostly represented by soluble products derived from decomposition of plankton, and is isotopically lighter (about -22‰ compared to PDB) than dissolved inorganic carbon (Boutton, 1991; Bauer et al., 1995).

Since the ocean, and particularly its dissolved inorganic carbon, is a much larger carbon pool than the atmosphere, the isotope ratio of atmospheric CO_2

is largely determined by the ocean-atmosphere CO_2 exchange together with fractionation during the CO_2 exchange with the terrestrial biosphere by photosynthesis and respiration. The isotopic composition of CO_2 would be, at equilibrium, depleted by about 7‰ in ^{13}C compared to the total inorganic pool dissolved in the surface ocean water (Mook, 1986). The isotope composition of atmospheric CO_2 varies seasonally, anti-parallel with fluctuation in CO_2 concentration, as a consequence of variation in photosynthesis and respiration (Keeling et al., 1996). In the Northern hemisphere, this fluctuation has a maximum in autumn and a minimum in spring (Mook et al., 1983). In the Southern hemisphere variations in atmospheric CO_2 isotope ratio are less pronounced because of greater activity in the tropics where the seasonality is less marked, and six months out of phase compared to the Northern hemisphere.

The carbon isotope ratio of atmospheric CO_2 is also subjected to a long-term decline associated with the increase in CO_2 concentration due to anthropogenic fossil fuel combustion and deforestation (Keeling et al., 1979; Mook et al., 1983; Francey, 1985; Friedli et al., 1986, 1987). The isotope composition of the CO_2 produced from human activities is invariably depleted in ^{13}C , compared to the atmospheric carbon isotope ratio. Consequently, fossil fuel combustion and deforestation result in the progressive dilution of the ^{13}C content of the atmosphere.

Atmospheric methane and carbon monoxide represent much smaller carbon pools than CO_2 . The atmospheric concentration of CH_4 has more than doubled in the past 200 years with a strong covariation of CO_2 and CH_4 concentrations (Battle et al., 1996). Changes in concentrations are reflected in a moderate increase of the ^{13}C content of CH_4 , with δ being presently around -48‰ (Craig et al., 1988; Lowe et al., 1993). Sources, sinks and fluxes of atmospheric CO are poorly known. Concentrations and isotope compositions of CO are highly variable; nevertheless the global CO concentration increased during the 1980s because of human activities (car exhaust, agricultural waste and burning of savanna). Recently, it has been reported that global CO concentration has started to decline, possibly because of a reduction in emissions (Khalil and Rasmussen, 1994). Moderate decreases in emission would be reflected in a decreased global CO concentration because of its short atmospheric lifetime (2–3 months). Oxidation of CH_4 and CO by atmospheric chemistry produces

CO_2 depleted in ^{13}C . Hence the effect on the isotopic composition of atmospheric CO_2 , although small, would be toward more negative δ values.

After photosynthetic CO_2 fixation by autotrophic organisms carbon is then cycled through heterotrophic organisms and detritus. During this trophic cycling the isotope ratios of carbon are maintained (Gearing, 1991). This is the meaning of the saying that, 'You are what you eat,' although a trend toward enrichment in ^{13}C with increasing trophic levels of about 1‰ per level does exist (De Niro and Epstein, 1978). Hence, the isotope composition of the entire biosphere is largely determined by the fractionation processes occurring during photosynthesis.

III. Definitions

A. Isotope Composition

Carbon isotope composition is generally measured on CO_2 by isotope ratio mass spectrometers. The material of interest is converted to CO_2 and then injected into the inlet of the mass spectrometer. Plant material is usually converted to CO_2 by combustion, and increasingly, using elemental analyzers. The latter are often coupled to mass spectrometers for automated combustion and analysis. Mass spectrometric analysis gives high precision measurements of the isotope abundance ratio R , defined as

$$R = ^{13}\text{CO}_2 / ^{12}\text{CO}_2 \quad (1)$$

However, absolute isotope composition is not easily measured directly, and mass spectrometers measure the deviation of the sample isotope ratio from that of a known standard. Hence the isotope composition $\delta^{13}\text{C}$ is defined by:

$$\delta^{13}\text{C} = R_p / R_s - 1, \quad (2)$$

where R_p is the isotope ratio in plant samples and R_s is the ratio of the standard. The internationally accepted standard for carbon isotope measurements is CO_2 produced from a Cretaceous belemnite from the Pee Dee formation in South Carolina, and referred to as PDB. Early measurements of the isotope ratio of PDB indicated a value of 0.01124 (Craig, 1957), while recent analysis suggests that a value of 0.01118 is more appropriate (Bakke et al., 1991). Isotope composition is usually referred relative to PDB, with

the deviation often conveniently expressed as parts per thousand (i.e. $\times 10^{-3}$ or ‰, 'per mil'). However, the supply of PDB standard has been exhausted and, consequently, the Commission on Atomic Weights and Isotopic Abundances of the International Union of Pure and Applied Chemistry has recently recommended (Coplen, 1995) that isotopic abundances for carbon be reported relative to VPDB (Vienna Pee Dee Belemnite). This is defined by adopting a $\delta^{13}\text{C}$ value of +1.95‰ for NBS 19 carbonate relative to VPDB. Nevertheless, PDB is currently the widely adopted standard in studies of carbon isotope composition.

B. Isotope Effects

Variation of isotope ratios and isotope compositions among different compounds is due to isotope fractionation during physical, chemical and biological processes. The isotope fractionations during such processes are described as isotope effects, the ratio of the rate constants for reactions of different isotopic species. Isotope effects are often divided into kinetic isotope effects and thermodynamic or equilibrium isotope effects. Kinetic effects are those in which a substrate or source is converted into a product with fractionation against the heavier isotope ^{13}C and, therefore, the product is isotopically lighter than the substrate. The kinetic isotope effect can be described as the ratio of the rate constants for the substrate containing ^{12}C and ^{13}C , k^{12} and k^{13} . When the source is a reservoir large enough to be not appreciably affected by product formation, then the isotope effect, α_k , is equal to the ratio of isotope ratios of source and product:

$$\alpha_k = k^{12} / k^{13} = R_s / R_p, \quad (3)$$

where R_s and R_p are the isotope ratios of the source and of the product, respectively. Similarly, the equilibrium isotope effect is defined as the ratio of the equilibrium constants K^{12} and K^{13} , for the molecules containing ^{12}C and ^{13}C , respectively.

Because equilibrium isotope effects are the resultant of opposing kinetic isotope effects, the kinetic isotope effects are generally larger than equilibrium ones. Fractionation associated with ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is a kinetic isotope effect. Another kinetic isotope effect is represented by the difference in the binary diffusivities of $^{13}\text{CO}_2$ -air and $^{12}\text{CO}_2$ -air, with

$^{12}\text{CO}_2$ diffusing faster in air than the heavier isotopic species. Examples of equilibrium isotope effects are the fractionation between CO_2 in air and in solution, and the effect associated with hydration of CO_2 to HCO_3^- . It is important to note that in a multistep fractionation sequence, equilibrium effects are additive while kinetic effects are not (O'Leary, 1993). A detailed discussion of isotope effects can be found in Farquhar et al. (1989a).

C. Carbon Isotope Discrimination

Isotope composition is not always a convenient way for expressing results. Indeed, Farquhar and Richards (1984) proposed for convenience the use of carbon isotope discrimination (Δ) defined as the deviation of isotope effects α from unity, because the isotope effect usually exceeds unity by a small number. Hence, it is more convenient to express the results as

$$\Delta = \alpha - 1. \quad (4)$$

In the case of plant processes and photosynthesis the source is represented by air CO_2 with isotope abundance ratio R_s , while the product is plant material or photosynthetic products with isotope ratio R_p . Hence, from Eq. (3)

$$\Delta = R_s/R_p - 1. \quad (5)$$

The value of Δ is calculated from the measured carbon isotope compositions of air CO_2 (δ_a) and of plant material (δ_p). Therefore, using Eq. (5) and the definition of Eq. (2) for air CO_2 ($\delta_a = R_s/R_s - 1$) and for plant material ($\delta_p = R_p/R_s - 1$), carbon isotope discrimination is given by

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p} \quad (6)$$

In plants, carbon isotope composition is a negative value, while discrimination is generally positive. That is, plants generally discriminate against ^{13}C . The value of Δ is independent of the isotope composition of the standard used in measurements of isotope composition. Furthermore, Δ has also the advantage of being independent of the source air CO_2 composition, and it is a measure of the intrinsic isotopic fractionation by plants. This is especially relevant for comparing experiments where the isotope compositions of the air CO_2 differ, such as with free

atmospheric CO_2 and, by contrast, industrial CO_2 produced from fuel combustion. It has been pointed out (O'Leary, 1981) that the simultaneous use of isotope composition and discrimination may be confusing because of the opposite sign. Hence, as previously suggested (Farquhar et al., 1989a), it is always preferable that results are expressed as discrimination Δ . In cases where the atmospheric CO_2 composition is not known, the use of δ_p is acceptable. However, if the uncertainty in δ_a is small, it may be less confusing to state an assumed value for δ_a and express the results in terms of Δ .

It is noteworthy that both δ and Δ are dimensionless. For both isotopic composition and discrimination, results are expressed for numerical convenience as the value times 10^{-3} or \% (per mil). For example a Δ value of 0.025 is usually presented as 25×10^{-3} or 25\% . However, these notations are not strictly units as often mistakenly stated in the literature. Nevertheless, in certain applications involving the global carbon cycle, for example, it is convenient to work with the product of a flux and either an abundance or a discrimination, for which the notation, say, of $\text{Gt } \text{\% a}^{-1}$ is convenient.

D. Instantaneous 'On Line' Measurements of Δ During Photosynthesis

Since the photosynthetic process generally discriminates against $^{13}\text{CO}_2$, when the source CO_2 for a plant or a leaf is not an unlimited reservoir, there also will be an effect of this fractionation process on the CO_2 in the air. Therefore, the CO_2 remaining after photosynthetic CO_2 uptake by the leaf will be enriched in the heavier isotope. Hence, if the isotope composition of the air is measured before and after this enrichment has occurred, it is possible to calculate the discrimination during photosynthesis.

This principle can be used either in a closed or in an open system. In the former method, a plant or a leaf is enclosed in a closed container with a given amount of CO_2 and known δ_a . The plant is allowed to take up CO_2 for a short period and then the new isotope composition of the remaining CO_2 is measured. The earliest application of this method was reported by O'Leary et al. (1986), and the fractionation during photosynthesis was calculated solving a series of equations. The open system has been more extensively applied, using open gas exchange systems and well-stirred leaf chambers, as it overcomes the problem of continuous changes in

CO_2 concentration and humidity. In this case, the isotope composition of the air is measured before (δ_o) and after (δ_e) it passes over the leaf. From the measured concentrations of CO_2 entering (c_e) and leaving (c_o) the leaf chamber, is therefore possible to calculate 'on-line' Δ as (Evans et al., 1986)

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)}, \quad (7)$$

where $\xi = c_e/(c_e - c_o)$. This method has been extensively used and tested in a large number of species with different photosynthetic pathways. An expression for on-line Δ accounting for CO_2 respired and photorespired and subsequently refixed has been used by Gillon and Griffiths (1997).

Measurement of Δ on-line gives an instantaneous estimate of all the fractionation processes associated with net CO_2 uptake and has the advantage of being non-destructive. Hence, discrimination in an individual leaf can be measured several times and in different conditions to estimate the effects of environmental factors.

E. Carbon isotope Composition of Source CO_2

The carbon source for the terrestrial photosynthetic process is the CO_2 of the surrounding air. This CO_2 after several physical and chemical processes is carboxylated into photosynthetic products. In the case of field grown plants, the source CO_2 is represented by atmospheric CO_2 , after possible changes due to variability in turbulent mixing within the canopy boundary layer. The isotope composition of atmospheric CO_2 at present is close to $-8\text{\textperthousand}$, with slight variations depending on the measuring stations and the time of the year (Francey et al., 1995; Keeling et al., 1995). Carbon isotope composition of atmospheric CO_2 is closely correlated with the CO_2 concentration of the atmosphere (Fig. 1), although climatic events such as El Niño/Southern Oscillation may induce changes in CO_2 concentration not reflected in the isotope record (Francey et al., 1995). As already pointed out above, atmospheric CO_2 concentration and isotope composition are subjected to variation, both seasonally and with latitude. Seasonal variation is mostly attributable to variation in the balance between photosynthesis and respiration. The amplitude of such variation in δ_a can be as wide as 1\textperthousand , and is dependent on latitude, being greatest at

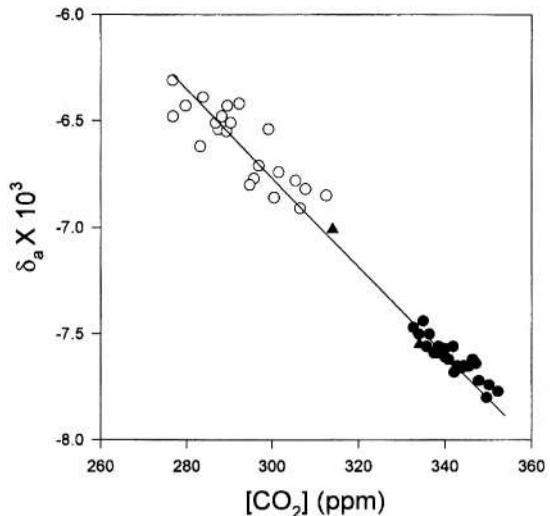


Fig. 1. Relationship between the carbon isotope composition (δ_a) and the concentration of atmospheric CO_2 . Data from direct measurements (closed symbols) are from Roeloffzen et al. (1991, circles, squares) and from Keeling et al. (1979, triangles). Open circles represent data from ice core measurements (Friedli et al., 1986).

northern latitude. There is a decreasing trend of fluctuation in δ_a going from the measurement station at Point Barrow (71°N), to those at Mauna Loa (21°N), Samoa (14°S), Cape Grim (41°S), and South Pole (90°S), in that order (Francey et al., 1995). There is also a gradient in the average δ_a with latitude, with most negative values occurring at northern latitudes, where the majority of combustion of fossil fuel occurs.

There is also a long-term decline in δ_a associated with the increasing CO_2 concentration of the atmosphere due to fossil fuel burning and deforestation. This has been demonstrated (Fig. 1) from long term measurements of concentration and isotope composition (Keeling et al., 1979; Roeloffzen et al., 1991) and from ice core records (Friedli et al., 1986). The isotope composition of the atmosphere was about $-6.4\text{\textperthousand}$ before the industrial revolution and has gradually become more depleted in ^{13}C because of human activities. Reconstruction of past isotope composition of the atmosphere has also been performed using the isotope composition of C_4 plants (Marino and McElroy, 1991; Marino et al., 1992), based on the assumption that discrimination in these plants is relatively constant across a range of environmental conditions. As discussed later, this assumption is not always valid.

Because of spatial and temporal variations in the

isotopic composition of atmospheric CO_2 , it is sometimes important to measure the actual δ_a in each experimental condition. This is especially relevant in experiments where plants are grown in artificial atmospheres, such as in growth chambers, open top chambers and experiments under free atmosphere CO_2 enrichment (FACE). In these conditions, the isotope composition is usually dependent on the relative mixing proportion of atmospheric CO_2 and tank CO_2 , which can be derived from different sources such as fossil fuel combustion or gas from natural CO_2 springs having quite different isotopic composition (Panichi and Tongiorgi, 1975; Gleason and Kyser, 1984). Estimates of exact source isotope composition are also important in the study of long-lived organisms, herbarium specimens and tree rings (Tans and Mook, 1980; Peñuelas and Azcón-Bieto, 1992; Maguas and Brugnoli, 1996).

Relevant variations in δ_a can also occur in the proximity of vegetation. Within or above plant canopies the isotopic composition can change substantially, particularly when turbulent transfer is poor, as a consequence of photosynthetic activity and plant and soil respiration. Photosynthetic fractionation tends to increase the content in ^{13}C of the air within the canopy. On the other hand, respiration and decomposition of organic material release CO_2 strongly depleted in ^{13}C . These gradients in δ_a are inversely related to gradients in CO_2 concentration. Measurements of such gradients have been used to study and to model fluxes of CO_2 within and above canopies and at the global level (Ciais et al., 1995; Flanagan et al., 1996; Lloyd et al., 1996; Yakir and Wang, 1996; Buchmann et al., 1997)

The value of δ_a can be measured directly from CO_2 cryogenically purified and injected into the mass spectrometer. As an alternative, it has been proposed (Beerling and Woodward, 1995; Picon et al., 1997) to assess δ_a from the isotope composition of C_4 plants growing in the same environment, based on the method of Marino and McElroy (1991). However, this application should be used with extreme caution since C_4 plants can show large variation in Δ in response to changes in environmental conditions (Henderson et al., 1992; Buchmann et al., 1996).

Isotope composition of source CO_2 can be also changed artificially for conducting labeling studies of plant metabolism (Osmond et al., 1988; Sharkey et al., 1991; Deléens et al., 1994; Loreto et al., 1996). While the use of pure isotopes is restricted to short-term studies because of the high cost, it is relatively

easy to find commercial CO_2 tanks with widely different δ_a . Therefore, switching from a certain composition to another can allow the labeling patterns to be followed in different metabolites (Schnyder, 1992).

IV. Photosynthetic Fractionation of Carbon Isotopes

Plants are generally depleted in ^{13}C compared to the source atmospheric CO_2 , with limited exceptions. Fractionation occurs during diffusion, with $^{13}\text{CO}_2$ diffusing slower than $^{12}\text{CO}_2$ in air, the binary diffusivities being inversely proportional to the square root of the reduced masses of CO_2 and air (Mason and Marrero, 1970). In addition, there are substantial fractionations associated with enzymatic reactions catalyzing carboxylation and decarboxylation. The kinetic isotope effects associated with carboxylations show a wide range, depending on the enzyme involved and thus on the photosynthetic pathway. Indeed, the largest variation in δ_p occurs among plants with different photosynthetic pathway, with differences in Δ around 14–15‰ between C_3 and C_4 plants. This large difference is due to the fact that ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and phosphoenolpyruvate (PEP) carboxylase, the primary carboxylating enzymes in C_3 and C_4 plants, respectively, show different intrinsic kinetic isotope effects. Furthermore, while the substrate for Rubisco is gaseous CO_2 , that for PEP carboxylase is HCO_3^- , with a significantly different isotope composition because of isotope effects during hydration of CO_2 to HCO_3^- . Plants exhibiting CAM metabolism show largely variable isotope composition, depending on the proportion of total carbon fixed by conventional C_3 photosynthesis during the day.

Variations are more pronounced among C_3 plants, than among C_4 species. The difference in behavior among plants with different CO_2 fixation pathways is specifically linked to the kinetic properties of the primary carboxylating enzymes involved and to consequent differences in the dependency of Δ on diffusive components, while variation within species is attributable to genetic and environmental effects. Hence, within a certain range of the ratio of intercellular and atmospheric partial pressures of CO_2 , C_3 species show higher variability in Δ than C_4 species.

A. Discrimination During C₃ Photosynthesis

Carbon isotope discrimination in C₃ species is determined by several fractionation processes occurring during diffusion of gaseous CO₂ from the free atmosphere, through boundary layers (canopy and leaf boundary layers) and through stomata, to the intercellular air spaces. Subsequently, fractionation occurs during dissolution and liquid phase diffusion to the sites of carboxylation, and during carboxylation itself. Various kinetic and equilibrium isotope effects are involved in these steps. Several authors have developed mathematical models describing the fractionation associated with C₃ photosynthesis (Vogel, 1980; O'Leary, 1981; Farquhar et al., 1982a). These models are similar in concept and structure and divide the fractionation associated into diffusive and carboxylation components. The diffusion processes are always reversible to some extent, while the carboxylation step is irreversible. Hence, carbon isotope discrimination can take place only before the carboxylation reaction occurs. Carbon isotope discrimination is conveniently described by the model of Farquhar et al. (1982a), which has been extensively tested in various species and experimental conditions. Carbon isotope discrimination is given by

$$\Delta = a_b \frac{p_a - p_i}{p_a} + a \frac{p_s - p_i}{p_a} + (e_s + a_l) \frac{p_i - p_c}{p_a} + b \frac{p_c - \frac{eR_d + f\Gamma^*}{k}}{p_a} \quad (8)$$

where p_a, p_s, p_i and p_c are the CO₂ partial pressures in the free atmosphere, at the leaf surface, in the intercellular air spaces before it enters in solution, and at the sites of carboxylation, in that order; a_b is the discrimination occurring during diffusion in the boundary layer (2.9‰, Farquhar, 1980); a is the fractionation occurring during diffusion in air (4.4‰, Craig, 1954); e_s is the fractionation occurring when CO₂ is dissolved in solution (1.1‰, at 25 °C, Vogel, 1980); a_l is the fractionation occurring during diffusion in the liquid phase (0.7‰, O'Leary, 1984); b is the net discrimination occurring during carboxylations in C₃ plants; e and f are the fractionations possibly occurring during dark respiration (R_d) and photorespiration, respectively; k is the carboxylation efficiency and Γ^* is the CO₂

compensation point in the absence of dark respiration (Brooks and Farquhar, 1985).

The value of b in Eq. (8) is not simply that of discrimination associated with carboxylation by Rubisco, because even in C₃ plants a variable proportion of the carbon is fixed by PEP carboxylase (Nalborczyk, 1978; Farquhar and Richards, 1984) and by carboxylases other than Rubisco (Raven and Farquhar, 1990). These carboxylases would operate in parallel with Rubisco affecting the isotopic composition of the overall C fixed. Anaplerotic CO₂ fixation by PEP carboxylase is the most significant contribution other than Rubisco, to the total carbon budget in C₃ species, but other carboxylases may also account for small amounts of carbon fixed. Raven and Farquhar (1990) indicated that, in addition to Rubisco and PEP carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC), acetyl CoA carboxylase and carbamyl phosphate synthetase (CPS) may also account each for at least 1% of total carbon acquisition in at least some O₂-evolving eukaryotes. Hence, taking into account that β is the relative proportion of carbon fixed by PEP carboxylase, γ is that fixed by CPS and ϵ is that fixed by Acetyl CoA carboxylase, with associated fractionation factors b₁ for Rubisco, b₄ for PEP carboxylase, b₅ for CPS and b₆ for Acetyl CoA carboxylase, then the discrimination b associated with all carboxylation reactions is given by

$$b = b_1(1 - \beta - \gamma - \epsilon) + b_4\beta + b_5\gamma + b_6\epsilon. \quad (9)$$

This equation would allow the study of the effects of different carboxylases on Δ of plants, taking into account the extent of organic acid synthesis and the influence of different nitrogen source for growth (Raven and Farquhar, 1990). Nevertheless, while Eq. (9) can be used for theoretical considerations about the true value of b, determination of the extent of organic acid synthesis as proposed by Raven and Farquhar (1990) would only be valid provided that subsequent decarboxylations have no effect on Δ of such products. However, decarboxylation reactions may have large effects on organic acid Δ (O'Leary et al., 1992, see below), particularly if the C atom added, for example, by PEP carboxylase is subsequently decarboxylated. In such a case, the resulting carbon skeleton would purely show a C₃ type isotopic signature. However, based on the above assumptions, Raven and Farquhar (1990) calculated a decrease in Δ due to these enzyme activities ranging

between 0.24‰ for NH_4^+ assimilation, and 2.80‰ for NO_3^- assimilation and reduction in the shoot with organic acid salt synthesis for acid-base balance and for further free organic acid requirements. Recent studies have shown that variation in isotope composition related to the source of N nutrition are not always in the predicted direction (Martinez-Carrasco et al. 1998, Yin and Raven, 1998). In addition, nitrogen source also induces changes in p_i/p_a with evident complication in the interpretation of results.

Among non-Rubisco carboxylases, the contribution by PEP carboxylase is probably predominant in higher plants. Therefore, if the contribution of carboxylases other than Rubisco and PEP carboxylase is ignored, then Equation (9) can be written in a simpler form as

$$b = b_3(1 - \beta) + b_4\beta = b_3 - (b_3 - b_4)\beta, \quad (10)$$

which is the net discrimination during carboxylations in C_3 plants as proposed by Farquhar and Richards (1984). The isotope effect associated with carboxylation by Rubisco was measured by Roeske and O'Leary (1984). They reported a value of $\alpha = 1.029 \pm 0.001$ with respect to dissolved CO_2 . If this value is corrected for the isotope effect during dissolution of CO_2 , then b_3 is approximately 30‰ compared to gaseous CO_2 (Brugnoli et al. 1988; Farquhar et al. 1989b). The isotope effect associated with Rubisco carboxylation was also found to be pH dependent (Roeske and O'Leary, 1984), being 1.026 at pH 9.0 and 1.030 at pH 7.0. However, it is unlikely that variations in stromal pH induce significant variation in discrimination by the enzyme. Guy et al. (1993) found a value of $b_3 = 30.3\text{‰}$ for spinach enzyme, while the values for *Rhodospirillum rubrum* and *Anacystis nidulans* enzymes ranged between 19.6 and 23.0‰, depending on reaction conditions, and especially on Mg^{2+} concentration. A lower isotope effect for Rubisco from *R. rubrum* was also reported by Roeske and O'Leary (1985). It is probable that the lower values are associated with form II of Rubisco, found in some cyanobacteria (Robinson and Cavanaugh, 1995).

The value of b_4 is a function of temperature, because of the temperature dependence of the equilibrium isotope effect between gaseous CO_2 and HCO_3^- . At 25 °C $b_4 = -5.7\text{‰}$ (Farquhar, 1983). Hence, the value of b is dependent on the amount of **β -carboxylations** and on the difference $b_3 - b_4$, which is approximately 36.0‰. The value of β may be

variable in C_3 plants depending on intrinsic (Nalborczyk, 1978) and environmental factors such as the N source for nutrition (Raven and Farquhar, 1990). An upper limit of $\beta = 0.1$ has been proposed by the latter authors, while an average value of about $\beta = 0.05$ or lower appears realistic for most C_3 plants (Farquhar et al., 1989b). Using the latter value, Eq. (10) would give a value of $b = 28.2\text{‰}$, which is 1.8‰ lower than the discrimination by Rubisco only. Nevertheless, as discussed above, if a proportion of carbon fixed by PEP carboxylase is subsequently decarboxylated, the true value of b would fall between 28.2‰ and 30‰. Further studies are needed on this subject.

Other sources of uncertainty in the determination of Δ are represented by the fractionation processes associated with respiration and photorespiration, the terms e and f of Eq. (8). Direct measurements of e and f are difficult, particularly in the light during photosynthesis. Indirect estimates using on-line Δ indicated that e is not significantly different from zero (von Caemmerer and Evans, 1991). Recently, the possible fractionation associated with dark respiration was measured on mesophyll protoplasts of bean and corn, using different culture solutions containing glucose, fructose and sucrose with known $\delta^{13}\text{C}$ (Lin and Ehleringer, 1997). From the comparison of $\delta^{13}\text{C}$ of the substrate and respired CO_2 it was shown that, at least in isolated protoplasts, there is no significant fractionation during mitochondrial dark respiration. Consistent results have also been reported by Gillon and Griffiths (1997). However, recent experiments on intact bean leaves have shown a significant enrichment in ^{13}C by about 6‰ in the CO_2 respired in the dark compared to leaf sucrose, indicating a substantial fractionation associated with respiration (Duranceau et al., 1999).

The value of f was estimated to be 7‰, measuring the air isotope composition at compensation point (Rooney, 1988). von Caemmerer and Evans (1991) also suggested that f may be non-zero and pointed out that the intrinsic problem in on-line Δ estimates is that a value of $f = 5\text{‰}$ would lead to a measurable effect of only 0.8‰. Recently, Gillon and Griffiths (1997) have used on-line measurements manipulating δ_a either during growth or during Δ measurements to obtain differences in δ between CO_2 respired and photorespired and that of ambient air. They found f to be variable among species, 3.3‰ in wheat and 0.5‰ in bean. However, subsequent measurements by the same authors (J. Gillon and H. Griffiths,

personal communication) gave values nearer to that estimated by Rooney (1988) and more constant across different species. Nevertheless, the fractionation during CO_2 release by plants is not yet well characterized and more quantitative studies are needed.

The formulation of Eq. (8) takes into account, step by step, the different fractionation factors associated with diffusion of CO_2 in the gas phase from still air to the intercellular air spaces, then the isotope effects associated with dissolution and liquid phase diffusion and with carboxylations and those occurring during photorespiration and dark respiration. However, in many studies a simplified version of Eq. (8) is used taking into account that the effects on discrimination associated with CO_2 transfer from the intercellular air spaces to the sites of carboxylation and those associated with photorespiration and dark respiration are often small and can be neglected (Farquhar et al., 1989a; O'Leary 1993). Then the discrimination in C_3 plants is given by

$$\Delta = a + (b - a) \frac{p_i}{p_a} \quad (11)$$

This simplification may also work roughly when the drop in $p\text{CO}_2$ from the intercellular spaces to the sites of carboxylation is not small, provided the source of variation in Δ is stomatal. Eq. (11) is a useful approximation and predicts a linear relationship between Δ and p_i/p_a (Fig. 2). It shows that when p_i/p_a is small, Δ is dominated by diffusional fractionation (i.e. 4.4‰), while when p_i/p_a is large then Δ is dominated by fractionation due to carboxylations (i.e. about 28‰). It is noteworthy that p_i/p_a is determined by the balance between photosynthetic capacity and stomatal conductance, i.e., the balance between the demand and the supply functions for photosynthesis (Farquhar and Sharkey, 1982). Therefore, a low value of p_i/p_a can be either determined by low conductance or by relatively high biochemical photosynthetic capacity, or both.

B. Discrimination During C_4 Photosynthesis

Carbon isotope discrimination during photosynthesis in C_4 plants is more complex, since PEP carboxylase is the primary carboxylating enzyme, and CO_2 , after conversion to HCO_3^- , is fixed through this pathway in mesophyll cells. After the transfer of C_4 compounds to the bundle sheath cells and subsequent decarboxylation, CO_2 enters the C_3 pathway via Rubisco

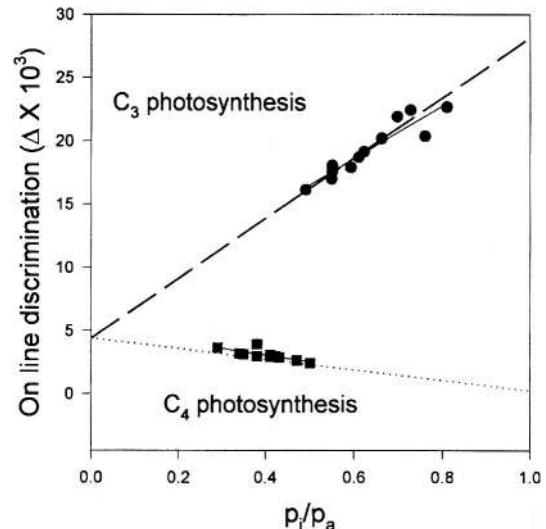


Fig. 2. Relationships between carbon isotope discrimination (Δ) and the ratio of intercellular and atmospheric partial pressures of CO_2 in plants possessing the C_3 or the C_4 photosynthetic pathways. Data on C_3 species are unpublished results on *Oryza sativa* from E. Brugnoli, M. Lauteri and A. Scartazza, while data on C_4 are from Henderson et al. (1992). Solid lines represent regression equations. The dashed line is the C_3 relationship $\Delta = a + (b - a)p_i/p_a$, with $b = 28.2\text{\textperthousand}$. The dotted line represent the relationship for C_4 photosynthesis with $\Delta = a + (b_4 + \phi(b_3 - s)a)p_i/p_a$, with $b_4 = -5.7\text{\textperthousand}$, $\phi = 0.21$. Regression equations: for C_3 $y = 6.1 + 20.8 \times (r = 0.93)$; for C_4 $y = 5.2 - 5.34 \times (r = 0.76)$.

carboxylation. If the bundle sheath cells were absolutely gas tight, all the CO_2 produced from decarboxylation would be subsequently refixed by Rubisco and there would be no opportunity for discrimination in the carboxylation of RuBP. In fact, the system is not perfected since some CO_2 leaks out from bundle sheath and, as a consequence, Rubisco can discriminate against $^{13}\text{CO}_2$. Several models have been developed to describe discrimination during C_4 photosynthesis (Peisker, 1982; Deleens et al., 1983; Farquhar, 1983; Peisker and Henderson, 1992). As in C_3 plants, fractionation also occurs in C_4 leaves as CO_2 diffuses from the atmosphere to the sites of primary carboxylation. After gas phase diffusion, CO_2 enters in solution and is converted to HCO_3^- , which is the substrate for PEP carboxylase. As stated above, the associated fractionation processes are temperature dependent. At equilibrium, during dissolution of CO_2 into water at 25 °C, $^{13}\text{CO}_2$ concentrates in the gas phase by 1.1‰ (Mook et al., 1974, O'Leary, 1984). On the other hand, at equilibrium ^{13}C concentrates in HCO_3^- by 9.0‰ (Mook et al. 1974). Hence the net equilibrium

fractionation during dissolution and hydration of CO_2 at 25°C is $-7.9\text{\textperthousand}$, while $-8.5\text{\textperthousand}$ at 20°C and $-7.4\text{\textperthousand}$ at 30°C (Farquhar, 1983). Discrimination by PEP carboxylase is $2.2\text{\textperthousand}$ against $\text{H}^{13}\text{CO}_3^-$, and therefore the net fractionation with respect to gaseous CO_2 is $b_4 = -5.7\text{\textperthousand}$ (at 25°C), which actually favors ^{13}C . A simple expression for calculating b_4 as function of temperature was presented by Henderson et al. (1992). Discrimination by PEP carboxylase is nearly independent of the phosphorylation state and of pH (Chollet et al., 1996). A further possibility for discrimination is that associated with Rubisco carboxylation (b_3) in bundle sheath cells, because a proportion ϕ of CO_2 released by decarboxylation can actually leak out of the bundle sheath. Finally, discrimination can occur during leakage of CO_2 from bundle sheath. By analogy with the model for C_3 photosynthesis (Eq. (10)), discrimination in C_4 plants (Farquhar, 1983; Henderson et al., 1992) can be written as

$$\Delta = a_b \frac{p_a - p_s}{p_a} + a \frac{p_s - p_i}{p_a} + (e_s + a_l) \frac{p_i - p_m}{p_a} + (b_4 + b_3\phi - s\phi) \frac{p_m}{p_a}, \quad (12)$$

where, p_a , p_s , p_i , and p_m are the CO_2 partial pressures in air, in the boundary layer, in the intercellular air spaces and in the mesophyll cytoplasm (assumed here to be in equilibrium with bicarbonate), in that order; b_4 and b_3 are the fractionations associated with PEP and RuBP carboxylations, respectively, and s is the fractionation during leakage. Assessment of an appropriate value for s is complicated (Farquhar 1983), but Henderson et al. (1992) took the value to be close to the sum ($e_s + a_l$) (i.e., $=1.8\text{\textperthousand}$).

Another model for discrimination during C_4 photosynthesis proposed by Peisker and Henderson (1992), although considering four types of limitations to the overall photosynthesis rate, is conceptually similar to that of Eq. (12). Carboxylations by Rubisco and PEP carboxylase were considered to be limited either by maximum enzyme activities or by maximum substrate regeneration capacities, but these limitations are still described by Eq. (12). The authors used this model to try to interpret the effects of environmental and genetic determinants on discrimination during C_4 photosynthesis.

A simplified version of Eq. (12) ignores the isotope effects due to CO_2 transfer in the boundary layer and during transfer from the intercellular air

spaces to the mesophyll cells, since these effects were thought to be relatively small (Farquhar, 1983; Henderson et al., 1992). Then the discrimination occurring in C_4 photosynthesis is given by

$$\Delta = a + (b + \phi(b_3 - s) - a) \frac{p_i}{p_a}. \quad (13)$$

The slope of the relationship between Δ and p_i/p_a (Fig. 2) is therefore dependent on the relative amount of leakage from bundle sheath cells. Henderson et al. (1992) estimated ϕ in a range of species using this relationship with instantaneous on-line measurements of Δ . They found values of ϕ of about 0.2 for several species, including NAD-ME (malic enzyme) dicots, NADP-ME dicots, and monocots either of the NADP-ME and NAD/PCK (phosphoenolpyruvate carboxykinase) types. The species analyzed by these authors also included two species possessing and four species lacking a suberized lamella in the bundle sheath cells. On the other hand, they found significantly higher values of ϕ ranging from 0.25 to 0.30 for the PCK monocot *Chloris gayana* with suberized lamella, and the NAD-ME monocot *Eleusine coracana*, lacking the suberization of bundle sheath. Hence, it was concluded that leakiness cannot unequivocally be associated with different decarboxylation pathways or with the presence of a suberized lamella in the bundle sheath, probably because leakiness is determined not only by the physical conductance of bundle sheath to CO_2 but also by the relative activities of PEP carboxylase and Rubisco. This report contrasts partially with previous views indicating a causal relationship between the proportion of leakiness, or the quantum yield of CO_2 assimilation, and the presence of a suberized lamella (Ehleringer and Pearcy, 1983; Ohsugi et al., 1988; Farquhar et al., 1989a). Variation in ϕ had been also attributed to different decarboxylation pathways, with NAD-ME showing greater leakage than NADP-ME and PCK type C_4 grasses (Farquhar et al., 1989a). Such variation in ϕ among different decarboxylation pathways was not observed in the work by Henderson et al. (1992). These authors also found that the value of ϕ estimated from on-line Δ measurements differed significantly from that estimated from dry matter Δ , which gave much higher ϕ values ranging between 0.31 to 0.47. The latter range is similar to that found by previous authors (Hattersley, 1982; Farquhar, 1983). The difference between the two estimates was attributed to fractionation occurring after photosynthesis, and possibly during night respiration (Lin and Ehleringer,

1997; Gillon and Griffiths, 1997; Duranceau et al., 1999). Possibly, other fractionation processes occurring during decarboxylation and secondary metabolism may affect these results.

In the foregoing it has been assumed that CO_2 and bicarbonate are in equilibrium in the cytosol. However, this is unlikely to be precisely the case as it would require that the ratio, ρ , of PEP carboxylation to HCO_3^- dehydration (by carbonic anhydrase) be zero. In C_3 plants (Farquhar and Lloyd, 1993; Williams et al., 1996), by analogy, the ratio of RuBP carboxylation to CO_2 hydration is thought to be about 0.05. To take this into account Farquhar (1983) noted that b_4 would need to be replaced by $[b_4 + (1.1 + x - b_4)\rho]$, where x (%) is the fractionation associated with hydration of CO_2 . At that stage, x was unknown, but has since been measured as 1.1% (Paneth and O'Leary, 1985). Thus the above estimates of ϕ need to be reduced by $\rho(1.1 + 1.1 - b_4)/(b_3 - s)$, i.e. by about 0.27 ρ . With ρ of 0.1, say, the reduction would be 0.03, making ϕ about 0.17. However, even after this correction, estimates of ϕ from on-line Δ are higher than those estimated from incorporation of $^{14}\text{CO}_2$ (Hatch et al., 1995) that fall in the range between 0.08 and 0.14 for nine species of Gramineae. To match these findings a value for ρ of about 0.3 would be required. Nevertheless, in the latter report high values of leakiness (0.27) were found in two NADP-malic enzyme-type species of *Flaveria*. Another issue related to carbonic anhydrase (CA) is the value of fractionation during leakage from the bundle sheath (s). The estimate of 1.8% for s assumes there is no efflux of bicarbonate, and may be reasonable if there is no CA in the bundle sheath. However, there would only need to be traces there for s to change its value (Farquhar, 1983; von Caemmerer et al., 1998). Indeed, it could even become negative, which would lead to an overestimate of ϕ when using Eq. (13). Farquhar (1983) did not consider any limitations associated with dissolution of CO_2 in the mesophyll. It would be useful to have estimates of these limitations and of the opposing kinetic fractionations that go together to produce the 1.1% associated with CO_2 dissolution in the mesophyll, in case the assumption of equilibrium is causing errors here.

In other experiments, estimates from instantaneous Δ showed ϕ values of 0.26 and 0.20 for *Zea mays* and *Andropogon glomeratus*, respectively, with significant diurnal changes in ϕ induced by drought and salinity stress (Bowman et al., 1989). Salinity stress induced increases in Δ , attributed to increased ϕ , were also

observed in sugarcane genotypes (Meinzer et al., 1994). These changes in ϕ were attributed to decrease in Rubisco activity relative to the C_4 cycle capacity, rather than to changes in physical characteristics of bundle sheath cells. Similar results were obtained on several sugarcane clones exposed to different irrigation, with drought induced decline in the ratio of Rubisco to PEP carboxylase activity explaining most of the decrease in Δ and ϕ (Saliendra et al., 1996). Genetic variation in Δ attributable either to variation in ϕ or in p_v/p_a or both, was also observed in *Sorghum bicolor* (Hubick et al., 1990). On the contrary, increasing salinity was found to induce a decline in Δ and in the proportion of leakiness in several *Atriplex* species growing along a gradient of soil salinity (Walker and Sinclair, 1992).

In a recent study (Buchmann et al., 1996), large variations in Δ induced by different light and water availability were reported for several C_4 grasses with different decarboxylation pathways. Discrimination was found to increase with decreasing light at PFD lower than $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ and with drought stress. These changes in Δ were explained by dramatic changes in the estimated ϕ , ranging between 0.22 to 0.75. The highest proportion of leakage was calculated for grasses of NAD-ME type, followed by PCK grasses, while those of the NADP-ME showed the lowest ϕ values. Nevertheless, it is possible that the observed variation in Δ was not entirely attributable to increased ϕ but also to changes in p_v/p_a .

Increased Δ and ϕ and reduced photosynthetic efficiency and quantum yield induced by nitrogen stress in sugarcane were accompanied by decreased ratio of Rubisco to PEP carboxylase activities, leading to an imbalance between C_3 and C_4 cycles (Ranjith et al., 1995; Meinzer and Zhu, 1998). In contrast with this observation, decreased Δ , suggesting also a decreased ϕ under low N nutrition, was observed in *Echinochloa frumentacea* (Wong and Osmond, 1991), though p_v/p_a was not measured in this study. Indeed, to estimate changes in ϕ it is absolutely necessary to measure possible variation in p_v/p_a in order to avoid misleading interpretations.

Most of the reported variations in Δ and in ϕ in C_4 plants were explained by variations in Rubisco activity leading to a decrease in the ratio of Rubisco to PEP carboxylase activities. Recently, von Caemmerer et al. (1997) have shown that the reduction of Rubisco content in transgenic *Flaveria bidentis*, with an antisense gene directed against the Rubisco mRNA, caused an increase in Δ mostly mediated by an

increase of bundle sheath CO_2 partial pressure and consequent increase in ϕ . Although the p_i/p_a ratio also increased, ϕ for transgenic plants was 0.37, in comparison with 0.24 in control plants. This increase in ϕ was evident from on-line Δ measurements and from the isotope composition measured directly on bulk leaf material. An imbalance between C_3 and C_4 cycles may be responsible of the increased ϕ , though, a down regulation of the C_4 cycle may also have occurred through a reduction in PEP regeneration rate (von Caemmerer et al., 1997).

In other studies, increasing Δ values from the base to the tip of corn leaves were attributed to an accompanying increase in PEP carboxylase content (Sasakawa et al., 1989), although an increase in Rubisco content from the base to the tip was also found. In sugarcane (Meinzer and Saliendra, 1997) a similar pattern of longitudinal variation in leaf Δ was entirely attributed to variation in p_i/p_a , with ϕ being constant over the leaf. Hence, coordinated variations in Rubisco and PEP carboxylase activities, were correlated with the prevailing light intensity, and did not affect the leakiness from bundle sheath cells. Hence, it is clear that, in addition to anatomical characteristics, the ratio of Rubisco to PEP carboxylase activities controls carbon isotope discrimination and leakiness in C_4 plants. Therefore, variation in the amounts and activities of the enzymes, if coordinated, will not affect ϕ .

C. Discrimination in CAM Plants

Carbon isotope discrimination in plants possessing Crassulacean acid metabolism has been reviewed previously (O'Leary, 1988; Farquhar et al., 1989a; Griffiths, 1992). Modeling carbon isotope discrimination in CAM plants is complicated by the presence of C_3 and C_4 cycles, temporally separated. Furthermore, PEP and RuBP carboxylations may sometimes partially coexist during short periods of the day. Diffusion limitation imposed by partial stomatal closure can have a substantially different impact on fractionation depending when in the CAM cycle it occurs. It may lead to an increased Δ during nocturnal CO_2 fixation, and to a smaller Δ during the C_3 phase, according to the models presented above. During dark CO_2 fixation by PEP carboxylase (phase I, Osmond, 1978), with storage of malate in the vacuole, carbon isotope discrimination would be essentially the same as for C_4 plants, with no leakage (Farquhar, 1983). However, at the end of the dark period, and

beginning of the daytime, there is a shift in carboxylation from PEP carboxylase to Rubisco (phase II). The former enzyme is gradually deactivated by malate efflux from the vacuole and by light, preventing futile cycling of decarboxylation products (Osmond et al., 1988), and the increased CO_2 concentration leads to stomatal closure by the end of phase II. During this phase, discrimination is expected to show values intermediate between those typical for C_3 and C_4 photosynthesis. Subsequently, CO_2 originating from decarboxylation of malic acid is refixed by Rubisco (Phase III), with a process analogous to that occurring in the bundle sheath in C_4 species. Hence, if no efflux of CO_2 occurred with tightly closed stomata, there would be no opportunity foreexpressing the fractionation by Rubisco. However, there is always some leakage of CO_2 , because stomata are not completely closed, and therefore discrimination by Rubisco occurs. Because of this discrimination, the CO_2 lost is expected to be very heavy (O'Leary, 1988). The discrimination in such cases would be $\phi(b_3 - s)$ leading to an enrichment in the CO_2 leaking out by $(b_3 - s)$. It is noteworthy that p_i can be as high as 2–4 kPa (Osmond, 1978, Griffiths, 1992).

In the late afternoon, toward the end of the light period, when all of the malic acid associated with the CAM cycle has been decarboxylated, stomata may re-open. Then CO_2 fixation may occur directly through Rubisco (phase IV, Osmond, 1978). This C_3 activity can contribute substantially to the total carbon gain, especially under optimal conditions. A further complication in quantitative approaches to discrimination in CAM plants is represented by the contribution of respiratory CO_2 refixed, particularly during phase I (Griffiths, 1992). It has been reported that in *Kalanchoe tubiflora*, at least 15% of the total malate was formed from respiratory CO_2 at 15 °C, while this contribution increased to 49% at 25 °C (Kalt et al., 1990). Furthermore, randomization of the isotope signature in malate by fumarase can have significant and variable effects on the isotope ratio of CO_2 decarboxylated (O'Leary, 1988; Osmond et al., 1988; Kalt et al., 1990). Measurements of instantaneous Δ during the various phases of CAM activity in *Tillandsia utriculata* (Griffiths et al., 1990) showed values of Δ ranging between 4.4 and 6.6‰ during phase I. More recently, on-line Δ measurements during phase I in *Clusia minor*, a C_3 -CAM intermediate, indicated values of Δ ranging from -6‰ with wide open stomata, to 4.4‰ with closed

stomata (Roberts et al., 1997). These values are close to the theoretical limits of Δ set for p_i/p_a values of 1 and 0, respectively. Subsequently, as expected, a shift in Δ toward C_3 values has been reported for phase II (Griffiths et al., 1990). During this phase Δ was initially around 10‰, indicating an immediate contribution of Rubisco, which became proportionally more pronounced as CO_2 fixation declined, as indicated by a Δ of about 21‰ at the end of phase II. A surprisingly high discrimination was indeed found in this study during phase III, with a Δ of about 64‰. This would indicate that the CO_2 leaking out of the leaf is highly enriched in ^{13}C (δ of about +52‰). However, this Δ value is much greater than predicted from current knowledge, with the estimated value of leakage of about 0.012, and it is rather difficult to explain. The intrinsic difficulty and inaccuracy in the on-line Δ measurements because of low CO_2 exchange rate during this phase may partly explain these results. Finally, the same authors reported values characteristic of the C_3 pathway during phase IV, because of direct Rubisco contribution to CO_2 uptake. However, recent studies have shown that the relative contribution of Rubisco and PEP carboxylase to the isotope signature of carbon during phase II and IV can vary significantly among CAM and C_3 -CAM intermediates. In the latter a substantial C_4 contribution has been reported during these phases, although also matched by an increased C_3 capacity compared to constitutive CAM (Borland and Griffiths, 1997).

Hence, carbon isotope discrimination can give information on the C fixation pathway being used, showing the relative importance of C_3 photosynthesis and CAM engagement. This is particularly relevant for studying the induction of CAM in C_3 -CAM intermediates (Borland et al. 1994).

Recent experiments with the obligate CAM *Kalanchoe daigremontiana* (Maxwell et al., 1997) have shown that the leaf internal conductance to CO_2 in CAM can be as low as $0.05 \text{ mol m}^{-2} \text{s}^{-1}$, much lower than the lowest limit found in C_3 species (see Chapter 14, Evans and Loreto). This low conductance, measured during C_3 fixation in phase IV, was explained as a consequence of the leaf or stem succulence, considered an adaptation for water conservation and a mean to increase the buffering capacity to store C_4 acids in vacuoles. Hence there would be a tradeoff for CO_2 diffusion and fixation by both PEP carboxylase and Rubisco (phases I, II, IV)

and the succulent mesophyll required for C_4 acid storage (Maxwell et al. 1997).

D. Discrimination in C_3 - C_4 Intermediates

Physical and biochemical factors underlying carbon isotope discrimination in C_3 - C_4 intermediates have been recently studied in detail, and models have been developed describing the discrimination during photosynthesis in these plants (Farquhar et al., 1989a; von Caemmerer, 1989). These aspects have been recently covered by an excellent review (von Caemmerer, 1992).

In C_3 - C_4 intermediates carbon fixation is performed by Rubisco in the mesophyll and in the bundle sheath cells. While the discrimination by Rubisco is expressed in both cell types, the source CO_2 may be rather different. The source CO_2 in the mesophyll cells is comparable to that of C_3 leaves, with effects of diffusion fractionation with respect to CO_2 in external air. In the bundle sheath cells, the source CO_2 is partly derived from decarboxylation of glycine and C_4 compounds, working as a shuttle between mesophyll and bundle sheath cells. In addition, similarly to C_4 photosynthesis, the expression of Rubisco fractionation in the bundle sheath is dependent on the amount of CO_2 leakage, ϕ . Hence, Δ is given by (von Caemmerer, 1992)

$$\Delta = a + (b_3 - a) \frac{p_i}{p_a} + \frac{A_s}{A} \left[(b_3 - s)\phi + (b_4 - b_3) \frac{V_p}{S} \right] \frac{p_i}{p_a}, \quad (14)$$

where, A is the overall net assimilation rate by the leaf, A_s is the assimilation rate in the bundle sheath, V_p is the rate of PEP carboxylations and S is the rate of CO_2 supply to the bundle sheath from the mesophyll and is the sum of glycine and C_4 acid decarboxylations. This equation shows that Δ in C_3 - C_4 intermediate is the sum of Δ in C_3 photosynthesis and a second term which depends on the relative proportion of CO_2 fixation in the bundle sheath, on the proportion of leakiness and on the relative contribution of glycine and C_4 acid decarboxylation in the bundle sheath.

Experimental results have shown that Δ in C_3 - C_4 intermediates is C_3 like (Hattersley et al., 1986; von Caemmerer and Hubick, 1989; Araus et al., 1991)

because only a small proportion of total carbon is fixed in the bundle sheath using the C₄ pathway (von Caemmerer, 1992). In this case, Eq. (14) is equivalent to that introduced by Farquhar et al. (1989a).

E. Discrimination in Lichens and Mosses

Although several authors have studied Δ in lichens and bryophytes, the knowledge of the mechanism of isotope fractionation in these organisms is still scanty. The range of variation in Δ in non-vascular plants is quite large. Variation in Δ in mosses (e.g., Rundel et al., 1979; Teeri, 1981; Proctor et al., 1992) has been attributed partly to the effect of water content. Particularly, variation in surface water films induces changes in the resistance to CO₂ uptake (Rice and Giles, 1994; Williams and Flanagan, 1996). Increasing thickness of such water films would therefore increase the diffusional fractionation component leading to lower Δ (Cowan et al., 1992). Changes in anatomical properties can also affect the isotope composition of mosses. Studies with mosses of the genus *Sphagnum* (Rice and Giles, 1996) showing different anatomical properties, indicated that the variation in Δ due to surface water films was higher than that attributable to difference in anatomy. A mechanistic model of gas exchange in mosses, with conductance to CO₂ being a function of water content, has been developed recently (Williams and Flanagan, 1998).

Large variation in ¹³C/¹²C ratios is also found in lichens. Such changes in Δ have been attributed to the type of photobiont partners and to diffusive resistances to CO₂ diffusion. Lichens with green algae (phycobiont) can assimilate CO₂ in the presence of high air humidity, while those with cyanobacteria require liquid water, and then will have a greater diffusion limitation and lower Δ (Lange et al., 1988). Recently, it has been shown that Δ in lichens falls into three categories: lichens with a phycobiont plus a cyanobacterium limited to structures of the thallus called cephalodia, with Δ around 24–28‰; lichens with a phycobiont alone and lichens with a cyanobiont alone with two distinct ranges of Δ , both around 15‰ (Máguas et al., 1995). Variation in Δ may be due to lower discrimination by Rubisco in cyanobacteria compared to algae (Guy et al. 1993). It has also been demonstrated that cyanobacterial lichens possess a CO₂-concentrating mechanism (CCM) similar to that of free living cyanobacteria, affecting photosynthetic

efficiency and carbon isotope discrimination. The existence of a CCM would therefore cause a marked reduction in Δ of those organisms (Raven et al., 1990; Máguas et al., 1993). The presence of a CCM has been confirmed by gas exchange studies and on-line Δ measurements (Palmquist et al., 1994; Máguas et al., 1995; Smith and Griffiths, 1996), and it has been shown to be related to the presence of chloroplast pyrenoids, structures highly enriched in Rubisco, present in some microalgae (Smith and Griffiths, 1996). In addition to the effects of the CCM, thallus structure, morphology and water content have also been shown to play a significant role in determining differences in CO₂ diffusion resistance and consequent changes in Δ in lichens (Máguas et al., 1995; Máguas and Brugnoli, 1996). Interestingly, differences in CO₂ diffusion resistance can cause variation in Δ within the same thallus (Máguas and Brugnoli 1996; Máguas et al. 1997).

Notwithstanding the increasing interest in this subject, the mechanisms determining Δ in lichens are still debated. More information is needed on the contribution of CCM activity and of gas diffusion limitation on fractionation, since both processes will invariably cause a decrease in the observed Δ .

F. Discrimination in Aquatic Plants and Algae

Carbon isotope composition in plants in the aquatic environment is extremely variable, with values of δ between -40 and -50‰. These large variations reflect changes in the carbon source for photosynthesis, plant physiological and ecological features, as well as environmental changes such as temperature, pH, salinity and substrate concentration. Despite the large variations in discrimination, most plants of the aquatic habitat possess the C₃ photosynthetic pathway, with very limited exceptions (Descolas-Gros and Fontugne, 1990; Farquhar et al., 1989a). Differences in isotope fractionation among these organisms are also caused by the presence or absence of a CCM (Yoshioka, 1997), as discussed for lichens. Carbon isotope discrimination in aquatic organism has been the object of recent reviews (Descolas-Gros and Fontugne, 1990; Keeley and Sandquist, 1992; Raven, 1992; Fry, 1996) and will be not addressed in further details here.

V. Variation in Isotope Composition Within the Plant

A. Fractionation of Carbon Isotopes in Different Metabolites

Carbon isotope composition can vary substantially among different metabolites and in different organs within the plant. Such differences should be taken into account when analyzing Δ either in the bulk dry matter or in individual metabolites, to study, for example, the diffusional limitation of photosynthesis and variation in the p_i/p_s ratio. In such cases, variation in Δ due to different organs or chemical composition could be erroneously attributed to variation in p_i/p_s . While the nature of the differences in isotope composition among some plant metabolites has been elucidated in detail, other differences are not clearly understood.

Differences among plant metabolites are found in all plant species, with different photosynthetic pathways. Figure 3 shows the pattern of variation of Δ in various metabolites in comparison with that in the bulk dry matter, in C₃ species.

One of the most striking differences in isotope composition is found in lipids, which are significantly depleted (5–10‰) in ¹³C compared to bulk plant material. Such differences were first observed in the pioneering work by Park and Epstein (1960). It is now well established that the ¹³C depletion of fatty acids, and lipids generally, is due to secondary fractionation associated with decarboxylation of pyruvic acid by pyruvate dehydrogenase (De Niro and Epstein, 1977; O'Leary, 1981; Melzer and Schmidt, 1987). Similarly, fractionation associated with formation of acetyl-CoA by pyruvate dehydrogenase is responsible for the high Δ found in isoprenoids like **β -carotene** and isoprene (Sharkey et al., 1991). A slight difference in Δ among isoprenoids and fatty acids (Fig. 3) was attributed to the fact that only the carbonyl carbon of acetyl-CoA was depleted in the heavier isotope (Sharkey et al., 1991).

Other relevant differences in Δ among metabolites are found in *amino acids*, showing an extremely wide range of variation among individual amino acids; Values of Δ ranging between about 6‰ for aspartic acid and serine to about 23‰ for leucine were reported by Abelson and Hoering (1961). Different fractionation processes cause such variation. Anaplerotic CO₂fixation by PEP carboxylase is responsible for the enrichment in ¹³C of aspartic acid

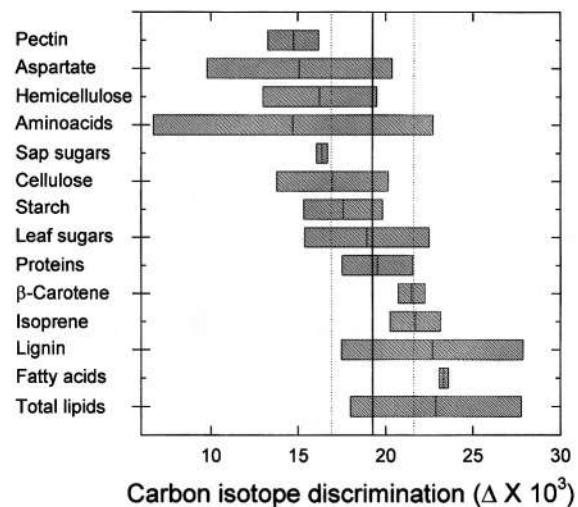


Fig. 3. Carbon isotope discrimination in different plant compounds and metabolites in C₃ species. The boxes in the plot represent the range of variation found in the literature for each compound. The dotted lines represent Δ for the bulk leaf material with the relative range of variation. Where needed, Δ was calculated using the value of δ_{a} for the corresponding year (Keeling et al., 1979; Friedli et al., 1986). Data are from: Abelson and Hoering (1961), Deines (1980), Galimov (1985), Leavitt and Long (1985), Melzer and O'Leary (1987), Brugnoli et al. (1988), Rossmann et al. (1990), Rossmann et al. (1991), Sharkey et al. (1991), Melzer and O'Leary (1991), Gleixner et al. (1993), Goñi and Eglinton (1996), Schmidt and Gleixner (1997), E. Brugnoli, O. Björkman, M. Lauteri and A. Scartazza (unpublished).

(Melzer and O'Leary, 1987, 1991). This is also the reason for the low Δ of organic acids compared to bulk material in C₃ plants (Raven and Farquhar, 1990; Brugnoli et al., 1997).

In general, secondary plant products show higher Δ than primary products (Schmidt and Gleixner, 1997). It is known that lignin, a secondary product, is strongly depleted in ¹³C compared to cellulose synthesized from primary photosynthetic products (Galimov, 1985; Gleixner et al., 1993). It has been demonstrated that differences among primary and secondary products can be attributed mainly to the isotope effects associated with fructose-1,6-bisphosphate aldolase reaction (Gleixner et al., 1993; Gleixner and Schmidt, 1997) and possibly to the triose phosphate isomerase reaction (Brugnoli et al., 1988). Differences may also arise due to fractionation during transport of metabolites.

A similar explanation can be found for the difference in Δ between sucrose and starch found in several plant species (Deléens-Provent and Schwobel-Dugué, 1987; Brugnoli et al., 1988; Gleixner et al.,

1993), with starch being enriched in ^{13}C compared to sucrose (Fig. 3). There could be an equilibrium isotope effect at triose phosphate, with lighter monomers being preferentially exported from the chloroplast to the cytosol. Hence, sucrose would be more depleted in ^{13}C than starch accumulated in the chloroplast (Brugnoli et al., 1988). Similarly, there is an equilibrium isotope effect on the aldolase reaction (Gleixner et al., 1993), which may explain the difference between starch and sucrose. In addition, it has been recently demonstrated (Gleixner and Schmidt, 1997) that fractionation during the aldolase reaction is the reason for the non-statistical isotope distribution in the glucose molecule, with a relative enrichment in ^{13}C in positions C-3 and C-4 compared to C-1 and C-6 positions, previously reported (Ivlev et al., 1987; Rossmann et al., 1991).

Other decarboxylation reactions also involve fractionation processes in secondary metabolism, and are responsible for the observed differences in Δ in plant metabolites. Such reactions include decarboxylation of glutamate, arginine, acetoacetate, isocitrate, histidine, aspartate and malate with associated fractionation factors quite variable between $-1.1\text{\textperthousand}$ (isocitrate) and $31\text{\textperthousand}$ (histidine, O'Leary et al., 1992).

In addition to variation in the isotopic signature of different metabolites, a difference between primary and secondary carbohydrates has been observed. For example it has been found that storage carbohydrate such as sucrose extracted from sugar beet roots (Gleixner et al., 1993) or from rice internodes (A. Scartazza and E. Brugnoli, unpublished) are less discriminated than primary sucrose extracted from leaves. These differences could be attributed to fractionation processes during transport or to fractionation during enzymatic reactions, as discussed above.

The isotope composition of sucrose and starch extracted and purified from leaves should reflect the discrimination of initial C_3 products, because the purification removes the products of PEP carboxylase, such as organic acid and amino acids. Hence, the Δ of sucrose and starch should be higher than the discrimination of total CO_2 uptake by an offset of $\beta(b_3 - b_4)$, with the same slope of the relationship between Δ and p_i/p_s , as discussed by Brugnoli et al. (1988, 1997). However, this expected offset was not observed in several experimental situations, indicating indeed that Δ in sucrose is not significantly different from Δ measured on-line (Brugnoli et al.,

1988, 1997; A. Scartazza and E. Brugnoli, unpublished). These results may be explained by counteracting effects of other fractionation processes considered in Eq. (10) but not in the simplified version of Eq. (11) (Brugnoli et al., 1988). Another possibility is that the carbon of initial C_3 products and that of products of β -carboxylation are not independent and are rapidly interconverted and randomized during the course of the day. Also subsequent decarboxylation of products of PEP carboxylations may explain these findings. Such effects would cause the Δ values of the two pool to converge. In the same experiments, Δ of leaf starch was significantly smaller than that of sucrose and on-line Δ , probably because of isotope effects at the equilibrium of triose phosphate or during the aldolase reaction, as discussed above. Certainly, further studies are needed to understand the possible source of fractionation in plant metabolites and carbon pools.

Variation in carbon isotope composition among biochemical fractions of CAM or C_3 -CAM intermediate plants have been studied by Deléens and Garnier-Dardart (1977), O'Leary and Osmond (1980), Borland et al. (1994). These variation is complicated by variable contributions of C_3 photosynthesis to individual metabolites, in addition to possible further fractionation processes, occurring after carboxylations. These variations have been reviewed previously (O'Leary, 1988, Griffiths, 1992).

B. Δ Variation in Different Anatomical Compartments

Variation in isotope composition may also occur among different plant organs and compartments. The earliest report of variation among plant parts concerned differences in δ among leaves and wood of several tree species (Craig, 1953). Subsequently, differences in Δ among organs were reported for a wide range of species. Table 1 summarizes some examples of studies showing differences in Δ among different anatomical parts. It is evident that there is a general trend toward an enrichment in ^{13}C in certain organs such as seeds, in comparison with leaves. Different Δ values between seeds and leaves were observed in a large range of species, including C_3 and C_4 species, monocot and dicot crop species. Such differences are rather variable ranging from $0.5\text{\textperthousand}$ in sunflower to about 5\textperthousand in wheat. Carbon isotope discrimination in stems is much closer to that in leaves, although in several cases stem Δ is slightly

Table I. Carbon isotope discrimination in different plant organs in several plant species. Where available, the mean \pm SE or the range of variation is shown. Values of Δ were calculated using the estimated value of δ_c for the appropriate year (Keeling et al., 1979; Friedli et al., 1986).

Species	Carbon isotope discrimination (‰)							Reference
	Leaves	Stems	Roots	Seeds	Tuber	Wood	Twigs	
Corn	5.2			3.7				Gleixner et al., 1993
Corn	3.8			3.6				Troughton, 1972
Wheat	18.9 \pm 0.6	19.0 \pm 0.6		16.4 \pm 1.0				Farquhar and Richards, 1984
Wheat	20.5 \pm 1.0			15.4 \pm 1.9				Farquhar and Richards, 1984
Wheat	19.8 - 20.05	19.3 - 20.0		17.9 - 18.6				Condon et al., 1992
Rice	20.1 \pm 0.2	19.4 \pm 0.1	19.0 \pm 0.2	18.7 \pm 0.1				Scartazza et al., 1998
Rice	19.6 \pm 0.2	18.7 \pm 0.2	18.5 \pm 0.2	18.3 \pm 0.2				A. Scartazza and E. Brugnoli, unpublished
Barley	17.0 - 21.5	17.2 - 21.5		15.0 - 17.2				Acevedo, 1993
Tomato	19.1	18.3	17.5					Park and Epstein, 1960
Tomato	18.0	17.7	17.6					Park and Epstein, 1960
Sunflower	21.5 \pm 0.1	21.5 \pm 0.1	21.0 \pm 0.1	21.0 \pm 0.1				E. Brugnoli and M. Lauteri, unpublished
Bean	20.6 \pm 0.1	20.4 \pm 0.2		17.6 \pm 0.3				Brugnoli and Lauteri, 1991
Peanut	21.5	21.9	21.1	20.6				Hubick and Farquhar, 1989
Cowpea	19.3 \pm 0.96			16.5 \pm 0.56				Hall et al., 1990
Beet	20.6		19.3					Gleixner et al., 1993
Potato	19.9				18.4			Gleixner et al., 1993
Potato	23.1				20.7			Troughton, 1972
Cotton	18.0 - 21.0			16.0 - 17.8				Brugnoli and Lauteri, 1991
Desert plants	18.7 - 21.0					17.6 - 20.1		Ehleringer et al., 1992
<i>Phragmites australis</i>	20.8 \pm 0.1	20.5 \pm 0.2	18.3 \pm 0.2					M. Lauteri, A. Augusti and E. Brugnoli, unpublished
<i>Picea Abies</i>	19.8 - 21.7		19.0			17.7 - 20.4		Gebauer and Schulze, 1991
<i>Abies lasiocarpa</i>	18.6 - 22.6				17.2 - 21.3			Craig, 1953
<i>Pinus contorta</i>	20.4				20.5			Craig, 1953
Maple	21.1				20.4			Craig, 1953
Willow	19.8				18.4			Craig, 1953

lower than that of leaves (Table 1). On the other hand, root Δ is intermediate between that of leaves and that of seeds, being about 1‰ lower than leaf Δ . A further difference is observed between tuber and leaves in potato, with Δ values 1.5–2.4‰ lower in the former than in the latter organs (Troughton, 1972; Gleixner et al., 1993). Differences between wood and leaves in tree species are less striking, and rather variable.

There are several possible explanations for the differences in Δ observed in various anatomical compartments. One possibility is that fractionation may occur during export, phloem loading and unloading and transport of carbohydrates from photosynthetic to storage organs such as seeds and tubers. These fractionation processes might be related to glycolysis with branching at some metabolite and subsequent equilibrium isotope effects, which in turn favor the transport of one isotope species compared to the other. Different chemical composition of different organs may also be responsible for the differences in Δ . For example, some organs may have a higher lipid content than others, leading to a relative depletion in ^{13}C . Different contents in lignin (lighter) and cellulose (heavier) in the wood may explain the range of variation reported for wood Δ . Similarly, differences in Δ among needles and twigs in *Picea abies* (Gebauer and Schulze, 1991), were attributed to changes in lipid, protein, hemicellulose and cellulose contents. On the other hand, differences in Δ among leaves and photosynthetic twigs in desert plants can be due to differences in diffusion limitation between organs, with twigs showing lower stomatal conductance, and hence lower Δ , than leaves (Farquhar et al., 1989a; Ehleringer et al., 1992). It is known that some succulent plants fix CO_2 by C_3 photosynthesis in leaves while engaging CAM in the stems, leading to a significant shift in Δ .

A further explanation of the differences in Δ among different plant parts is that some plant organs and structures may be formed at different times and developmental stages, when the contribution of diffusion and carboxylation limitation may be significantly different. For example, seeds are formed late during the plant life cycle. Often plants experience more unfavorable conditions in the late stage of development, with frequent stress imposition because of decreased soil water availability and/or increased vapor pressure deficit. This effect has been described for glasshouse- and field-grown wheat, with difference of about 5‰ between early-formed leaves

and grain (Condon et al., 1992, 1993), attributed to drought-induced stomatal closure. Hence, during such stresses p_i/p_a would be lower, and the carbon fixed at this time would be isotopically heavier. Furthermore, aging and senescence could contribute to variation in p_i/p_a and Δ . In addition, variation in other physiological characteristics such as mesophyll conductance to CO_2 diffusion during ontogeny, have been observed in some species (Loreto et al., 1994; Brugnoli et al., 1997), and this may contribute to the observed decrease in Δ during the ontogeny of plants (Scartazza et al., 1998). In addition, possible differences in dark respiration rate and in the resulting fractionation among organs may contribute to variation in Δ across different plant parts.

All the above effects could simultaneously contribute in different ways to variation in Δ of various organs. For example, it is peculiar that the smallest difference between leaf and seed Δ was observed in sunflower (Table 1), where seeds are especially rich in lipids. Hence, the isotope composition of sunflower seed could be the result of two counteracting effects: increased diffusional limitation after flowering tends to decrease the fractionation against ^{13}C , while the high lipid content would tend to increase the Δ value of the bulk seed material.

Regardless of the underlying causes, variations in Δ among metabolites and anatomical compartments should be considered in studies devoted to quantitative analysis of physiological and ecophysiological parameters in plants.

VI. Carbon Isotope Discrimination and the Ratio of Intercellular and Atmospheric Partial Pressures of CO_2

Models of photosynthetic carbon isotope discrimination predict linear relationships between Δ and p_i/p_a . As already discussed, the dependency of Δ on p_i/p_a is maximum in C_3 species, while is least in C_4 species, where it also depends strongly on bundle sheath leakiness.

A. Agreement Between Models and Experimental Results

Several experimental studies have confirmed the linear relationship between Δ and p_i/p_a , especially in C_3 plants (Fig. 2). These have integrated Δ and p_i/p_a ,

over different time scales. Earliest reports showed linear relationships between Δ measured in the plant dry matter and p_i/p_a in several species under a wide range of experimental conditions (Winter, 1981; Farquhar et al., 1982b; Bradford et al., 1983; Downton et al., 1985; Ehleringer et al., 1985; Seemann and Chritchley, 1985; Flanagan and Jefferies, 1989). Although the source isotope composition was not measured in these studies, all the relationships were reasonably close to that predicted from Eq. (11), with values of b ranging between 26‰ and 29‰.

The relationship between Δ and p_i/p_a has been demonstrated also in the short-term using on-line Δ measurements. Evans et al. (1986) showed linear relationships between on-line Δ and p_i/p_a in several C_3 and C_4 species. Subsequently, several authors, using on-line Δ measurements, showed results consistent with the theory, both for C_4 (Bowman et al. 1989; von Caemmerer and Hubick, 1989; Henderson et al., 1992; von Caemmerer et al., 1997) and for C_3 species (Farquhar et al., 1989a,b; Ehleringer et al., 1991, 1992; von Caemmerer and Evans, 1991; Evans et al., 1994). On-line results on C_3 - C_4 intermediate species (von Caemmerer and Hubick, 1989) showed that Δ was similar to that of C_3 species, with significant variations depending on CO_2 concentration and the species. Variation among species was attributed to the presence of a glycine shuttle, either coupled or not with the C_4 pathway.

More recently, several authors confirmed positive relationships between bulk dry matter Δ and p_i/p_a . Reasonably good agreement between the theoretical and the observed relationship was found in studies concerning wheat genotypes (Condon et al., 1990), accessions of *Eucalyptus camaldulensis* (Gibson et al., 1991), cotton exposed to salinity (Brugnoli and Lauteri, 1991), ozone sensitive and resistant populations of Jeffrey pine (Patterson and Rundel, 1993), the desert shrub *Larrea tridentata* (Rundel and Sharifi, 1993) and *Festuca arundinacea* (Johnson, 1993). In another study with several ecotypes of *Arabidopsis thaliana* (Masle et al., 1993), Δ and p_i/p_a were linearly correlated, according to Eq. (11) with b values ranging between 27 and 30‰.

Carbon isotope discrimination in bulk plant or leaf dry matter gives an assimilation-weighted integration of p_i/p_a over the entire period during which the carbon forming that dry matter was fixed. Hence, this integration time can encompass the entire plant lifespan. Conversely, on-line Δ measurements give an instantaneous estimate of physiological events

and fractionation processes associated with net photosynthetic CO_2 uptake. It has been demonstrated (Brugnoli et al., 1988) that carbon isotope discrimination measured in leaf soluble sugars synthesized in a diurnal course correlates with an assimilation weighted average of p_i/p_a over the entire day. The fitted regression equation gave values of $a = 3.9\text{‰}$ and $b = 25.5\text{‰}$, in good agreement with the theoretical prediction, although higher Δ values would be expected for initial C_3 products (Brugnoli et al., 1997, see also Section V above). In the same experiment Δ of leaf starch was significantly smaller than that of sugars, although still strongly correlated with p_i/p_a (Brugnoli et al., 1988). The possible causes of these differences may be further fractionation processes during carbohydrate synthesis, as discussed above.

There are several other cases where the relationship between Δ measured in leaf sugars and p_i/p_a was confirmed. Figure 4 (upper panel) shows the relationships between Δ measured in leaf sugars and p_i/p_a . These results were obtained on cotton plants exposed to well-watered conditions, or to drought or to 200 mM salinity stress, and in rice plants exposed to fully irrigated conditions or to drought. It is shown that Δ in leaf sugars is strongly correlated with changes in p_i/p_a , in agreement with Eq. (11) with a value of $b = 25\text{‰}$. In agreement with this evidence, in a previous experiment with cotton plants exposed to continuous salinity stress, Δ in leaf sugars was correlated with p_i/p_a calculated from spot gas exchange measurements, taken during the same day on which sugars were sampled (Brugnoli and Björkman, 1992). The relationship observed was in reasonable agreement with that predicted from theory. Hence, Δ in leaf soluble sugars is a reliable indicator of changes in p_i/p_a induced by environmental stresses such as drought or salinity, imposed either in the short-term or in the long-term.

B. Complication in the Relationship Between Δ and p_i/p_a

Despite experimental evidence having confirmed the relationship between Δ and p_i/p_a , there are several known complications in the use of the model, especially that described by Eq. (11). As stated above, Eq. (11) does not take into account possible effects of limitation to CO_2 diffusion between the substomatal cavities and the sites of carboxylation, and possible fractionation processes during respiration and

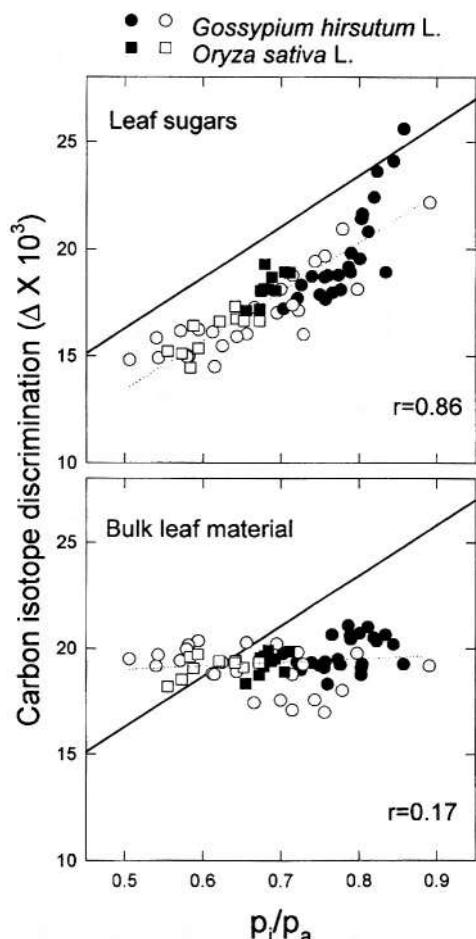


Fig. 4. Relationships between p_i/p_a and carbon isotope discrimination measured in leaf soluble sugars (upper panel) and in the bulk leaf material (lower panel) in cotton (circles) and rice (squares). The p_i/p_a ratio is the average of several (4–8) spot gas exchange measurements taken during the day on fully expanded leaves. Soluble sugars were extracted from the same leaves sampled at the end of the day. Plants grown under optimal conditions (closed symbols) are compared with plants subjected to drought and salinity stress (open symbols). Data from E. Brugnoli and O. Björkman (unpublished) and A. Scartazza and E. Brugnoli (unpublished). The regression equation for soluble sugars is $y = 2.0 + 22.9x$; that for bulk dry matter is $y = 18.2 + 1.7x$. The solid lines represent the theoretical relationship $\Delta = a + (b - a)p_i/p_a$, with $b = 28.2\%$.

photorespiration. When these components are substantial, the observed results will show a deviation from the predicted value. While the effect of dark respiration is thought to be close to zero (but see Duranteau et al., 1999), the fractionation associated with photorespiration may be substantial (von Caemmerer and Evans, 1991; Gillon and Griffiths, 1997). The effect of low internal conductance to CO_2

diffusion, sometimes called mesophyll or wall conductance (g_m), and the consequent drop in CO_2 partial pressure between the substomatal cavities and the chloroplast may be even more important in the estimation of Δ (Evans et al., 1986; Chapter 14, Evans and Loreto). Indeed, most of the deviations between observed results and Δ expected on the basis of Eq. (11), found in numerous reports, are most likely attributable to low mesophyll conductance. Certainly, this can also explain slight deviations in the relationship between Δ and p_i/p_a in Fig. 4 (upper panel) and those reported previously (Brugnoli et al., 1988; Brugnoli and Björkman, 1992; Scartazza et al., 1998). A significant internal diffusion limitation may explain the deviation of the relationship from Eq. (11) observed with gap and understory plants in a tropical forest (Jackson et al., 1993), although underestimation of δ_a could also contribute to this evidence. As discussed above, δ_a in a closed tropical forest canopy can show large variations because of the relative effects of photosynthesis and respiration. Similarly, different values of a and b were reported for *Agropyron desertorum* growing in two different sites (Read et al., 1992). Once again, such differences may be attributed to variation in fractionation processes during liquid phase diffusion and, possibly, to differences in the source CO_2 composition, not measured in this experiment.

Evans et al. (1986) developed a method to estimate mesophyll conductance based on the deviation between measured on-line Δ and that calculated (Δ_i) using Eq. (8) with p_i set equal to p_c at the same p_i/p_a measured from gas exchange. This method is described in detail in Chapter 14 (Evans and Loreto, 1997).

On the basis of on-line Δ analysis it has been shown that g_m can be variable and quite low, particularly in woody species (Evans et al., 1986; von Caemmerer and Evans, 1991; Lloyd et al., 1992). However, the low g_m usually found in woody species is roughly matched by low photosynthetic capacity and by low stomatal conductance, as confirmed by the fact that there is usually a near-linear relationship between g_m and the assimilation rate and, consequently, between g_m and g_s across different species. If this relationship were perfectly linear the drop in CO_2 pressure between p_i and p_c would be constant among herbaceous and woody species (Chapter 14, Evans and Loreto). However, there is significant variation in the slope of the relationship between A and g_m with consequent variation in the drop between

p_i and p_e . This may reflect an adaptive significance of low g_m in woody species.

In the above reports, on-line A measurements were used to determine g_m . More recently, it has been shown that the deviation between Δ and that measured in leaf sucrose can also be used to estimate g_m (Brugnoli et al., 1997; Lauteri et al., 1997; Scartazza et al., 1998). This method has been tested in several C₃ species in different conditions, giving a good agreement with previous reports using the on-line method (see review by Evans and von Caemmerer, 1996). In agreement with previous reports, a linear relationship between assimilation rate and g_m has been observed across different species and under different environmental conditions (Brugnoli et al., 1997).

The estimate of g_m using sugar Δ gives a longer integration time of the physiological features involved, compared to that using on-line A. Several samples can be collected in the same day, for subsequent sugar extraction and mass spectrometric analysis. Hence, this method can be especially useful in ecophysiological applications in the field where on-line measurements are difficult. Certainly the effects of mesophyll conductance should be taken into account in studies on isotope discrimination, and especially in cases where g_m tends to be low relative to stomatal conductance. Further studies are needed to elucidate the ecological significance of g_m in terms of photosynthetic efficiency and water conservation under unfavorable conditions.

C. Other Sources of Variation in the Relationship Between Δ and p_i/p_e

In addition to the complication discussed above, other factors can interfere with measurements of Δ and its relationship with p_i/p_e . One possible problem is due to the fact that measurements of p_i/p_e by gas exchange can be affected by systematic errors. For example, a salinity induced decrease in Δ accompanied by an increase in p_i/p_e was observed in bean plants (Brugnoli and Lauteri, 1991). These results were explained by stress induced heterogeneity of stomatal conductance over the leaf surface, causing an overestimation of p_i/p_e as far as Δ is concerned (Terashima et al., 1988). Heterogeneous stomatal aperture would therefore offset the relationship between p_i/p_e and Δ and/or decrease the significance level for the best fit regression. The effect of heterogeneity of stomatal aperture on the relationship

between Δ and p_i/p_e has been discussed in detail by Farquhar (1989). Briefly, Δ records the assimilation rate-weighted value of p_i/p_e while gas exchange estimates are of the conductance-weighted value. Thus heterogeneity lowers the value of p_i/p_e seen by Δ compared to that estimated by conventional gas exchange.

A further possible source of uncertainty in the estimation of p_i/p_e is given by the existence of a substantial cuticular conductance to water vapor. In conventional gas exchange studies, it is assumed that such conductance is close to zero both for CO₂ and H₂O. However, while the cuticular conductance to CO₂ should be insignificant, the conductance to water vapor may be more important. This has been shown recently in grape leaves (Boyer et al., 1997). Under stress conditions, when stomata are closed, the relative contribution of cuticular conductance to total leaf conductance may be relatively high, causing an overestimation of p_i (Meyer and Genty, 1996). Hence, this would also affect the relationship between Δ and p_i/p_e if the latter were not measured properly.

Differences in chemical composition and in the relative Δ of the material analyzed can also affect the relationship between Δ of bulk material and p_i/p_e . A correlation between Δ and p_i/p_e was shown for cotton plants exposed to salinity (Brugnoli and Lauteri, 1991), although the slope of the relationship was higher for Δ measured in cotton fiber (mostly cellulose) than for that in leaf dry matter. Differences in the relationship between Δ and p_i/p_e were also found among sun and shade leaves of *Coffea arabica* (Gutiérrez and Meinzer, 1994) and attributed to differences in the chemical composition or in the translocation of carbohydrates between sun and shade leaves. Furthermore, as discussed above, different metabolites or organs may show very different Δ because of difference in turnover time and in the time when the relative carbon is fixed. This effect is clearly shown in Fig. 4, where Δ in bulk leaf material (lower panel) is almost unaffected by changes in p_i/p_e induced by salinity or drought, while Δ in leaf sugars (upper panel) is strongly correlated with p_i/p_e , with a value of $b=25\%$, in reasonable agreement with Eq. (11). These results are explained by the fact that when stress is imposed leaf structural carbon is already formed and its Δ is not affected by changes in p_i/p_e , whereas leaf soluble sugars are rapidly turned over and their Δ is strongly affected by short-term changes in p_i/p_e . In a previous experiment with cotton plants exposed to continuous salinity stress, Δ in leaf

sugars was slightly more sensitive to salinity-induced changes in p_i/p_a than was Δ in the insoluble fraction (Brugnoli and Björkman, 1992). In agreement with these results, strong relationships were demonstrated between leaf sugar Δ and p_i/p_a in different sunflower genotypes grown either under well-watered or drought conditions (Lauteri et al., 1993). The slopes of the relationships between leaf sugar Δ and p_i/p_a were always higher than those for Δ in the non-soluble fraction or for bulk leaf material. The value of b for the relationship between soluble sugar Δ and p_i/p_a ranged between 24 to 25.5‰ (Lauteri et al., 1993), indicating that the observed Δ was always lower than that expected on the basis of Eq. (11), possibly because of low (finite) mesophyll conductance.

Recently, Picon et al. (1997) working on the combined effects of elevated CO_2 and drought on *Quercus robur*, reported a drought induced decrease in leaf soluble sugar Δ accompanied by a decrease in the ratio of CO_2 assimilation to stomatal conductance measured from gas exchange. Therefore, these results imply that Δ and p_i/p_a were negatively correlated. These results were in agreement with those obtained previously on bulk leaf material Δ in *Quercus petraea* (Picon et al., 1996). They explained these unexpected results arguing that gas exchange measurements were not representative of the daily integrated assimilation-weighted p_i/p_a , because of possible significant CO_2 fixation early in the morning, at significantly different p_i/p_a (Picon et al. 1997). Certainly, this possibility

may partly explain these results. Nevertheless, possible occurrence of heterogeneity of stomatal aperture and a significant cuticular conductance to water vapor may also contribute to the inverse relationship between Δ and p_i/p_a .

Despite the latter reports, it is now clearly evident that carbon isotope discrimination in leaf soluble sugars is correlated with an assimilation-weighted average value of p_i/p_a over a period of about one-two days. Leaf sugar Δ gives an intermediate integration time between that of bulk dry matter and on-line Δ analyses. Hence, this analysis can be extremely useful in ecophysiological studies to analyse fluctuations in environmental parameters affecting the rate of photosynthesis.

In recent experiments with several rice genotypes, it has been demonstrated that soluble carbohydrates accumulated in the stem, and specially in the uppermost internode and peduncle, have a longer turnover than that of leaf sugars. The analysis of Δ in such sugars was then compared with different integration times of p_i/p_a . Figure 5 shows that while peduncle sugar Δ was correlated with the p_i/p_a value determined during flowering and during grain filling, the best fit regression was obtained with the assimilation-weighted value of p_i/p_a over a period of about 15 days. Hence, the analysis of Δ in sugars extracted from the peduncle gives a longer integration time compared to that of leaf sugars. Differences in Δ among peduncles and other organs such as leaves

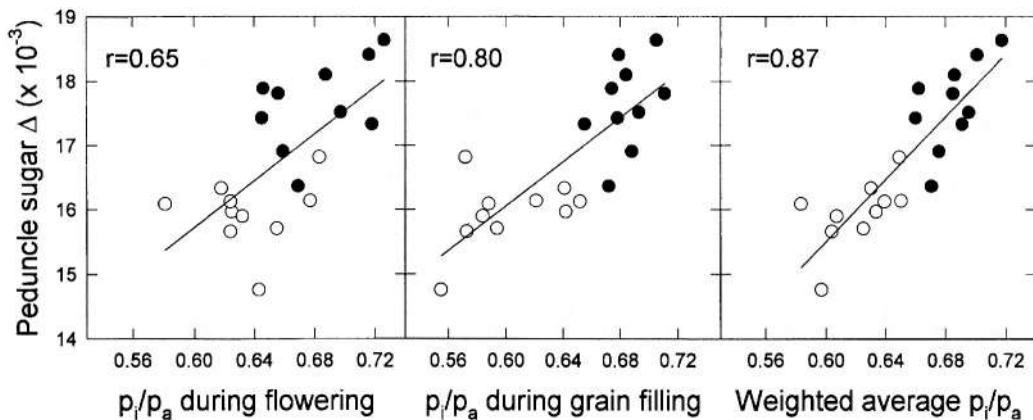


Fig. 5. Correlations between carbon isotope discrimination in *Oryza sativa* peduncle sugars and p_i/p_a measured during flowering, during grain filling, and the assimilation weighted average of p_i/p_a , on a 15 day period from flowering. Measurements of p_i/p_a for flowering and grain filling represent the average of measurements taken during a single day, while the weighted average is an integrated assimilation weighted average over a period of 15 days from flowering to early grain filling stage. Plants subjected to fully irrigated conditions are compared with plants subjected to drought starting from panicle-emergence. Data from A. Scartazza, M. Lauteri and E. Brugnoli (unpublished). Regression equations are: $y = 4.8 + 18.2x$, flowering; $y = 5.7 + 17.2x$, grain filling; $y = 0.91 + 24.32x$, the weighted average over 15 days from flowering to early grain filling.

were previously reported by Condon et al. (1992), but they measured Δ of bulk peduncles rather than soluble sugars from these organs. Therefore, they obtained a relatively longer integration of Δ .

In conclusion, carbon isotope discrimination gives a good estimate of p_i/p_a , provided that other sources of variation and anomalies discussed above are negligible. The ratio of p_i/p_a can be estimated at different increasing integration times, from minutes to the entire plant life cycle, using Δ measured online, on leaf soluble sugars, on storage carbohydrates and on the bulk plant biomass, in that order. The association of all the above measurements can be useful for detecting variations in the photosynthetic performances of plants.

VII. Water Use-Efficiency, Productivity and Δ in C_3 Species

Water use efficiency (WUE), the ratio of biomass produced to water used, is thought to be a relevant parameter in determining crop productivity, at least when water is limiting. According to Passioura (1986), crop yield (Y) is given by

$$Y = WUE \times E \times HI, \quad (15)$$

where E is the water transpired (i.e., a fraction of total evapotranspiration) and HI is the harvest index, the ratio of economical production to total biomass produced. Therefore, breeding for improved WUE may be beneficial for increasing productivity, at least in drought-prone environments. The application of Δ to plant breeding has been previously reviewed by Hall et al. (1994).

On the basis of the positive correlation between Δ and p_i/p_a , carbon isotope discrimination has been proposed as a selection criterion in C_3 species for improving water-use efficiency (Farquhar et al., 1982a; Farquhar and Richards 1984). As discussed above, the p_i/p_a ratio represents the balance between photosynthesis and stomatal conductance to CO_2 . Because CO_2 and H_2O share the same path for gas exchange, through stomata, any increase in g_s will improve photosynthetic CO_2 uptake but will also increase the amount of water loss by transpiration. Indeed, the photosynthetic water-use efficiency (W), also called transpiration efficiency, is negatively related to p_i/p_a . According to Farquhar et al. (1982a), W is given approximately by

$$W = \frac{A}{E} = \frac{p_a \left(1 - \frac{p_i}{p_a} \right)}{1.6(e_i - e_a)}, \quad (16)$$

where A is the rate of net CO_2 assimilation and E is the transpiration rate, e_i and e_a are the water vapor pressures inside the leaf and in the surrounding atmosphere, respectively. The factor 1.6 is the ratio of the binary diffusivity of water vapor in air to that of CO_2 in air.

At the whole plant level, water-use efficiency (WUE) is determined not only by the rates of CO_2 uptake and water loss, but also by respiratory CO_2 release and further water losses not related to photosynthetic activity. A relative amount of carbon Φ_c is lost because of respiration either at night by the whole plant, or during the day by non-photosynthetic organs. In addition, a proportion (Φ_w) of total water loss is independent of CO_2 uptake, such as water lost at night through stomata or by other organs and cuticular transpiration during daytime. Hence, according to Farquhar et al. (1989a), WUE is given by

$$WUE = \frac{A}{E} \frac{(1 - \Phi_c)}{(1 + \Phi_w)}. \quad (17)$$

Combining Eqs. (16) and (17) with Eq. (8) it is evident that a negative relationship is expected between Δ and WUE in C_3 plants, given by

$$WUE = \frac{p_a \left(\frac{b - d - \Delta}{b - a} \right) (1 - \Phi_c)}{1.6(e_i - e_a)(1 + \Phi_w)}, \quad (18)$$

where the term d summarizes all possible fractionation processes associated with liquid phase diffusion, photorespiration and respiration (Farquhar et al., 1989a). In C_4 species, based on the negative relationship between Δ and p_i/p_a , the correlation between Δ and WUE should be positive as confirmed recently in sorghum (Henderson et al., 1998).

Experimental results on several C_3 species (see review by Hall et al., 1994) showed negative correlations between Δ and WUE, in reasonable agreement with theoretical expectations. More recently, similar results have been confirmed on tree species such as *Eucalyptus camaldulensis* (Hubick and Gibson, 1993) and *E. globulus* (Osorio and Pereira, 1994), *Pinus pinaster* and *Quercus petraea*

(Guehl et al., 1994, 1995), *Quercus robur* (Picon et al., 1996) and *Picea glauca* (Sun et al., 1996). In such experiments, negative correlations between Δ and WUE were either genetically or environmentally induced, or both. Environmentally induced increases in WUE, as under drought and other stresses, are expected to be accompanied by decreases in Δ , because under drought, changes in p_i/p_a and WUE will be largely due to stomatal closure.

Genotypic variation in Δ associated with variation in WUE has been explored both in controlled environments and under field conditions. Studies on several species including wheat, barley, rice, bean, peanut, cowpea, alfalfa and sunflower have shown genetic variation in Δ (see review by Hall et al., 1994). Genetic variation in Δ associated with variation in stomatal conductance has also been reported in bean (Ehleringer, 1990), *Gossypium barbadense* (Lu et al., 1996), *Pseudotsuga menziesii* and *Pinus ponderosa* (Zhang and Marshall, 1995). In addition, it has been demonstrated in several species that Δ is under genetic control and heritable, although variable results have been reported for different species and environmental conditions (Hall et al., 1994). Subsequently, it has been shown (Ismail and Hall, 1993) that inheritance of Δ and WUE is nuclear in cowpea, and no maternal effects were observed. No conclusive results were reported in this study about the possible presence of dominance. Low Δ appeared to show partial dominance in a pot experiment, while in the field high Δ was partially dominant. These authors attributed the contrasting evidence to differences in rooting conditions, but in a subsequent study they failed to demonstrate unequivocal correlation between Δ or WUE and xylem ABA concentration (Ismail et al., 1994), which they thought should represent the main hormonal root signal. Certainly many other factors may influence these results.

Genetic control of Δ has been demonstrated studying the association between genetic markers and variation in Δ . Martin et al. (1989) showed that most of the variation in Δ between two tomato species was associated with three restriction fragment length polymorphisms (RFLPs). Subsequent RFLP analysis of Δ in *Arabidopsis thaliana* (Masle et al., 1993) was hindered by genotype x environment interactions. Variation in Δ was related to genetic variation in 21 isozyme loci among Turkish populations of *Castanea sativa* (Villani et al., 1992). The variation in Δ among chestnut populations was mostly attributable to

changes in photosynthetic capacity and mesophyll CO_2 transfer conductance (Lauteri et al., 1997). Quantitative trait loci (QTL) analysis has been also used to study WUE and Δ in trees and crop species (Guehl J.M., personal communication, Mian et al., 1998).

Using wheat-barley disomic chromosome addition lines and wild barley populations, Handley et al. (1994) concluded that chromosome 4 controls at least some of the observed variation in Δ in barley. On the other hand, studies with chromosome substitution lines and dimonotelosomic lines in wheat indicated that all chromosomes were influencing Δ , suggesting that many genes are involved in the control of this trait (Hall et al., 1994; Ehdaie and Waines, 1997). Certainly new molecular techniques becoming available will greatly improve the knowledge about the genetic control of Δ and WUE.

The negative relationships between Δ and WUE reported in various C_3 species and the genetic control of these traits are encouraging for application in breeding programs for increased yield and yield stability, at least under water-limiting conditions. However, early reports showed that Δ may be directly related to biomass production or grain yield in wheat under field conditions (Condon et al., 1987). Similar results were reported for wheat (Ehdaie et al., 1991; Read et al., 1991a; Araus et al., 1993; Sayre et al., 1995), barley (Craufurd et al., 1991; Acevedo, 1993), and other grasses (Johnson et al., 1990). The positive relation between yield in good environments and Δ in C_3 grasses (and negative in the C_4 grass *Zea mays*) was reviewed by Farquhar et al. (1994). On the other hand, negative correlations between Δ and grain yield were reported for lines of wheat near-isogenic for dwarfing genes (Ehdaie and Waines, 1994). Similar results were also reported for the woody shrub *Chrysothamnus nauseosus* where biomass production and Δ were inversely related (Donovan and Ehleringer, 1994b). In other studies, the sign of correlations between Δ and yield or WUE were dependent on environmental conditions (e.g., irrigated or drought, Read et al., 1991b; Ehdaie and Waines, 1994). Similarly, a negative correlation between Δ and tree height has been found in *Picea mariana* on a dry site, whereas no correlation was observed in sites characterized by higher water availability (Flanagan and Johnsen, 1995). In some of the results reported, the correlation between Δ and yield was environmentally induced, while the genotypic correlation within species was non-significant or

with different slopes (Morgan et al., 1993, Johnson and Bassett, 1991).

There are several possible explanation of the conflicting results found in the literature. Under non-limiting water conditions, an improved WUE and low Δ would not be necessarily beneficial (Condon and Richards, 1993). Indeed, when the difference in Δ is mostly due to changes in stomatal conductance, genotypes with high Δ would show high conductance and photosynthesis rates and consequently high yield. Thus, a positive correlation between Δ and grain yield does not imply a positive correlation between Δ and WUE. Indeed positive correlations between Δ and yield accompanied by negative correlations between Δ and WUE have also been observed (Johnson et al., 1990; Brugnoli et al., 1997). Hence, under irrigated conditions WUE will not generally be an essential feature for high or stable yield. On the other hand, when differences in Δ are due to changes in photosynthetic capacity (Hubick et al., 1986), any improvement in WUE is expected to be beneficial, both under irrigated and drought conditions.

A further complication in the correlation between Δ and WUE is represented by the canopy structure, which may influence soil water losses and boundary layer resistance (reviewed by Farquhar et al., 1989a). In addition, the vapor pressure difference between leaf and atmosphere is not an independent variable and may be affected by changes in conductance and in leaf temperature. This may be relevant when comparing both environmental and genetic effects on Δ (Farquhar et al., 1989a; Ehleringer et al., 1992; Comstock and Ehleringer, 1993; Condon et al., 1993; Brugnoli et al., 1997). Further insights in this subject are expected from the study of oxygen isotopes in plants (Farquhar et al., 1997).

Other physiological, morphological and developmental parameters may be directly or indirectly related to Δ , influencing the relationship between Δ and WUE or yield. It has been shown that changes in carbon allocation patterns among roots, leaves and stems and relative growth rate are associated with changes in Δ in sunflower and other species (Farquhar et al., 1989a; Virgona et al., 1990; Virgona and Farquhar, 1996; Scartazza et al., 1998). Increased allocation to root may be beneficial for taking up water from deeper soil layers during drought. Root length density was positively correlated with Δ in bean genotypes (White et al., 1990). A negative correlation has also been reported between Δ and specific leaf area in peanut (Wright et al., 1994). In

addition, it has been shown that early flowering genotypes show higher Δ than late flowering ones (Hall et al., 1994). The correlation between Δ and earliness may represent a disadvantage when selecting drought 'tolerant' genotypes on the basis of Δ , because early flowering is a major drought 'avoidance' mechanism. The correlation is thought not to be causal (Hall et al., 1994).

Often, yield is strongly dependent on water availability during specific developmental stages, such as flowering. At this stage, drought is especially detrimental because it causes flower sterility and consequent yield losses. It is expected that an increased WUE during this stage may be useful for conferring 'drought tolerance'. In several studies on different upland rice genotypes, it has been demonstrated that Δ measured in ethanol soluble sugars (i.e., mainly sucrose) extracted from the upper internode and peduncles sampled 15 days after panicle emergence flowering, was negatively related to grain yield (B. Pinheiro, R.B. Austin, E. Brugnoli, A. Scartazza and M. do Carmo, unpublished). This genetic correlation was found both under irrigated and drought conditions, when drought was imposed at flowering. This correlation is explained by the relationship between Δ in stem sugars and the average of p_i/p_a over a period of about two weeks (Fig. 5), suggesting that this Δ value gives an estimate of WUE during the period of flowering and early grain filling. Hence, low Δ during this stage would imply higher WUE at leaf level, which in turn may be beneficial for flower fertility. On the other hand, a short period of drought at this stage does not affect significantly the Δ value of bulk dry matter, despite possible dramatic effects on grain yield. This may explain why correlations between bulk dry matter Δ and yield were less significant (E. Brugnoli, A. Scartazza, M.C. Monteverdi, M. Lauteri and A. Augusti, unpublished). Hence, the analysis of Δ in internodes and peduncle carbohydrates may be used to assess WUE during crucial periods for fertility and productivity. This method represents a significant improvement with respect to the use of Δ analysis in peduncle dry matter introduced by Condon et al. (1992).

Other possible mechanisms influencing plant WUE and Δ which need to be considered include leaf movements. Leaf rolling may affect plant WUE and Δ in rice (Dingkuhn et al., 1991). Similarly, active paraheliotropic leaf movements in bean may influence the leaf energy balance and temperature, affecting

the relationship between Δ and WUE (Ehleringer et al., 1991). Such mechanisms are expected to increase WUE. In addition, hormone signaling (e.g. ABA) between root and shoot may also play a role in regulating WUE. Nevertheless, the effects of either genetic or environmental variations in ABA content on Δ are not clear (Read et al., 1991a; Ismail et al., 1994; A. Scartazza and E. Brugnoli, unpublished). Further studies should investigate the complex effects of ABA on Δ , yield and drought tolerance.

In spite of the large amount of effort in the study of Δ in relation to water-use efficiency and productivity, our understanding of the complex mechanisms influencing these relationships is still rudimentary. More information is needed about interactions between roots and shoots, with special emphasis on root signals. Furthermore, the study of temporal variation in WUE during special stages of development, such as flowering, deserves more attention. New insight on this subject is expected from the study of carbon isotope discrimination in carbohydrate pools, which can give information on WUE at different integration times.

VIII. Carbon Isotope Discrimination and Physiological Ecology of Photosynthesis

The analysis of plant carbon isotope ratios has become a useful tool for assessing photosynthetic performance and relative water-use efficiency in ecophysiological and ecological studies. Recently this has involved the role of relative changes in photosynthetic capacity and stomatal conductance in the distribution of species, in population dynamics within species, and in adaptation to the environment.

Variations in Δ among plant populations in relation to the area of origin and to environmental parameters such as water availability, temperature, and altitude have been studied. The information available indicates a complex relationship between Δ and adaptation to stress conditions. For example, one would expect that populations of plant adapted to dry conditions have low Δ and high WUE. However, studies conducted with several populations and species grown in common gardens have shown that this may not always be the case. In *Nothofagus* species a negative correlation between Δ and summer rainfall in the area of origin was reported (Read and Farquhar, 1991). These results would imply that species from regions with summer-dry climates have lower WUE.

Other adaptive mechanisms, such as high capacity for water uptake and differential stomatal sensitivity, were invoked to explain these results. Recently, similar results have been demonstrated in chestnut populations grown in a common field, with populations originating from the driest environment showing higher Δ than those collected in relatively wet sites (Lauteri et al., 1997). Similar results were observed in *Faidherbia albida* provenances (Roupsard et al., 1998). In contrast, positive correlations between Δ and mean annual precipitation and seasonality of precipitation were reported for populations of 14 species of *Eucalyptus* (Anderson et al., 1996, but see Brodribb and Hill, 1998). In a different experiment in situ, i.e. combining genetic and direct environmental effects, a positive correlation between Δ and average rainfall was also reported across 348 species growing along a rainfall gradient, corresponding to an aridity gradient, in Southern Queensland (Stewart et al., 1995). A similar positive relationship between Δ and rainfall, but limited to the most arid conditions (rain below 475 mm) has been recently reported among trees along a transect through Northern Australia (Schulze et al., 1998). These results indicate that populations from more arid environment would be more efficient in the use of water. In a comparison of species of the Mediterranean macchia, it has been reported that evergreen species, which rely mostly on rain-water, have lower Δ and higher WUE than deciduous species depending almost exclusively on ground-water (Valentini et al., 1992). Similar results were reported among Larches and sympatric evergreen species (Kloppel et al., 1998).

The results available on this subject reflect different strategies for adaptation and survival in dry environments. It is possible that the best compromise between water conservation and photosynthetic CO_2 assimilation is dependent on the extent and duration of drought periods experienced. The lack of a simple correlation between WUE and growth and survival was also evident in natural shrub populations growing in arid and semi-arid environments (Donovan and Ehleringer, 1994a). In addition, in an elegant experiment Ehleringer (1993) has shown in *Encelia farinosa* populations that tradeoffs between conditions favoring high- or low- Δ genotypes may exist in these desert species. Genotypes with low Δ may be favored under long-term drought or high competitive pressure, while genotypes with high Δ and high photosynthetic rates will benefit most of period with high availability of resources.

Variation in Δ and WUE have been reported in relation to size and age classes in natural populations (Donovan and Ehleringer, 1992). Discrimination is also variable with life forms, with annual species discriminating more than perennials, and grasses discriminating less than forbs (Smedley et al., 1991). Sex-related differences in Δ , have been reported for the dioecious tree species *Acer negundo* (Dawson and Ehleringer, 1993). Male trees showed lower stomatal conductance, photosynthesis rate, p_i , and Δ , compared to female trees. This pattern of variation explains the sex-biased distribution in box elder, with male trees dominating droughted habitats and female trees dominating wet, streamside habitats. This pattern of distribution would likely optimize the pollination efficiency and the cost for reproduction. The opposite pattern was observed in *Phoradendron juniperinum*, a dioecious xylem-tapping mistletoe, with males showing higher photosynthesis rate and lower Δ , and dominating habitat with higher resource availability (Marshall et al., 1993). This may be explained by the different ecological distribution of epiphytes, and a different cost of reproduction in males in this species.

From an evolutionary point of view, it is clear that selective pressure may favor divergent genotypes and photosynthetic behaviors. Plants with low Δ and higher WUE may be favored in dry environments. On the other hand, plants with high Δ and high photosynthetic productivity may be favored under conditions (spatially or temporally distinct) of non-limiting resources. Hence plants with diverging Δ values may coexist in the same environment, increasing species plasticity and likelihood of survival.

IX. Concluding Remarks

Carbon isotope discrimination has contributed significantly in improving our understanding of photosynthesis and water-use efficiency in plants. More information is becoming available on fractionation processes associated with carbon metabolism and especially secondary metabolism. This will also aid in improving carbon isotope discrimination models, taking into account fractionation processes subsequent to photosynthetic carboxylation. Further studies will elucidate fractionation processes during decarboxylations to

understand the fate of carbon originating from β -carboxylations.

Carbon isotope discrimination has been increasingly used in global and ecosystem studies, in order to scale-up the understanding of the regulation of photosynthesis. Certainly, significant advancements are expected from the joint studies of fluxes of $^{18}\text{O}/^{16}\text{O}$ and H/D isotopes in addition to stable carbon isotopes in ecosystem studies. Recent technological advancements will greatly help such studies.

There is also an increasing interest in the study of carbon isotope discrimination in trees. However, there is still a gap of knowledge in this field, which is expected to be overcome in future studies. In particular, the study of mesophyll conductance in these plants deserves more attention for understanding the mechanisms and the significance of low mesophyll conductance.

The use of new molecular approaches is expected to give further insight and new possibilities to study the genetic control of Δ , photosynthetic efficiency and WUE and to assist the use of carbon isotope discrimination in breeding programs for improved crop yield in drought regions.

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References

- Abelson PH and Hoering TC (1961) Carbon isotope fractionation in formation of amino acids by photosynthetic organisms. Proc Natl Acad Sci 47: 623–632
- Acevedo E (1993) Potential of carbon isotope discrimination as a selection criterion in barley breeding. In: Ehleringer JR, Hall AE and Farquhar GD (eds) Stable Isotopes and Plant Carbon-Water Relations, pp 399–417. Academic Press, San Diego
- Anderson JE, Williams J, Kriedemann PE, Austin MP and Farquhar GD (1996) Correlations between carbon isotope discrimination and climate of native habitats for diverse eucalypt taxa growing in a common garden. Aust J Plant Physiol 23: 311–320
- Araus JL, Brown HR, Byrd GT and MD Serret (1991) Comparative effects of growth irradiance on photosynthesis and leaf anatomy of *Flaveria brownii* (C_4 -like) and *Flaveria linearis* ($\text{C}_3\text{-C}_4$) and their F_1 hybrid. Planta 183: 497–504
- Araus JL, Reynolds MP and Acevedo E (1993) Leaf posture,

- grain yield, growth, leaf structure, and carbon isotope discrimination in wheat. *Crop Sci* 33: 1273–1279
- Bakke EL, Beaty DW and Hayes JM (1991) The effect of different ion correction methodologies on $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ results. The Geological Society of America, Annual Meeting, San Diego
- Battle M, Bender M, Sowers T, Tans PP, Butler JH, Elkins JW, Ellis JT, Conway T, Zhang N, Lang P and Clarke AD (1996) Atmospheric gas concentrations over the past century measured in air from firn at the South Pole. *Nature* 383: 231–235
- Bauer JE, Reimers CE, Druffel ERM and Williams PM (1995) Isotopic constraints on carbon exchange between deep ocean sediments and sea water. *Nature* 373: 686–689
- Beerling DJ and Woodward FI (1995) Leaf stable carbon isotope composition records increased water-use efficiency of C_3 plants in response to atmospheric CO_2 enrichment. *Funct Ecol* 9: 394–401
- Bender MM (1968) Mass spectrometric studies of carbon-13 variations in corn and other grasses. *Radiocarbon* 10: 468–472
- Bender MM (1971) Variations in the $^{13}\text{C}/^{12}\text{C}$ ratios of plants in relation to the pathway of photosynthetic carbon dioxide fixation. *Phytochemistry* 10: 1239–1244
- Borland AM and Griffiths H (1997) A comparative study on the regulation of C_3 and C_4 carboxylation processes in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* and the $\text{C}_3\text{-CAM}$ intermediate *Clusia minor*. *Planta* 201: 368–378
- Borland AM, Griffiths H, Broadmeadow MSJ, Fordham MC and Maxwell C (1994) Carbon-isotope composition of biochemical fractions and the regulation of carbon balance in leaves of the C_3 -crassulacean acid metabolism intermediate *Clusia minor* L. growing in Trinidad. *Plant Physiol* 106: 493–501
- Boutton TW (1991) Stable carbon isotope ratios of natural materials: II. Atmospheric, terrestrial, marine, and freshwater environments. In: Coleman DC and Fry B (eds) *Carbon Isotope Techniques*, pp 173–185. Academic Press, San Diego
- Boyer JS, Wong SC and Farquhar GD (1997) CO_2 and water exchange across leaf cuticle (epidermis) at various water potentials. *Plant Physiol* 114: 185–191
- Bowman WD, Hubick KT, von Caemmerer S and Farquhar GD (1989) Short-term changes in leaf carbon isotope discrimination in salt- and water-stressed C_4 grasses. *Plant Physiol* 90: 162–166
- Bradford KJ, Sharkey TD and Farquhar GD (1983) Gas exchange, stomatal behavior, and $\delta^{13}\text{C}$ values of the *flacca* tomato mutant in relation to abscisic acid. *Plant Physiol* 72: 245–250
- Brodrribb T and Hill RS (1998) The photosynthetic drought physiology of a diverse group of Southern hemisphere conifer species is correlated with minimum seasonal rainfall. *Funct Ecol* 12: 465–471
- Brooks A and Farquhar GD (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* 165: 397–406.
- Brugnoli E and Björkman O (1992) Growth of cotton under continuous salinity stress: Influence on allocation pattern, stomatal and non-stomatal components of photosynthesis and dissipation of excess light energy. *Planta* 187: 335–347
- Brugnoli E and Lauteri M (1991) Effects of salinity on stomatal conductance, photosynthetic capacity, and carbon isotope discrimination of salt-tolerant (*Gossypium hirsutum* L.) and salt-sensitive (*Phaseolus vulgaris* L.) C_3 non-halophytes. *Plant Physiol* 95: 628–635
- Brugnoli E, Hubick KT, von Caemmerer S, Wong SC and Farquhar GD (1988) Correlation between the carbon isotope discrimination in leaf starch and sugars of C_3 plants and the ratio of intercellular and atmospheric partial pressures of carbon dioxide. *Plant Physiol* 88: 1418–1424
- Brugnoli E, Scartazza A, Lauteri M, Monteverdi MC and Máguas C (1998) Carbon isotope discrimination in structural and non-structural carbohydrates in relation to productivity and adaptation to unfavourable conditions. In Griffiths H (ed) *Stable Isotopes: Integration of Biological, Ecological and Geochemical Processes*, pp 133–146. BIOS, Oxford
- Buchmann N, Brooks JR, Rapp KD and Ehleringer JR (1996) Carbon isotope composition of C_4 grasses is influenced by light and water supply. *Plant Cell Environ* 19: 392–402
- Buchmann N, Guehl J-M, Barigah TS and Ehleringer JR (1997) Interseasonal comparison of CO_2 concentrations, isotopic composition, and carbon dynamics in an Amazonian rainforest (French Guiana). *Oecologia* 110: 120–131
- Chollet R, Vidal J and O'Leary MH (1996) Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 273–298
- Ciais P, Tans PP, Trolier M, White JWC and Francey RJ (1995) A large northern hemispheric terrestrial CO_2 sink indicated by $^{13}\text{C}/^{12}\text{C}$ ratio of atmospheric CO_2 . *Science* 269: 1098–1102
- Comstock J and Ehleringer J (1993) Stomatal response to humidity in common bean (*Phaseolus vulgaris*): Implications for maximum transpiration rate, water-use efficiency and productivity. *Aust J Plant Physiol* 20: 669–691
- Condon AG and Richards RA (1993) Exploiting genetic variation in transpiration efficiency in wheat: an agronomic view. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 435–450. Academic Press, San Diego
- Condon AG, Richards RA and Farquhar GD (1987) Carbon isotope discrimination is positively correlated with grain yield and dry matter production in field-grown wheat. *Crop Sci* 27: 996–1001
- Condon AG, Farquhar GD and Richards RA (1990) Genotypic variation in carbon isotope discrimination and transpiration efficiency in wheat. Leaf gas exchange and whole plant studies. *Aust J Plant Physiol* 17: 9–22
- Condon AG, Richards RA and Farquhar GD (1992) The effect of variation in soil water availability, vapour pressure deficit and nitrogen nutrition on carbon isotope discrimination in wheat. *Aust J Agric Res* 43: 935–947
- Condon AG, Richards RA and Farquhar GD (1993) Relationships between carbon isotope discrimination, water use efficiency and transpiration efficiency for dryland wheat. *Aust J Agric Res* 44: 1693–1711
- Coplen TB (1995) Discontinuance of SMOW and PDB. *Nature* 375: 285
- Cowan IR, Lange OL and Green TGA (1992) Carbon-dioxide exchange in lichens: Determination of transport and carboxylation characteristics. *Planta* 187: 182–294
- Craig H (1953) The geochemistry of the stable carbon isotopes. *Geochim Cosmochim Acta* 3: 53–92
- Craig H (1954) Carbon 13 in plants and the relationship between carbon 13 and carbon 14 variation in nature. *J Geol* 62: 115–149

- Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochim Cosmochim Acta* 12: 133–149
- Craig H, Chou C, Whelan J, Stevens C and Engelkeimer A (1988) The isotopic composition of methane in polar ice cores. *Science* 242: 1535–1539
- Craufurd PQ, Austin RB, Acevedo E and Hall MA (1991) Carbon isotope discrimination and grain-yield in barley. *Field Crops Res* 27: 301–313
- Dawson TE and Ehleringer JR (1993) Gender-specific physiology, carbon isotope discrimination, and habitat distribution in boxelder, *Acer negundo*. *Ecology* 74: 798–815
- De Niro MJ and Epstein S (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* 197: 261–263
- De Niro MJ and Epstein S (1978) Influence of diet on the distribution of carbon isotopes in animals. *Geochim Cosmochim Acta* 42: 495–506
- Deines P (1980) The isotopic composition of reduced organic carbon. In: Fritz P and Fontes JC (eds) *Handbook of Environmental Isotope Geochemistry*, Vol. I, The Terrestrial Environment, pp 329–406. Elsevier Scientific, Amsterdam
- Deléens E and Garnier-Dardart J (1977) Carbon isotope composition of biochemical fractions isolated from leaves of *Bryophyllum daigremontianum* Berger, a plant with crassulacean acid metabolism: Some physiological aspects related to CO₂ dark fixation. *Planta* 135: 241–248
- Deléens E, Ferhi A and Queiroz O (1983) Carbon isotope fractionation by plants using the C₄ pathway. *Physiol Veg* 21: 897–905
- Deléens E, Cliquet J-B, and Prioul J-L (1994) Use of ¹³C and ¹⁵N plant label near natural abundance for monitoring carbon and nitrogen partitioning. *Aust J Plant Physiol* 21: 133–146
- Deléens-Provent E and Schwebel-Dugué N (1987) Demonstration of autotrophic state and establishment of full typical C₄ pathway in maize seedlings by photosynthate carbon isotope composition. *Plant Physiol Biochem* 25: 567–572
- Descolas-Gros C and Fontugne M (1990) Stable carbon isotope fractionation by marine phytoplankton during photosynthesis. *Plant Cell Environ* 13: 207–218
- Dingkuhn M, Farquhar GD, De Datta SK and O'Toole JC (1991) Discrimination of ¹³C among upland rices having different water use efficiencies. *Aust J Agric Res* 42: 1123–1131
- Donovan LA and Ehleringer JR (1992) Contrasting water-use patterns among size and life-history classes of a semi-arid shrub. *Funct Ecol* 6: 482–488
- Donovan LA and Ehleringer JR (1994a) Carbon isotope discrimination, water-use efficiency, growth, and mortality in a natural shrub population. *Oecologia* 100: 347–354
- Donovan LA and Ehleringer JR (1994b) Potential for selection on plants for water-use efficiency as estimated by carbon isotope discrimination. *Am J Botany* 81: 927–935
- Downton WJS, Grant WJR and Robinson SP (1985) Photosynthetic and stomatal responses of spinach leaves to salt stress. *Plant Physiol* 77: 85–88
- Duranceau M, Ghashghaei J, Badeck F, Deléens E and Cornic G (1999) δ¹³C of CO₂ respired in the dark in relation to δ¹³C of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant Cell Environ*, in press
- Ehdaie B and Waines JG (1994) Growth and transpiration efficiency of near-isogenic lines for height in a spring wheat. *Crop Sci* 34: 1443–1451
- Ehdaie B and Waines JG (1997) Chromosomal location of genes influencing plant characters and evapotranspiration efficiency in bread wheat. *Euphytica* 96: 363–375
- Ehdaie B, Hall AE, Farquhar GD, Nguyen HT and Waines JG (1991) Water-use efficiency and carbon isotope discrimination in wheat. *Crop Sci* 31: 1282–1288
- Ehleringer JR (1990) Correlations between carbon isotope discrimination and leaf conductance to water vapor in common beans. *Plant Physiol* 93: 1422–1425
- Ehleringer JR (1993) Variation in leaf carbon isotope discrimination in *Encelia farinosa*: implications for growth, competition, and drought survival. *Oecologia* 95: 340–346
- Ehleringer JR and Pearcy RW (1983) Variation in quantum yield for CO₂ uptake among C₃ and C₄ plants. *Plant Physiol* 73: 555–559
- Ehleringer JR, Schulze E-D, Ziegler H, Lange OL, Farquhar GD and Cowan JR (1985) Xylem-tapping mistletoes: Water or nutrient parasites? *Science* 227: 1479–1481
- Ehleringer JR, Klassen S, Clayton C, Sherrill D, Fuller-Holbrook M, Fu Q and Cooper TA (1991) Carbon isotope discrimination and transpiration efficiency in common bean. *Crop Sci* 31: 1611–1615
- Ehleringer JR, Phillips SL and Comstock JP (1992) Seasonal variation in the carbon isotopic composition of desert plants. *Funct Ecol* 6: 396–404
- Evans JR and von Caemmerer S (1996) Carbon dioxide diffusion inside leaves. *Plant Physiol* 110: 339–346
- Evans JR, Sharkey TD, Berry JA and Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO₂ diffusion in leaves of higher plants. *Aust J Plant Physiol* 13: 281–292
- Evans JR, von Caemmerer S, Setchell BA and Hudson GS (1994) The relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with reduced content of Rubisco. *Aust J Plant Physiol* 21: 475–495
- Farquhar GD (1980) Carbon isotope discrimination by plants and the ratio of intercellular and atmospheric CO₂ concentrations. In: Pearman GI (ed) *Carbon Dioxide and Climate: Australian Research*, pp 105–110, Australian Academy of Science, Canberra
- Farquhar GD (1983) On the nature of carbon isotope discrimination in C₄ plants. *Aust J Plant Physiol* 10:205–226
- Farquhar GD (1989) Models of integrated photosynthesis of cells and leaves. *Phil Trans R Soc Lond B* 323: 357–367
- Farquhar GD and Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 47–70. Academic Press, San Diego
- Farquhar GD and Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Aust J Plant Physiol* 11: 359–352
- Farquhar GD and Sharkey TD (1982) Stomatal conductance and photosynthesis. *Annu Rev Plant Physiol* 33: 317–345
- Farquhar GD, O'Leary MH and Berry JA (1982a) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust J Plant Physiol* 9: 121–137
- Farquhar GD, Ball MC, von Caemmerer S and Roksandic Z (1982b) Effect of salinity and humidity on δ¹³C value of

- halophytes-Evidence for diffusional isotope fractionation determined by the ratio of intercellular/atmospheric partial pressure of CO_2 under different environmental conditions. *Oecologia* 52: 121–124
- Farquhar GD, Ehleringer JR and Hubick KT (1989a) Carbon isotope discrimination and photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* 40: 503–537
- Farquhar GD, Hubick KT, Condon AG and Richards RA (1989b) Carbon isotope fractionation and water-use efficiency. In: Rundel PW, Ehleringer JR and Nagy KA (eds) *Stable Isotopes in Ecological Research*. Ecological Studies, V 68, pp21–40, Springer-Verlag New York.
- Farquhar GD, Condon AG and Masle J (1994) On the use of carbon and oxygen isotope composition and mineral ash content in breeding for improved rice production under favorable, irrigated conditions. In: Cassman KG (ed) *Breaking the Yield Barrier*. International Rice Research Institute, pp 95–101
- Farquhar GD, Barbour MM and Henry BK (1998) Interpretation of oxygen isotope composition of leaf material. In Griffiths H (ed) *Stable Isotopes: Integration of Biological, Ecological and Geochemical Processes*, pp 27–62. BIOS, Oxford
- Flanagan LB and Jefferies RL (1989) Effect of increased salinity on CO_2 assimilation, O_2 evolution and the $\delta^{13}\text{C}$ values of leaves of *Plantago maritima* L. developed at low and high NaCl levels. *Planta* 178: 377–384
- Flanagan LB and Johnsen KH (1995) Genetic variation in carbon isotope discrimination and its relationship to growth under field conditions in full-sib families of *Picea mariana*. *Can J For Res* 25: 39–47
- Flanagan LB, Brooks JR, Varney GT, Berry SC and Ehleringer JR (1996) Carbon isotope discrimination during photosynthesis and the isotope ratio of respired CO_2 in boreal forest ecosystem. *Global Biogeochemical Cycles* 10: 629–640
- Francey RJ (1985) Cape Grim isotope measurements—A preliminary assessment. *J Atmos Chem* 3: 247–260
- Francey RJ, Tans PP, Allison CE, Enting IG, White JWC and Trolier M (1995) Changes in oceanic and terrestrial carbon uptake since 1982. *Nature* 373:326–330
- Friedli H, Lotscher H, Oeschger H, Siegenthaler U and Stauffer B (1986) Ice core recorded of the $^{13}\text{C}/^{12}\text{C}$ ratio of atmospheric CO_2 in the past two centuries. *Nature* 324: 237–238
- Friedli H, Siegenthaler U, Rauber D., Oeschger H (1987) Measurements of concentration, $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ ratios of tropospheric carbon dioxide over Switzerland. *Tellus* 39B: 80–88
- Fry B (1996) $^{13}\text{C}/^{12}\text{C}$ fractionation by marine diatoms. *Marine Ecology Progress Series* 134: 283–294
- Galimov EM (1985) The biological fractionation of isotopes. Academic Press, New York
- Gearing JN (1991) The study of diet and trophic relationships through natural abundance ^{13}C . In: Coleman DC and Fry B (eds) *Carbon Isotope Techniques*, pp 201–218. Academic Press, San Diego
- Gebauer G and Schulze E-D (1991) Carbon and nitrogen isotope ratios in different compartments of a healthy and declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. *Oecologia* 87: 198–207
- Gibson A, Hubick KT and Bachelard EP (1991) The effects of water stress on the morphology and gas exchange characteristics of *Eucalyptus camaldulensis* seedlings. *Aust J Plant Physiol* 18: 153–163
- Gillon JS and Griffiths H (1997) The influence of (photo)respiration on carbon isotope discrimination in plants. *Plant Cell Environ*, 20: 1217–1230
- Gleason JD and Kyser TK (1984) Stable isotope composition of gases and vegetation near naturally burning coal. *Nature* 307: 254–257
- Gleixner G and Schmidt H-L (1997) Carbon isotope effects on the fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical ^{13}C distributions in carbohydrates. *J Biol Chem* 272: 5382–5387
- Gleixner G, Danier H-J, Werner RA, and Schmidt H-L (1993) Correlations between the ^{13}C content of primary and secondary plant products in different cell compartments and that in decomposing basidiomycetes. *Plant Physiol* 102: 1287–1290
- Goñi MA and Eglinton TI (1996) Stable carbon isotopic analysis of lignin-derived CuO oxidation products by isotope ratio monitoring-gas chromatography-mass spectrometry (irm-GC-MS). *Org Geochem* 24: 601–615
- Griffiths H (1992) Carbon isotope discrimination and the integration of carbon assimilation pathways in terrestrial CAM plants. *Plant Cell Environ* 15: 1051–1062
- Griffiths H, Broadmeadow MSJ, Borland AM and Hetherington CS (1990) Short-term changes in carbon-isotope discrimination identify transitions between C_3 and C_4 carboxylation during crassulacean acid metabolism. *Planta* 181: 604–610
- Guehl J-M, Picon C, Aussenac G and Gross P (1994) Interactive effects of elevated CO_2 and soil drought on growth and transpiration efficiency and its determinants in two European forest tree species. *Tree Physiol* 14: 707–724
- Guehl J-M, Fort C and Ferhi A (1995) Differential response of leaf conductance, carbon isotope discrimination and water-use efficiency to nitrogen deficiency in maritime pine and pedunculate oak plants. *New Phytol* 131: 149–157
- Gutiérrez MV and Meinzer FC (1994) Carbon isotope discrimination and photosynthetic gas exchange in coffee hedgerows during canopy development. *Aust J Plant Physiol* 21: 207–219
- Guy RD, Fogel ML and Berry JA (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. *Plant Physiol* 101: 37–47
- Hall AE, Mutters RG, Hubick KT and Farquhar GD (1990) Genotypic differences in carbon isotope discrimination by cowpea under wet and dry field conditions. *Crop Sci* 30: 300–305
- Hall AE, Richards RA, Condon AG, Wright GC and Farquhar GD (1994) Carbon isotope discrimination and plant breeding. *Plant Breeding Reviews* 4: 81–113
- Handley LL, Nevo E, Raven JA, Martínez-Carrasco R, Scrimgeour CM, Pakniyat H and Forster BP (1994) Chromosome 4 controls potential water use efficiency ($\delta^{13}\text{C}$) in barley. *J Exp Botany* 45: 1661–1663
- Hatch MD and Slack CR (1966) Photosynthesis by sugarcane leaves: A new carboxylation reaction and the pathway of sugar formation. *Biochem J* 101: 103–111
- Hatch MD, Agostino A and Jenkins CLD (1995) Measurements of the leakage of CO_2 from bundle-sheath cells of leaves during C_4 photosynthesis. *Plant Physiol* 108: 173–181
- Hattersley PW (1982) $\delta^{13}\text{C}$ values of C_4 types in grasses. *Aust J Plant Physiol* 9: 139–154
- Hattersley PW, Wong SC, Perry S and Roksandic Z (1986) Comparative ultrastructure and gas exchange characteristics

- of the C₃-C₄ intermediate *Neurachne minor* S.T. Blake (Poaceae). *Plant Cell Environ* 9: 217–233
- Henderson SA, von Caemmerer S and Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C₄ species. *Aust J Plant Physiol* 19: 263–285
- Henderson SA, von Caemmerer S, Farquhar GD, Wade L and Hammer G (1998) Correlation between carbon isotope discrimination and transpiration efficiency in lines of the C₄ species *Sorghum bicolor* in the glasshouse and the field. *Aust J Plant Physiol* 25: 111–123
- Hubick KT and Farquhar GD (1989) Carbon isotope discrimination and the ratio of carbon gain to water lost in barley cultivars. *Plant Cell Environ* 12: 795–804
- Hubick KT and Gibson A (1993) Diversity in the relationship between carbon isotope discrimination and transpiration efficiency when water is limited. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 311–325. Academic Press, San Diego
- Hubick KT, Farquhar GD and Shorter R (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. *Aust J Plant Physiol* 13: 803–816
- Hubick KT, Hammer GL, Farquhar GD, Wade LJ, von Caemmerer S and Henderson SA (1990) Carbon isotope discrimination varies genetically in C₄ species. *Plant Physiol* 91: 534–537
- Ismail AM and Hall AE (1993) Inheritance of carbon isotope discrimination and water-use efficiency in cowpea. *Crop Sci* 33: 498–503
- Ismail AM, Hall AE, and Bray EA (1994) Drought and pot size effects on transpiration efficiency and carbon isotope discrimination of cowpea accessions and hybrids. *Aust J Plant Physiol* 21: 23–35
- Ivlev AA, Apin AV and Brizanova LY (1987) Distribution of carbon isotopes in the glucose of maize starch. *Fiziologica Rastenij* 34: 493–498
- Jackson PC, Meinzer FC, Goldstein, Holbrook NM, Cavelier J and Rada F (1993) Environmental and physiological influences on carbon isotope composition of gap and understory plants in a lowland tropical forest. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 131–140. Academic Press
- Johnson DA, Asay KH, Tieszen LL, Ehleringer JR and Jefferson PG (1990) Carbon isotope discrimination: Potential in screening cool-season grasses for water-limited environments. *Crop Sci* 30: 338–343
- Johnson RC (1993) Carbon isotope discrimination, water relations, and photosynthesis in tall fescue. *Crop Sci* 33: 169–174
- Johnson RC and Bassett LM (1991) Carbon isotope discrimination and water use efficiency in four cool-season grasses. *Crop Sci* 31: 157–162
- Kalt W, Osmond B and Siedow JN (1990) Malate metabolism in the dark after ¹³CO₂ fixation in the crassulacean plant *Kalanchoë tubiflora*. *Plant Physiol* 94: 826–832
- Keeley JE and Sandquist DR (1992) Carbon: Freshwater plants. *Plant Cell Environ* 15: 1201–1035
- Keeling CD, Mook WG and Tans PP (1979) Recent trends in the ¹³C/¹²C ratio of atmospheric carbon dioxide. *Nature* 277: 121–123
- Keeling CD, Whorf TP, Wahlen M and van der Plicht J (1995) Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980. *Nature* 375: 666–670
- Keeling CD, Chin JFS and Whorf TP (1996) Increased activity of northern vegetation inferred from atmospheric CO₂ measurements. *Nature* 382: 146–149
- Khalil MAK and Rasmussen RA (1994) Global decrease in atmospheric carbon monoxide concentration. *Nature* 370: 639–641
- Kloepfel BD, Gower ST, Treichel IW and Kharuk S (1998) Foliar carbon isotope discrimination in *Larix* species and sympatric evergreen conifers: A global comparison. *Oecologia* 114: 153–159
- Kortschak HP, Hartt CE and Burr GO (1965) Carbon dioxide fixation in sugarcane leaves. *Plant Physiol* 40: 209–213
- Lange OL, Green TGA and Ziegler H (1988) Water status related photosynthesis and carbon isotope discrimination in species of the lichen genus *Pseudocyphellaria* with green or blue-green photobionts and in photosymbiodemes. *Oecologia* 75: 494–501
- Lauteri M, Brugnoli E and Spaccino L (1993) Carbon isotope discrimination in leaf soluble sugars and in whole-plant dry matter in *Helianthus annuus* L. grown under different water conditions. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 93–108. Academic Press, San Diego
- Lauteri M, Scartazza A, Guido MC and Brugnoli E (1997) Genetic variation in photosynthetic capacity, carbon isotope discrimination and mesophyll conductance in provenances of *Castanea sativa* adapted to different environments. *Funct Ecol*, 11: 675–683
- Leavitt SW and Long A (1985) Stable-carbon isotopic composition of maple sap and foliage. *Plant Physiol* 78: 427–429
- Lin G and Ehleringer JR (1997) Carbon isotope fractionation does not occur during dark respiration in C₃ and C₄ plants. *Plant Physiol* 114: 391–394
- Lloyd J, Syvertsen JP, Kriedemann PE and Farquhar GD (1992) Low conductances for CO₂ diffusion from stomata to the sites of carboxylation in leaves of woody species. *Plant Cell Environ* 15: 873–899.
- Lloyd J, Kruijt B, Hollinger DY, Grace J, Francy RJ, Wong SC, Kelliher FM, Miranda AC, Farquhar GD, Gash JHC, Vygodskaya NN, Wright IR, Miranda HS and Schulze E-D (1996) Vegetation effects on the isotopic composition of atmospheric CO₂ at local and regional scales: Theoretical aspects and a comparison between rain forest in Amazonia and a Boreal forest in Siberia. *Aust J Plant Physiol* 23: 371–399
- Loreto F, Harley PC, Di Marco G and Sharkey TD (1994) Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. *Photosynth Res* 41: 397–403
- Loreto F, Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Fabozzi C and Tricoli D. (1996) Evidence of the photosynthetic origin of monoterpenes emitted by *Quercus ilex* L. leaves by ¹³C labelling. *Plant Physiol* 110: 1317–1322
- Lowe DC, Breninkmeijer CAM, Manning MR, Brailsford GW, Lassey KR and Gomez AJ (1993) Carbon isotopic composition of atmospheric methane in New Zealand and Antarctica. In: *Isotope Techniques in the Study of Past and Current Environmental Changes in the Hydrosphere and the Atmosphere*, pp 43–51. International Atomic Energy Agency, Vienna
- Lu Z, Chen J, Percy RG, Sharifi MR, Rundel PW and Zeiger E

- (1996) Genetic variation in carbon isotope discrimination and its relation to stomatal conductance in Pima cotton (*Gossypium barbadense*). *Aust J Plant Physiol* 23: 127–132
- Máguas C and Brugnoli E (1996) Spatial variation in carbon isotope discrimination across the thalli of several lichen species. *Plant Cell Environ* 19: 437–446
- Máguas C, Griffiths H, Ehleringer JR and Serodio J (1993) Characterization of photobiont associations in lichens using carbon isotope discrimination techniques. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 201–212. Academic Press, San Diego
- Máguas C, Griffiths H and Broadmeadow MSJ (1995) Gas exchange and carbon isotope discrimination in lichens: Evidence for interactions between CO_2 concentrating mechanisms and diffusion limitations. *Planta* 196: 95–102
- Máguas C, Valladares F and Brugnoli E (1997) Effects of thallus size on morphology and physiology of foliose lichens: New findings with a new approach. *Symbiosis* 23: 149–164
- Marino BD and McElroy MB (1991) Isotopic composition of atmospheric CO_2 inferred from carbon in C_4 plant cellulose. *Nature* 349: 127–131
- Marino BD, McElroy MB, Salawitch RJ and Spaulding WG (1992) Glacial-to-interglacial variations in the carbon isotopic composition of atmospheric CO_2 . *Nature* 357: 461–466
- Marshall JD, Dawson TE and Ehleringer JR (1993) Gender-related differences in gas exchange are not related to host quality in the xylem-tapping mistletoe, *Phoradendron juniperinum* (Viscaceae). *Am J Botany* 80: 641–645
- Martin B, Nienhuis J, King G and Schaefer A (1989) Restriction fragment length polymorphisms associated with water use efficiency in tomato. *Science* 243: 1725–1728
- Martínez-Carrasco R, Pérez P, Handley LL, Scrimgeour CM, Igual M, Martin del Molino I and Sánchez de la Puente (1998) Regulation of growth, water-use efficiency and $\delta^{13}\text{C}$ by nitrogen source in *Casuarina equisetifolia* Forst & Forst. *Plant Cell Environ* 21: 531–534
- Masle J, Shin JS and Farquhar GD (1993) Analysis of restriction fragment length polymorphisms associated with carbon isotope discrimination among ecotypes of *Arabidopsis thaliana*. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 371–386. Academic Press, San Diego
- Mason EA and Marrero TR (1970) The diffusion of atoms and molecules. *Adv At Mol Phys* 6: 155–232
- Maxwell K, von Caemmerer S and Evans J (1997) Is a low internal conductance to CO_2 diffusion a consequence of succulence in plants with Crassulacean acid metabolism? *Aust J Plant Physiol* 24: 777–786
- Meinzer FC and Saliendra NZ (1997) Spatial patterns of carbon isotope discrimination and allocation of photosynthetic activity in sugarcane leaves. *Aust J Plant Physiol.* 24: 769–775
- Meinzer FC and Zhu J (1998) Nitrogen stress reduces the efficiency of the C_4 CO_2 concentrating system, and therefore quantum yield, in *Saccharum* (sugarcane) species. *J Exp Botany* 49: 1227–1234
- Meinzer FC, Plaut Z and Saliendra NZ (1994) Carbon isotope discrimination, gas exchange, and growth of sugarcane cultivars under salinity. *Plant Physiol* 104: 521–526
- Melzer E and O'Leary MH (1987) Anapleurotic CO_2 fixation by phosphoenolpyruvate carboxylase in C_3 plants. *Plant Physiol* 84: 58–60
- Melzer E and O'Leary MH (1991) Aspartic-acid synthesis in C_3 plants. *Planta* 185: 368–371
- Melzer E and Schmidt H-L (1987) Carbon isotope effects on the pyruvate dehydrogenase reaction and their importance for relative Carbon-13 depletion in lipids. *J Biol Chem* 262: 8159–8164
- Meyer S and Gentz B (1996) Mapping intercellular CO_2 molar fraction (C_i) in rosa leaf fed with ABA. Significance of C_i estimated from leaf gas exchange. In: Mathis P (ed) *Photosynthesis: From Light to Biosphere*, Vol V, pp 603–606, Kluwer Academic Publishers, Dordrecht
- Mian MAR, Ashley DA and Boerma HR (1998) An additional QTL for water-use efficiency in soybean. *Crop Sci* 38: 390–393
- Mook WG (1986) ^{13}C in atmospheric CO_2 . *Netherlands J Sea Res* 20: 211–223
- Mook WG, Bommerman JC and Staverman WH (1974) Carbon isotope fractionation between dissolved carbonate and gaseous carbon dioxide. *Earth Planet Sci Lett* 22: 169–176
- Mook WG, Koopmans M, Carter AF and Keeling CD (1983) Seasonal, latitudinal and secular variations in the abundance and isotopic ratios of atmospheric carbon dioxide. 1. Results from land stations. *J Geophys Res* 88: 10915–10933
- Morgan JA, LeCain DR, McCaig TN and Quick JS (1993) Gas exchange, carbon isotope discrimination, and productivity in winter wheat. *Crop Sci* 33: 178–186
- Nalborczyk E (1978) Dark carboxylation and its possible effect on the value of $\delta^{13}\text{C}$ in C_3 plants. *Acta Physiol Plant* 1: 53–58
- O'Leary MH (1981) Carbon isotope fractionation in plants. *Phytochemistry* 20: 553–567
- O'Leary MH (1984) Measurement of the isotope fractionation associated with diffusion of carbon dioxide in aqueous solution. *J Phys Chem* 88: 823–825
- O'Leary MH (1988) Carbon isotopes in photosynthesis. *BioSciences* 38: 328–336
- O'Leary MH (1993) Biochemical basis of carbon isotope fractionation. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) *Stable Isotopes and Plant Carbon-Water Relations*, pp 19–28. Academic Press, San Diego
- O'Leary MH and Osmond CB (1980) Diffusional contribution to carbon isotope fractionation during dark CO_2 fixation in CAM plants. *Plant Physiol* 66: 931–934
- O'Leary MH, Treichel I and Rooney M (1986) Short-term measurement of carbon isotope fractionation in plants. *Plant Physiol* 80: 578–582
- O'Leary MH, Madhavan S, and Paneth P (1992) Physical and chemical basis of carbon isotope fractionation in plants. *Plant Cell and Environ* 15: 1099–1104
- Ohsugi R., Samejima M., Chonan N and Murata T (1988) $\delta^{13}\text{C}$ values and the occurrence of suberized lamellae in some *Panicum* species. *Annals of Botany* 62: 53–59
- Osmond CB (1978) Crassulacean acid metabolism: A curiosity in context. *Annu Rev Plant Physiol* 29: 379–414
- Osmond CB, Holtum JAM, O'Leary MH, Roeske C, Wong OC, Summons RE and Avadhani PN (1988) Regulation of malic-acid metabolism in crassulacean-acid-metabolism plants in the dark and light: in-vivo evidence from ^{13}C -labeling patterns after $^{13}\text{CO}_2$ fixation. *Planta* 175: 184–192
- Osório J and Pereira JS (1994) Genotypic differences in water use efficiency and ^{13}C discrimination in *Eucalyptus globulus*.

- Tree Physiol 14: 871–882
- Palmqvist K, Mágus C, Badger MR and Griffiths H (1994) Assimilation, accumulation and isotope discrimination of inorganic carbon in lichens: Further evidence for the operation of a CO_2 concentrating mechanism in cyanobacterial lichens. Crypt Bot 4: 218–226
- Paneth P and O'Leary MH (1985) Carbon isotope effect on dehydration of bicarbonate ion catalyzed by carbonic anhydrase. Biochemistry 24: 5143–5147
- Panichi C and Tongiorgi E (1975) Carbon isotopic composition of CO_2 from springs, fumaroles, moffettes, and travertines of central and southern Italy: A preliminary prospection method of geothermal area. Proceedings of the Second United Nations Symposium on the development and use of geothermal resources, Vol 1, pp 815–825
- Park R and Epstein S (1960) Carbon isotope fractionation during photosynthesis. Geochim Cosmochim Acta 21: 110–126
- Park R and Epstein S (1961) Metabolic fractionation of ^{13}C and ^{12}C in plants. Plant Physiol 36: 133–138
- Passioura JB (1986) Resistance to drought and salinity: avenues for improvement. Aust J Plant Physiol 13: 191–201
- Patterson MT and Rundel PW (1993) Carbon isotope discrimination and gas exchange in ozone-sensitive and -resistant populations of Jeffrey pine. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) Stable Isotopes and Plant Carbon-Water Relations, pp 213–225. Academic Press, San Diego
- Peisker M (1982) The effect of CO_2 leakage from bundle sheath cells on carbon isotope discrimination in C_4 plants. Photosynthetica 16: 533–541
- Peisker M and Henderson SA (1992) Carbon: terrestrial C_4 plants. Plant Cell Environ 15: 987–1004
- Peñuelas J and Azcon-Bieto J (1992) Changes in leaf $\Delta^{13}\text{C}$ of herbarium plants species during the last 3 centuries of CO_2 increase. Plant Cell Environ 15: 485–489
- Peterson BJ and Fry B (1987) Stable isotopes in ecosystem studies. Annu Rev Ecol Syst. 18: 293–320
- Picon C, Guehl J-M and Ferhi A (1996) Leaf gas exchange and carbon isotope composition responses to drought in a drought-avoiding (*Pinus pinaster*) and a drought-tolerant (*Quercus petraea*) species under present and elevated CO_2 concentrations. Plant Cell Environ 19:182–190
- Picon C, Ferhi A and Guehl J-M (1997) Concentration and $\delta^{13}\text{C}$ of leaf carbohydrates in relation to gas exchange in *Quercus robur* under elevated CO_2 and drought. J Exp Botany 48: 1547–1556
- Proctor MCF, Raven JA and Rice SK (1992) Stable carbon isotope discrimination measurements in *Sphagnum* and other bryophytes: Physiological and ecological implications. J Bryology 17: 193–202
- Ranjith SA, Meinzer FC, Perry MH and Thorn M (1995) Partitioning of carboxylase activity in nitrogen stressed sugarcane and its relationship to bundle sheath leakiness to CO_2 , photosynthesis and carbon isotope discrimination. Aust J Plant Physiol 22: 903–911
- Raven JA (1992) Present and potential uses of the natural abundance of stable isotopes in plant science, with illustrations from the marine environment. Plant Cell Environ 15: 1083–1091
- Raven JA and Farquhar GD (1990) The influence of N metabolism and organic acid synthesis on the natural abundance of isotopes of carbon in plants. New Phytol 116: 505–529
- Raven JA, Johnston AM, Handley LL and McInroy SG (1990) Transport and assimilation of inorganic carbon by *Lichina pygmaea* under emersed and submersed conditions. New Phytol 114: 407–417
- Read JJ and Farquhar GD (1991) Comparative studies in *Nothofagus* (Fagaceae). I. Leaf carbon isotope discrimination. Funct Ecol 5: 684–695
- Read JJ, Johnson RC, Carver BF and Quarrie SA (1991a) Carbon isotope discrimination, gas exchange, and yield of spring wheat selected for abscisic acid content. Crop Sci 31: 139–146
- Read JJ, Johnson DA, Asay KH and Tieszen LL (1991b) Carbon isotope discrimination, gas exchange, and water-use efficiency in crested wheatgrass clones. Crop Sci 31: 1203–108
- Read JJ, Johnson DA, Asay KH and Tieszen LL (1992) Carbon isotope discrimination: Relationship to yield, gas exchange and water-use efficiency in field-grown crested wheatgrass. Crop Sci 32: 168–175
- Rice SK and Giles L (1994) Climate in the Pleistocene. Nature 371: 111
- Rice SK and Giles L (1996) The influence of water content and leaf anatomy on carbon isotope discrimination and photosynthesis. Plant Cell Environ 19: 118–124
- Roberts A, Borland AM and Griffiths H (1997) Discrimination processes and shift in carboxylation during the phases of crassulacean acid metabolism. Plant Physiol 113: 1283–1292
- Robinson JJ and Cavanaugh C.M. (1995) Expression of form I and form II Rubisco in chemoautrophic symbioses: Implications for the interpretation of stable carbon isotope values. Limnol Oceanogr 40, 1496–1502
- Roeloffzen JC, Mook WG and Keeling CD (1991) Trends and variations in stable carbon isotopes of atmospheric carbon dioxide. In: Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental Studies, pp 601–618. International Atomic Energy Agency, Vienna
- Roeske CA and O'Leary MH (1984) Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose bisphosphate. Biochemistry 23: 6275–6284
- Roeske CA and O'Leary MH (1985) Carbon isotope effect on carboxylation of ribulose bisphosphate catalyzed by ribulosebisphosphate carboxylase from *Rodospirillum rubrum*. Biochemistry 23: 6275–6284
- Rooney MA (1988) Short term carbon isotopic fractionation in plants. Ph.D. Thesis, University of Wisconsin, Madison
- Rossmann A, Rieth W and Schmidt H-L (1990) Möglichkeiten und ergebnisse der kombination von messungen der verhältnisse stabiler wasserstoff-und kohlenstoff-isotope mil resultaten konventioneller analysen (RSK-werte) zum nachweis des zuckerzusatzes zu fruchtsäften. Z Lebensm Unters Forsch 191: 259–264
- Rossmann A, Butzenlechner M and Schmidt H-L (1991) Evidence for nonstatistical carbon isotope distribution in natural glucose. Plant Physiol 96: 609–614
- Roupsard O, Joly HI and Dreyer E (1998) Variability of initial growth, water-use efficiency and carbon isotope discrimination in seedlings of *Faidherbia albida* (Del.) A. Chev., a multipurpose tree of semi-arid Africa—Provenance and drought effects. Ann Sci For 55: 329–348
- Rundel PW and Sharifi MR (1993) Carbon isotope discrimination and resource availability in the desert shrub *Larrea tridentata*. In: Ehleringer JR, Hall AE and Farquhar GD (Eds) Stable Isotopes and Plant Carbon-Water Relations, pp 173–185.

- Academic Press, San Diego
- Rundel PW, Stichler W, Zander RH and Ziegler H (1979) Carbon and hydrogen isotope ratios of bryophytes from arid and humid regions. *Oecologia* 44: 91–94
- Salinera NZ, Meinzer FC, Perry MH and Thom M (1996) Associations between partitioning of carboxylase activity and bundle sheath leakiness to CO_2 , carbon isotope discrimination, photosynthesis, and growth in sugarcane. *J Exp Botany* 47: 907–914
- Sasakawa H, Sugiharto B, O'Leary MH and Sugiyama T (1989) $\delta^{13}\text{C}$ values in maize leaf correlate with phosphoenolpyruvate carboxylase levels. *Plant Physiol* 90: 582–585
- Sayre KD, Acevedo E, and RB Austin (1995) Carbon isotope discrimination and grain yield for three bread wheat germoplasm groups grown at different levels of water stress. *Field Crop Res* 41: 45–54.
- Scartazza A, Lauteri M, Guido MC and Brugnoli E (1998) Carbon isotope discrimination in leaf and stem sugars, water-use efficiency and mesophyll conductance during different developmental stages in rice subjected to drought. *Aust J Plant Physiol* 25: 489–498
- Schmidt H-L and Gleixner G (1998) Carbon isotope effects on key reactions in plant metabolism and ^{13}C -patterns in natural compounds. In: Griffiths H (ed) Stable Isotopes: Integration of Biological, Ecological and Geochemical Processes, pp 13–25. BIOS, Oxford
- Schnyder H (1992) Long-term steady-state labelling of wheat plants by use of natural $^{13}\text{CO}_2/^{12}\text{CO}_2$ mixtures in an open, rapidly turned-over system. *Planta* 187: 128–135
- Schulze E-D, Williams RJ, Farquhar GD, Schulze W, Langridge J, Miller JM and Walker BH (1998) Carbon and nitrogen isotope discrimination and nitrogen nutrition of trees along a rainfall gradient in Northern Australia. *Aust J Plant Physiol* 25: 413–425
- Seemann JR and Critchley C (1985) Effects of salt stress on the growth, ion content, stomatal behaviour and photosynthetic capacity of a salt-sensitive species, *Phaseolus vulgaris* L.. *Planta* 164: 151–162
- Sharkey TD, Loreto F, Delwiche CF, and Treichel IW (1991) Fractionation of carbon isotopes during biogenesis of atmospheric isoprene. *Plant Physiol* 97: 463–466
- Siegenthaler U and Sarmiento JL (1993) Atmospheric carbon dioxide and the ocean. *Nature* 365: 119–125
- Smedley MP, Dawson TE, Comstock JP, Donovan LA, Sherrill DE, Cook CS and Ehleringer JR (1991) Seasonal carbon isotope discrimination in a grassland community. *Oecologia* 85: 314–320
- Smith BN and Epstein S (1971) Two categories of $^{13}\text{C}/^{12}\text{C}$ ratios for higher plants. *Plant Physiol* 47: 380–384
- Smith EC and Griffiths H (1996) The occurrence of the chloroplast pyrenoid is correlated with the activity of a CO_2 -concentrating mechanism and carbon isotope discrimination in lichens and bryophytes. *Planta* 198: 6–16
- Stewart GR, Turnbull MH, Schmidt S and Erskine PD (1995) ^{13}C natural abundance in plant communities along a rainfall gradient: A biological integrator of water availability. *Aust J Plant Physiol* 22: 51–55
- Sun ZJ, Livingston NJ, Guy RD and Ethier GJ (1996) Stable carbon isotopes as indicators of increased water use efficiency and productivity in white spruce (*Picea glauca* (Moench) Voss) seedlings. *Plant Cell Environ* 19: 887–894
- Tans PP and Mook WG (1980) Past atmospheric CO_2 levels and the $^{13}\text{C}/^{12}\text{C}$ ratios in tree rings. *Tellus* 32: 268–283
- Teeri JA (1981) Stable carbon isotope analysis of mosses and lichens growing in xeric and moist habitats. *Bryologist* 84: 82–84
- Terashima I, Wong SC, Osmond CB and Farquhar GD (1988) Characterization of non-uniform photosynthesis induced by abscisic acid in leaves having different mesophyll anatomies. *Plant Cell Physiol* 29: 385–394
- Troughton JH (1972) Carbon isotope fractionation by plants. In: Proc. 8th Intl Conf Radiocarbon Dating, Wellington, NZ, pp E20-E57. The Royal Society of New Zealand
- Valentini R, Scarascia-Mugnozza GE and Ehleringer JR (1992) Hydrogen and carbon isotope ratios of selected species of a mediterranean macchia ecosystem. *Funct Ecol* 6: 627–631
- Villani F, Pigliucci M, Lauteri M, Cherubini M and Sun O (1992) Congruence between genetic, morphometric, and physiological data on differentiation of Turkish chestnut (*Castanea sativa*). *Genome* 35: 251–256
- Virgona JM and Farquhar GD (1996) Genotypic variation in relative growth rate and carbon isotope discrimination in sunflower is related to photosynthetic capacity. *Aust J Plant Physiol* 23: 227–236
- Virgona JM, Hubick KT, Rawson HM, Farquhar GD and Downes RW (1990) Genotypic variation in transpiration efficiency, carbon-isotope discrimination and carbon allocation during early growth in sunflower. *Aust J Plant Physiol* 17: 207–214
- Vogel JC (1980) Fractionation of the carbon isotopes during photosynthesis. In: Sitzungsberichte der Heidelberger Akademie der wissenschaften, mathematisch-naturwissenschaftliche Klasse Jahrgang 1980, 3, Abhandlung, pp 111–135. Springer-Verlag, Heidelberg, Berlin, New York
- von Caemmerer S (1989) A model of photosynthetic CO_2 assimilation and carbon-isotope discrimination in leaves of certain $\text{C}_3\text{-C}_4$ intermediates. *Planta* 178: 463–474
- von Caemmerer S (1992) Carbon isotope discrimination in $\text{C}_3\text{-C}_4$ intermediates. *Plant Cell Environ* 15: 1063–1072
- von Caemmerer S and Hubick KT (1989) Short-term carbon isotope discrimination in $\text{C}_3\text{-C}_4$ intermediate species. *Planta* 178: 475–481
- von Caemmerer S and Evans JR (1991) Determination of the average partial pressure of CO_2 in chloroplasts from leaves of several C_3 species. *Aust J Plant Physiol* 18: 287–305
- von Caemmerer S, Millgate A, Farquhar GD and Furbank RT (1997) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase by antisense RNA in the C_4 plant *Flaveria bidentis* leads to reduced assimilation rates and increased carbon isotope discrimination. *Plant Physiol* 113: 469–477
- von Caemmerer S, Ludwig M, Millgate A, Farquhar GD, Price D, Badger M and Furbank RT (1998) Isotope discrimination during C_4 photosynthesis: Insight from transgenic plants. *Aust J Plant Physiol* 24: 487–494
- Walker CD and Sinclair R (1992) Soil salinity is correlated with a decline in ^{13}C discrimination in leaves of *Atriplex* species. *Aust J Ecology* 17: 509–517
- White JW, Castillo JA and Ehleringer JR (1990) Associations between productivity, root growth and carbon isotope discrimination in *Phaseolus vulgaris* under water deficit. *Aust J Plant Physiol* 17: 189–198
- Wickman FE (1952) Variations in the relative abundance of the carbon isotopes in plants. *Geochim Cosmochim Acta* 2: 243–

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- Williams TG and Flanagan LB (1996) Effect of changes in water content on photosynthesis, transpiration and discrimination against $^{13}\text{CO}_2$ and $\text{C}^{18}\text{O}^{16}\text{O}$ in *Pleurozium* and *Sphagnum*. *Oecologia* 108: 38–46
- Williams TG and Flanagan LB (1998) Measuring and modelling environmental influences on photosynthetic gas exchange in *Sphagnum* and *Pleurozium*. *Plant Cell Environm* 21: 555–564
- Williams TG, Flanagan LB and Coleman JR (1996) Photosynthetic gas exchange and discrimination against $^{13}\text{CO}_2$ and $\text{C}^{18}\text{O}^{16}\text{O}$ in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase. *Plant Physiol* 112: 319–326
- Winter K (1981) CO_2 and water vapour exchange, malate content and $\delta^{13}\text{C}$ value in *Cicer arietinum* grown under two water regimes. *Z Pflanzenphysiol* 101: 421–430
- Wong SC and Osmond CB (1991) Elevated atmospheric partial pressure of CO_2 and plant growth. III Interactions between *Triticum aestivum* (C_3) and *Echinochloa frumentacea* (C_4) during growth in mixed culture under different CO_2 , N nutrition and irradiance treatments, with emphasis on below-ground responses estimated using the $\delta^{13}\text{C}$ value of root biomass. *Aust J Plant Physiol* 18: 137–152
- Wright GC, Nageswara Rao RC and Farquhar GD (1994) Water-use efficiency and carbon isotope discrimination in peanut under water deficit conditions. *Crop Sci* 34: 92–97
- Yakir D and Wang X-F (1996) Fluxes of CO_2 and water between terrestrial vegetation and the atmosphere estimated from isotope measurements. *Nature* 380: 515–517
- Yin ZH and Raven JA (1998) Influences of different nitrogen sources on nitrogen- and water-use efficiency, and carbon isotope discrimination in C_3 *Triticum aestivum* L. and C_4 *Zea mays* L. plants. *Planta* 205: 574–580
- Yoshioka T (1997) Phytoplanktonic carbon isotope fractionation—Equations accounting for CO_2 concentrating mechanisms. *J Plankton Res* 19: 1455–1476
- Zhang JW and Marshall JD (1995) Variation in carbon isotope discrimination and photosynthetic gas exchange among populations of *Pseudotsuga menziesii* and *Pinus ponderosa* in different environment. *Funct Ecol* 9: 402–412

Chapter 18

C₄ Photosynthesis: Mechanism and Regulation

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Summary

The C₄ photosynthetic process is a combination of biochemical, anatomical and genetic specialization which concentrates CO₂ at the site of carbon fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase. This essentially suppresses photorespiration and allows carbon fixation to proceed at near CO₂ saturation. In this review we summarize the three diverse biochemical mechanisms underlying C₄ photosynthesis, describe the regulation of individual enzymes in the C₄ pathway, discuss the integrated regulation of C₄ photosynthesis and how these regulatory processes respond to environmental conditions.

I. Introduction

C₄ photosynthesis is a unique blend of modified biochemistry, anatomy and organelle function designed to avoid the worst outcomes of Rubisco's oxygenase reaction and the associated process of photorespiration. This elaboration of the conventional PCR cycle functions to concentrate CO₂ at the site of Rubisco operation, thereby suppressing the oxygenase

reaction and eliminating the need for the photorespiratory cycle.

It is several years since general aspects of the biochemistry and physiology of C₄ photosynthesis were reviewed (Edwards and Walker, 1983; Hatch, 1987). However, a comprehensive account of C₄ biology is currently being prepared for publication (Sage and Monson, 1999). The main purpose of the present chapter is to review developments in our

understanding of the regulation of C₄ photosynthesis. However, to provide a framework for this discussion we will briefly describe the biochemical processes operating to transfer CO₂ to bundle sheath cells and emphasize the more recent advances in the understanding of these mechanisms. Subsequent chapters will consider in detail the metabolite transport processes that are an integral part of the C₄ mechanism as well as developmental and ecophysiological aspects of this process.

II. Mechanism and Function of C₄ Photosynthesis

A. Mechanism

The biochemistry of C₄ photosynthesis and its function are intimately linked with a highly specialized leaf anatomy. Leaves of C₄ plants contain two distinct types of photosynthetic cells (Fig. 1). These are usually arranged radially around vascular tissue in two adjacent layers, the outer layer being termed mesophyll cells and the inner layer, bundle sheath cells. A critical and universal feature of this type of anatomy is that Rubisco and the PCR cycle, responsible for the ultimate assimilation of CO₂, occur only in the inner bundle sheath cell layer.

In all C₄ plants CO₂ is initially fixed in mesophyll cells via PEP carboxylase (PEPC). The inorganic carbon species used by this enzyme is HCO₃⁻ so that the first reaction is, in fact, the carbonic anhydrase-mediated hydration of CO₂ (Hatch and Burnell, 1990). The oxaloacetate formed in this carboxylation reaction is converted to larger pools of the C₄ acids malate and aspartate and one or other of these acids then diffuses to the bundle sheath cells where it is decarboxylated to release CO₂. This CO₂ is refixed by Rubisco and the PCR cycle and the 3-carbon compound remaining from the decarboxylation

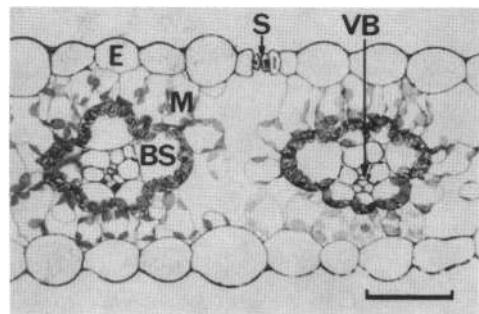


Fig. 1. Anatomy of a C₄ leaf showing epidermis (E), stomata (S), the outer layer of chlorophyll-containing mesophyll cells (M), and adjacent layer of bundle sheath cells (BS) surrounding the vascular bundle tissue (VB). Light micrograph prepared by Stuart Craig.

reaction is returned to mesophyll cells to serve as a precursor for the regeneration of PEP.

A most unexpected outcome in the C₄ story was the realization that three quite distinct biochemical mechanisms operate to concentrate CO₂ in the bundle sheath cells of different C₄ species. These mechanisms are outlined in Fig. 2. The key feature distinguishing these three biochemical options for C₄ photosynthesis is the mechanism of C₄ acid decarboxylation in the bundle sheath cells. These decarboxylases, NADP-ME, NAD-ME and PEPCK (see later sections) function in the 'NADP-ME-type', 'NAD-ME-type' and 'PCK-type' mechanisms, respectively, described in Fig. 2. A detailed account of these schemes is provided in an earlier review (Hatch, 1987). These mechanisms appear to account for photosynthesis in the majority of C₄ plants. However, it should be emphasized at the outset that some species may use processes which are intermediate between these 'classical' mechanisms. Examples of such intermediacy will be mentioned.

The first of these options to be recognized and resolved was the NADP-ME-type which accounts for photosynthesis in grass species like maize, sugar cane, and sorghum as well as several dicotyledonous species. In this process OAA formed by PEP carboxylase is reduced to malate in mesophyll chloroplasts by NADP-MDH and the malate is then transferred to bundle sheath cells (Fig. 2). After entering the chloroplasts, malate is decarboxylated by NADP-ME, generating both CO₂ and NADPH. The CO₂ is fixed by Rubisco and the PCR cycle, which are specifically located in these cells, and the

Abbreviations: CAM – Crassulacean acid metabolism; FBP – fructose 1,6-bisphosphate; G6P – glucose 6-phosphate; NADP-MDH – NADP malate dehydrogenase; NAD-ME – NAD malic enzyme; NAD-ME-type – NAD malic enzyme-type; NADP-ME – NADP malic enzyme; NADP-ME-type – NADP malic enzyme-type; OAA – oxaloacetate; PCK-type – phosphoenolpyruvate carboxykinase-type; PCR cycle – photosynthetic carbon reduction cycle; PEP-phosphoenolpyruvate; PEPC-phosphoenolpyruvate carboxylase; PEPC-PK – phosphoenolpyruvate carboxylase protein kinase; PEPCK – phosphoenolpyruvate carboxykinase; PGA – 3-phosphoglycerate; PPDK – pyruvate, Pi dikinase; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase

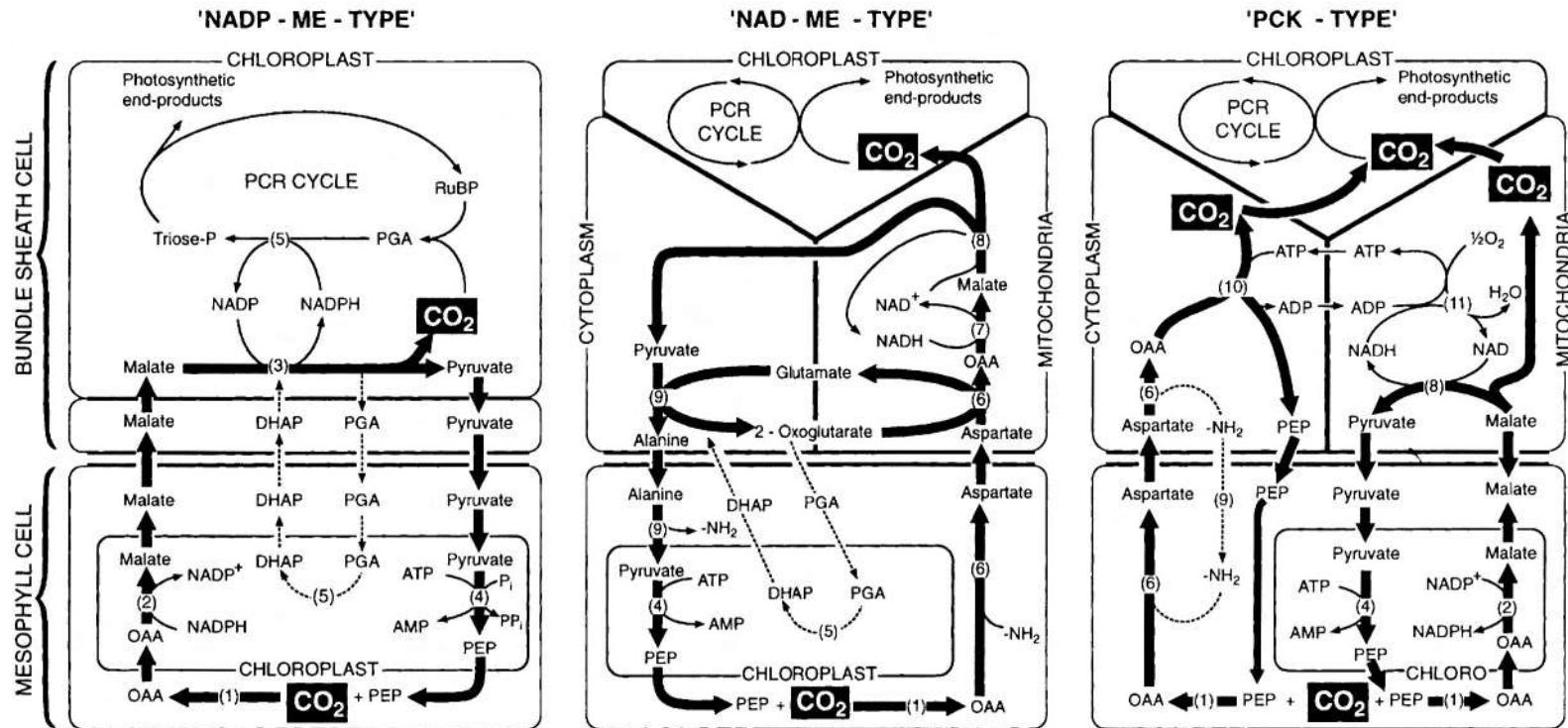


Fig. 2. Path of carbon assimilation and the inter- and intracellular location of reactions for the three biochemically distinct subgroups of C₄ species. The main path of carbon flow and associated metabolite transport processes are indicated by heavy arrows. The enzymes involved, indicated by the numbers in brackets, are: 1, phosphoenolpyruvate carboxylase; 2, NADP malate dehydrogenase; 3, NADP malic enzyme; 4, pyruvate Pi dikinase; 5, 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase; 6, aspartate aminotransferase; 7, NAD malate dehydrogenase; 8, NAD malic enzyme; 9, alanine aminotransferase; 10, phosphoenolpyruvate carboxykinase; 11, mitochondrial NADH oxidation systems. For the PCK-type mechanism the PGA/dihydroxyacetone-phosphate shuttle would also operate between cells (as shown for other types) and the cycling of amino groups between mesophyll and bundle sheath cells involves alanine and alanine aminotransferase (see text).

pyruvate remaining after decarboxylation is returned to the mesophyll chloroplasts and converted to the CO_2 acceptor PEP by pyruvate, P_i dikinase (PPDK). It should be noted that with this mechanism half the NADPH necessary for the subsequent reduction of PGA to triose phosphate is generated in the bundle sheath chloroplasts during malate decarboxylation. Particularly in those species where Photosystem II is almost totally absent from the bundle sheath chloroplasts, the remaining PGA must be transferred to mesophyll cells for reduction. Most NADP-ME-type grasses have very little Photosystem II activity in bundle sheath cells (Edwards et al., 1976; Chapman et al., 1980). However, there is substantial Photosystem II activity in the bundle sheath cells of some NADP-ME-type dicotyledons (Meister et al., 1996) and this is associated with increased transport of aspartate to bundle sheath cells which, of course, would not involve the transfer of reducing power.

A wide range of grasses and dicotyledonous species from various genera use the NAD-ME-type mechanism for C_4 photosynthesis. In this mechanism, the major C_4 acid transported to bundle sheath cells is aspartate (Fig. 2). There, aspartate enters the mitochondria together with 2-oxoglutarate and is converted through aspartate aminotransferase to OAA. The OAA is then reduced to malate and the malate decarboxylated by NAD-ME, specifically located in these mitochondria. It is important to note that where aspartate is the C_4 acid entering mitochondria, these latter two steps are coupled through an NAD/NADH cycle. However, an option under certain conditions is that malate from mesophyll cells may enter the mitochondria directly; in this case the NADH formed during the subsequent decarboxylation of malate is probably oxidized by non-coupled respiration (Agostino et al., 1996).

As with the NADP-ME-type mechanism, the pyruvate formed during the decarboxylation of malate by NAD-ME is returned to mesophyll cells where it serves as a precursor for the regeneration of PEP. However, in the NAD-ME-type species the C_4 compound actually transferred is apparently alanine. By this means, 2-oxoglutarate is regenerated in bundle sheath cells to sustain aspartate conversion to OAA and the deamination of alanine in mesophyll cells, giving glutamate as well as pyruvate, would be stoichiometrically linked with the continuing generation of aspartate (Fig. 2). As with NADP-ME-type species, the leaf's capacity for converting PGA to triose-P (by PGA kinase and NADP glyceraldehyde

3-P dehydrogenase) is distributed about equally between mesophyll and bundle sheath cells. From this it is assumed that a substantial part of the PGA generated in bundle sheath cells is transferred to mesophyll cells for reduction, thus ensuring that both cell types contribute to the Photosystem II-based generation of the required NADPH.

So far, the PCK-type mechanism has been identified only in a range of grass species. Although distinguished by the operation of PEP carboxykinase (PEPCK) for decarboxylation it will be seen that NAD-ME contributes to the decarboxylation of C_4 acids while serving to generate ATP in mitochondria to drive the PEPCK reaction. Thus, it is visualized that both aspartate and malate contribute to the transfer of C_4 acids to bundle sheath cells (Fig. 2). This view of the PCK-type mechanism was developed in a series of studies over the last decade (Burnell and Hatch, 1988; Hatch and Carnal, 1992; Carnal et al., 1993; Agostino et al., 1996).

In PCK-type species, OAA decarboxylated in the cytosol of bundle sheath cells by PEPCK is derived from aspartate transported from mesophyll cells. The PEPCK reaction uses ATP synthesized in the mitochondria during coupled oxidation of NADH generated by NAD-ME. PEP formed during this OAA decarboxylation is presumably transported back to mesophyll cells and used directly for the further assimilation of CO_2 by PEPC. An option, which can at least be demonstrated with isolated bundle sheath cells (Carnal et al., 1993), is that OAA generated in the bundle sheath cells may enter the mitochondria and then be decarboxylated through the coupled action of malate dehydrogenase and NAD-ME. This alternative route may be limited by the concentration of OAA that prevails *in vivo*.

To generate ATP for the PEPCK reaction, malate must be transported from mesophyll cells to the bundle sheath mitochondria (Fig. 2). Even assuming a near theoretical yield of three ATP generated through coupled NADH oxidation, this would still require one mole of malate transported and decarboxylated by NAD-ME for each three moles of OAA decarboxylated by PEPCK. In some species the potential for malate decarboxylation and respiratory oxidation of NADH by these bundle sheath mitochondria is greater than this. This has led to the suggestion that NAD-ME may make a greater contribution to C_4 acid decarboxylation under some circumstances (Carnal et al., 1993; Agostino et al., 1996). Where malate is decarboxylated by NAD-

ME, the product pyruvate would be converted back to PEP after transfer to the mesophyll chloroplasts. It should also be noted that available evidence clearly indicates a key role for alanine and alanine aminotransferase in maintaining the amino group balance between mesophyll and bundle sheath cells (Fig. 2) However, the exact stoichiometry of these reactions and their relation to the aspartate/PEP and malate/pyruvate cycles is uncertain.

The options for C_4 photosynthesis described in Fig. 2 involve a remarkable array of metabolite transport processes into and out of chloroplasts and mitochondria. There is, in addition, a variety of fluxes required between mesophyll and bundle sheath cells, all being required to proceed at rates matching overall rate of photosynthesis. These will be considered in Chapter 19 (Leegood).

B. Function and Efficiency

Viewed simply, the function of the special reactions of C_4 photosynthesis is to transfer CO_2 from mesophyll to bundle sheath cells. In fact, as already noted in the Introduction, the specific purpose is to concentrate CO_2 in bundle sheath cells with a view to eliminating the Rubisco-catalysed oxygenase reaction and associated photorespiration. For normal C_3 plants in air the oxygenase reaction proceeds at about half the rate of the carboxylation of RuBP. This results in a large decline in efficiency compared with the situation where there is negligible oxygenase. Measured in terms of quantum yield for CO_2 fixation, the decline is from about 0.08 mol CO_2 fixed/mol quanta to 0.05 at 30 °C, that is about 40%. By concentrating CO_2 in bundle sheath cells the oxygenase reaction and associated photorespiration are very largely eliminated in C_4 plants under normal conditions. However, there is an energetic cost in concentrating this CO_2 —essentially equivalent to the 2 ATP consumed in the PPDK reaction (somewhat more complicated in PCK-type species, see Hatch, 1987). Hence, the quantum yields for C_4 plants, in the range from 0.06 to 0.07 mol CO_2 /mol quanta for most C_4 plants at 30 °C (Ehleringer and Pearcy, 1983), are higher than the values for C_3 plants in air but less than the values for the latter plants measured in the absence of photorespiration. One of the factors that contributes to reducing the efficiency of photosynthesis in C_4 plants is the extent to which CO_2 leaks back to mesophyll cells instead of being refixed. This, and other aspects of the function and efficiency

of C_4 photosynthesis, have been discussed in more detail previously (Farquhar, 1983; Hatch, 1987, 1992a, 1995; Furbank et al., 1990b; Ehleringer and Monson, 1993).

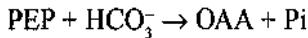
Largely as the result of almost abolishing photorespiration under normal ambient conditions, and the operation of Rubisco at or near its V_{max} , C_4 plants are capable of higher rates of photosynthesis at both limiting and saturating light compared with C_3 plants (Hatch, 1992a,b). However, these advantages are only apparent at higher temperatures, generally above 20–25 °C, the result of photosynthetic efficiency decreasing in C_3 plants as temperature increases (Ehleringer and Pearcy, 1983). The main basis of this effect is a disproportionate increase in Rubisco oxygenase activity relative to carboxylase activity as temperature rises, resulting in a greater increase in photorespiration relative to photosynthesis (Jordan and Ogren, 1984). A related feature of C_4 plants is higher water-use efficiency; at higher temperature they average about twice the carbon assimilation per unit of water loss compared with C_3 plants. Thus, at higher temperature and light the C_4 photosynthetic option provides plants with the potential for rapid growth and this can be manifested in terms of record rates of dry matter production (Hatch, 1992a,b). However, many C_4 species choose the option of conserving water instead, explaining their dominance in more arid situations (Stowe and Teeri, 1978; Hattersley, 1992). These aspects are considered in more detail in Chapter 22, Monson and Rawsthorne.

III. Regulation of Individual Enzymes

This section will deal with the details of the mechanisms operating to regulate some key enzymes of C_4 photosynthesis. For other enzymes implicated in the C_4 process, including the aminotransferases, adenylate kinase and pyrophosphatase, there is no evidence for significant regulation. Later sections will deal with the integrated regulation of C_4 photosynthesis and responses to environmental changes.

A. Phosphoenolpyruvate carboxylase

PEPC catalyses the primary photosynthetic carboxylation reaction in C_4 plants yielding the initial C_4 acid of the C_4 cycle in the mesophyll cytosol:



The enzyme plays a key role in regulation of the C₄ cycle and its coordination with the C₃ cycle. Various isoforms of the enzyme are ubiquitous in plants as well as in many microorganisms. However, the isoform of PEPC involved in C₄ photosynthesis is specifically synthesized in leaves in response to light and achieves very high levels of activity, between 15 to 30 times the activity in C₃ leaves and about five times the maximum rate of photosynthesis when assayed in vitro under optimal conditions. This clearly indicates that, *in vivo*, the enzyme is under constant 'restraint' which is effected via allosteric metabolite- and covalent modification regulatory mechanisms. Similarly, the form of the enzyme involved in CAM photosynthesis is thought to be closely regulated in a diurnal cycle and the regulatory mechanisms may be similar. PEPC is arguably the most-studied enzyme of those involved in C₄ photosynthesis and the properties, mechanism and regulation of the enzyme in plants have been reviewed in detail on a number of occasions (O'Leary, 1982; Andreo et al., 1987; Stiborova, 1988; Jiao and Chollet, 1991; Lepiniec et al., 1994; Gadal et al., 1996; Chollet et al., 1996).

Activity of PEPC is subject to activation or inhibition by metabolites and the possible physiological relevance of this was recognized in early studies using crude or partially-purified extracts of C₄ plants (Huber and Edwards, 1975; O'Leary, 1982). Activation occurs with phosphorylated metabolic intermediates, particularly G6P, triose-phosphate and fructose 6-phosphate, with G6P often considered the most effective (Doncaster and Leegood, 1987; Gao and Woo, 1996a). The enzyme from monocot C₄ species (but not dicots) is also activated by certain amino acids, notably glycine, serine, and alanine (Nishikido and Takanashi, 1973; Doncaster and Leegood, 1987; Gao and Woo, 1996a). PEPC activity is inhibited by the C₄ acids malate and aspartate which is of obvious importance in the present context since these are metabolites of the C₄ cycle. With the purification of the enzyme, initially from maize (Uden and Sugiyama, 1976) and subsequently from a range of C₄ species, the oligomeric structure was determined, allosteric regulatory properties were confirmed and responses to pH and metabolites examined in greater detail (O'Leary, 1982; Andreo et al., 1987; Stiborova, 1988). The enzyme occurs as a tetramer of 109 kD subunits, but it has more recently been recognized that these subunits may be

easily degraded by N-terminal deletion to approximately 105 kD products. This can be prevented by protease inhibitors, particularly chymostatin (McNaughton et al., 1989; reviewed by Chollet et al., 1996). Since the N-terminal region contains the regulatory phosphorylation site (see below), the significance of some earlier conclusions regarding regulatory properties may be doubtful. *In vitro*, maximum activities are observed around pH 8, the response to PEP concentration is usually hyperbolic, and at this pH the inhibitory effects of C₄ acids and stimulatory effects of phosphorylated metabolites are decreased. At the more physiologically relevant pH, around 7, activity is lower, cooperative binding of PEP occurs, and strong allosteric effects of activators and inhibitors are observed (Huber and Edwards, 1975; O'Leary, 1982; Andreo, 1987; Stiborova, 1988). Activation by G6P mainly alters the K_m(PEP) whereas glycine modifies both K_m(PEP) and V_{max}, with these types of activators considered to operate at separate sites. Malate inhibition appears competitive with PEP at pH 7 but not at pH 8. Whether malate acts at the substrate site or by conformational changes resulting from its binding at another site is not resolved. Binding site studies with chemical modification reagents earlier indicated that various residues may be involved in PEP and metabolite binding, but few of these have been assigned to specific domains (Jiao et al., 1990) and, more recently, the earlier findings have been questioned (Gao and Woo, 1996b). Specific site-directed mutagenesis and domain swapping studies are in progress which should clarify functional regions and residues of the protein. A three dimensional molecular structure is yet to emerge.

Findings more than a decade ago (Budde and Chollet, 1986; Huber and Sugiyama, 1986; Doncaster and Leegood, 1987) that the properties of the enzyme in leaf extracts were modified in response to previous light or dark pre-treatments, along with the observation that phosphorylation of the enzyme could occur, stimulated intensive efforts to characterize regulatory phosphorylation mechanisms. When measured under sub-optimal, but probably more physiological, conditions of low PEP concentration and low pH, enzyme in extracts from illuminated leaves is less sensitive to malate inhibition and activated to a greater extent by phosphorylated metabolites, than enzyme from darkened leaves. Both enzyme forms show similar activity under optimal *in vitro* conditions. It is now generally accepted that

phosphorylation of the enzyme plays an important regulatory role in relation to light-dark changes in both C₄ and CAM plants (Chollet et al., 1996), and phosphorylation of the enzyme in C₃ plants has also been shown to occur (Li et al., 1996). Both phosphorylated and dephosphorylated forms of the enzyme have been purified from C₄ plants (McNaughton et al., 1989; Arrio-Dupont et al., 1992).

The regulatory process as currently perceived to occur in C₄ plants has been reviewed in detail (Chollet et al., 1996; Gadot et al., 1996) and is outlined in Fig. 3. The dark form of the enzyme is activated by phosphorylation of a specific serine residue (residue 15 in the maize polypeptide, corresponding to residue 8 in the sorghum sequence) catalysed by a protein kinase, designated PEPC-PK. This serine residue is located in a conserved sequence motif present in all C₄ and CAM PEPC enzymes, and possibly all plant PEPCs, but absent from the bacterial enzymes, suggesting that it may have been acquired in evolution as a regulatory element. The phosphorylated enzyme exhibits greater activity at sub-saturating PEP concentration, lower sensitivity to malate inhibition, and greater activation by G6P. On illumination, phosphorylation and an increase of PEPC-PK activity occur in parallel, indicating that the protein kinase itself is subject to light regulation and that PEPC phosphorylation is subject to a regulatory cascade (Chollet et al., 1996). In darkened leaves PEPC is specifically dephosphorylated. The protein phosphatase involved is apparently active in the leaves during light and dark periods, and has been shown from inhibitor studies to resemble a mammalian type 2A protein phosphatase. Therefore, the light-dark phosphorylation status of PEPC is dependent on the mechanism and extent to which PEPC-PK activity is regulated. The focus of ongoing research is to understand the signal transduction pathway by which light-dark signals are related to enzyme phosphorylation by the PEPC-PK.

A number of protein kinase activities in plants, in addition to a number of mammalian protein kinases, are capable of phosphorylating PEPC, but only one or two are responsive to light in maize. Using a functional assay, a light-responsive PEPC-PK has been purified from maize and its properties characterized (Wang and Chollet, 1993). The native protein is a monomer of 30 kD which is not responsive to Ca²⁺ or chelating agents indicating that it is not a Ca²⁺/calmodulin-dependent type protein kinase. Later studies identified, in addition, a 37 kD protein kinase

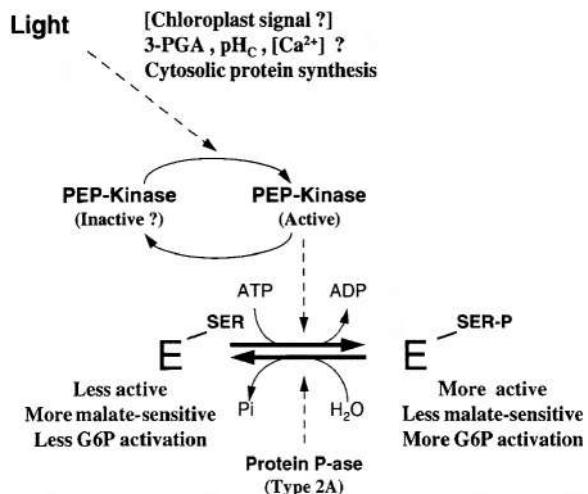


Fig. 3. Proposed mechanism of dark/light regulation of PEPC in C₄ leaves. The enzyme is denoted as E and the phosphorylated regulatory serine residue is shown (adapted from Chollet et al., 1996). For other details see text.

with similar properties (Li and Chollet, 1993). Whether these are separate enzymes or derivatives of the same protein has not been resolved.

The increase in PEPC-PK activity and maximum PEPC phosphorylation takes approximately 60 to 100 min to occur when darkened maize leaves are transferred to light, and dephosphorylation on darkening takes about the same time. A threshold light intensity of about 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ is required in maize with little response to changes in intensity above 500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (Nimmo et al., 1987), suggesting that this mechanism of regulation could be responsible for dark-light effects under normal diurnal conditions. Since the increase in PEPC-PK activity requires light and is prevented in the presence of photosynthetic inhibitors of either electron transport (Diuron or methyl viologen) or of the carbon reduction cycle (D,L-glyceraldehyde), a chloroplastic signal, which has been presumed to originate in the bundle sheath cells, has been invoked. Candidates for this signal are PGA and pyruvate (Duff et al., 1996; Giglioli-Guivarc'h et al., 1996). As the increase in PEPC-PK activity is also inhibited by cytosolic protein synthesis inhibitors, such as cycloheximide, protein synthesis is also involved. Evidence is also accumulating for a role of cytosolic pH, which becomes more alkaline on illumination (Giglioli-Guivarc'h et al., 1996). Recently, an effect of Ca²⁺ transport antagonists has been observed, leading to the suggestion that a Ca²⁺/calmodulin responsive

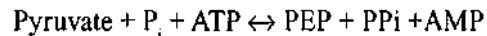
element may be involved (Duff et al., 1996). This has been proposed to act higher up the signal transduction pathway since, as noted above, the present view is that PEPC-PK is not **Ca²⁺/calmodulin-dependent** (Chollet et al., 1996).

Since these responses to changing light conditions are relatively slow, this phosphorylation-based mechanism is apparently not involved in the regulation of PEPC during short-term fluctuations of light intensity during the day. These responses are presumably brought about by changes in substrate and effector concentrations. Regulation of PEPC is considered necessary to coordinate the C₃ and C₄ cycles, to allow PEPC to operate in the high malate concentration prevailing in mesophyll cells during photosynthesis, and, in the dark, to prevent depletion of PEP arising from glycolysis. The light-dark phosphorylation mechanism may be most important for the latter two processes, with the sensitivity to malate inhibition high in darkened leaves but decreased in the light to allow relatively high PEPC activity in the presence of high malate concentration. A related process is thought to occur with the enzyme in CAM plants (Chollet et al., 1996). Also, as phosphorylation has been shown to occur in C₃ plants, the regulatory process may not be entirely specific to C₄ and CAM plants, although the regulatory signal transduction in C₃ species shows some differences (Li et al., 1996). It has been pointed out that the lower activity dephosphorylated form of PEPC could not support observed rates of photosynthesis at the concentrations of malate and PEP present in the mesophyll cytosol but that light induced phosphorylation and associated changes in metabolite control act synergistically to increase PEPC activity (Doncaster and Leegood, 1987; Gao and Woo, 1996a). Indeed, in contrast to C₃ plants, photosynthetic carbon dioxide assimilation was inhibited in C₄ plants when the phosphorylation state of PEPC, and the activity of PEPC-PK were decreased by short-term treatment with cytosolic protein synthesis inhibitors (Bakrim et al., 1993). This led to the suggestion that PEPC phosphorylation may be a cardinal event in regulation of C₄ photosynthesis.

B. Pyruvate, P_i Dikinase

PPDK was discovered during a search for an enzyme to account for the conversion of pyruvate to PEP in C₄ plants (Hatch and Slack, 1968). This enzyme catalyses a unique type of reaction in which two

substrates are phosphorylated using the γ and β phosphates of ATP:



Even with various precautions to maximize recovered activity, it has been difficult to demonstrate PPDK activities in C₄ leaf extracts much above the maximum photosynthesis rates for those leaves (Hatch, 1987). This, combined with the fact that the enzyme is subject to a complex mechanism of dark/light-based regulation, has led to the view that the PPDK reaction is a key site for regulation of C₄ photosynthesis. In this section we will consider the specific details of the regulation of this reaction.

Maize PPDK is a tetramer with subunit molecular weight of about 94 kDa (Sugiyama, 1973). Kinetic features are reviewed elsewhere (Ashton et al., 1990). Free Mg²⁺ is essential for activity in addition to Mg²⁺-ATP, and both the forward and reverse rates are stimulated several-fold by the monovalent cations NH₄⁺ or K⁺ (Jenkins and Hatch, 1985). The regulatory significance of these cation effects is uncertain. Also of possible regulatory significance is the fact that PPDK is reversibly inactivated below 10 °C, an effect prevented by PEP or pyruvate, and is inhibited by all three products of the reaction in the direction of PEP synthesis (Edwards et al., 1985; Jenkins and Hatch, 1985). The level of the enzyme in leaves also changes in response to changing light conditions during growth (Hatch, 1978). However, the most significant regulatory process operating on PPDK is the remarkably complex and rapid dark/light-mediated modulation of its activity (Burnell and Hatch, 1985a). Through this mechanism the enzyme is almost totally inactivated in darkened leaves, is partially activated in low light, and is completely activated after a few minutes illumination with high light. The down-regulation of PPDK in the dark presumably serves to prevent the unproductive ATP-dependent conversion of pyruvate to PEP. The same mechanism is apparently responsible for modulating PPDK activity in response to varying light in the range of up to half of full sunlight (Hatch 1981; Usuda et al., 1984). This would serve to maintain the supply of PEP in step with the prevailing light and associated demands for CO₂ assimilation and could have a rate limiting function (Usuda et al., 1984, see Section IV.C).

The processes responsible for this dark/light regulation have been described in detail (Burnell and Hatch, 1985a; Edwards et al., 1985) and the key

elements are summarized in Fig. 4. PPDK is active when a Thr residue, near a catalytic site His, is unphosphorylated. Inactivation results from the phosphorylation of this Thr but there are two remarkable features of this reaction. Firstly, ADP is the donor of the phosphate group in a reaction essentially without precedent in biological chemistry. Secondly, an absolute prerequisite for this ADP-mediated phosphorylation is that PPDK is already phosphorylated on the catalytic site His residue. In the normal course of catalysis this can occur by reacting with either ATP or PEP, thus explaining the requirement for both ADP and ATP for inactivation. Notably, pyruvate prevents inactivation by causing the removal of the catalytic site phosphate. Reactivation occurs by another unusual reaction, unique at least in terms of protein dephosphorylation. In this reaction the phosphate is removed from the regulatory Thr residue by phosphorolytic cleavage to yield inorganic pyrophosphate. Activation is much more rapid with the inactive enzyme free of the histidine phosphate and the reaction is inhibited by AMP, ADP and PP_i (Fig. 4). Final confirmation that this mechanism is the basis of the dark/light regulation in leaves was provided by the studies of Budde et al., (1985) showing that the regulatory threonine is phosphorylated with PPDK isolated from darkened leaves and is removed when leaves are illuminated. The maize PPDK gene has recently been expressed in *E. coli* opening the way for a site-directed analysis of the function of specific catalytic and regulatory residues (Chastain et al., 1996).

To add to the unique features of this dark/light mediated regulation of PPDK, these mechanistically different activation and inactivation reactions are catalysed by the same protein entity, termed PPDK regulatory protein. Such bifunctional activity is rare and its significance for the coordinated control of PPDK activity is uncertain, although some possibilities have been discussed (Burnell and Hatch, 1986). PPDK regulatory protein has been partially purified and some of its properties described (Burnell and Hatch, 1985b; Smith et al., 1994). There are some unresolved differences with regard to the purification of this protein but agreement that it is a most difficult enzyme to work with, has a monomeric molecular weight of about 45 kDa, and can exist as a dimer or tetramer. Chloroplast ADP levels may have a critical role in regulating PPDK activity since ADP is a strong inhibitor of the phosphorolytic activation, reaction as well as being the phosphate donor for

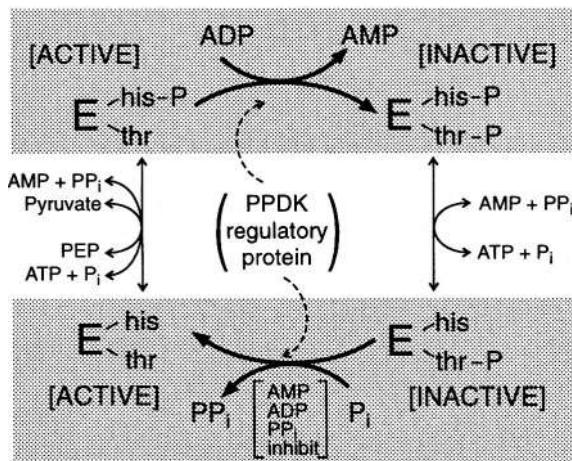


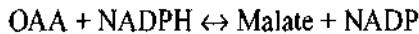
Fig. 4. Mechanism of dark/light regulation of PPDK in C₄ leaves. The enzyme is denoted as E and a catalytic site His residue and a regulatory Thr are shown. The key regulatory reactions are an ADP-mediated phosphorylation of the Thr residue to inactivate the enzyme and Pi-dependent phosphorolysis of this phosphate group for reactivation.

inactivation (Fig. 4; Burnell and Hatch, 1985a). PP_i also inhibits activation. Smith et al., (1994) could find no evidence for regulation of the PPDK regulatory protein by either covalent modification or a mechanism based on changes of chloroplast stromal pH with changing light intensity.

The precise mechanism by which changes in irradiance elicit changes in PPDK activity through the reactions shown in Fig. 4 remains uncertain. However, possible explanations have been advanced based on changes in adenylates, P_i and adenylate energy charge (Edwards et al., 1985; Budde et al., 1986; Nakamoto and Edwards, 1987; Roeske and Chollet, 1989). Pyruvate levels are also likely to exert a critical regulatory influence by maintaining the active enzyme in a non-inactivatable form (see Fig. 4, Budde et al., 1986; Burnell et al., 1986). The rates of both activation and inactivation of PPDK in leaves are dramatically reduced as temperature is reduced in the range from 30 to 10 °C (Edwards et al., 1985). This is one possible explanation for the cold sensitivity of C₄ plants.

C. NADP Malate Dehydrogenase

NADP-MDH has a critical role in NADP-ME-type C₄ plants where it catalyses the reduction of OAA to malate in mesophyll chloroplasts using light-generated NADPH (Fig. 2).



This enzyme was first discovered in leaves of NADP-ME-type C₄ species where its activity is 10 to 20 times that found in other C₄ plants or in C₃ species (see Hatch, 1987). Earlier studies on the kinetic properties and regulation of NADP-MDH have been reviewed (Edwards et al., 1985; Ashton et al., 1990). NADP-MDH activity in the direction of OAA reduction is strongly inhibited by the product NADP providing simple feedback control (Ashton and Hatch, 1983). However, as discussed below, this effect may also interact synergistically with the dark/light-mediated regulation of NADP-MDH resulting from varying the NADPH to NADP ratio.

NADP-MDH is rapidly inactivated when leaves or isolated chloroplasts are darkened and is reactivated with a half time of a few minutes following subsequent illumination (Johnson and Hatch, 1970; Hatch, 1977). Activity also varies with varying light intensity in the range up to about 40% of full sunlight (Johnson and Hatch, 1970; Usuda et al., 1984). This modulation with varying light presumably serves to co-ordinate the supply of malate with the prevailing potential for CO₂ assimilation. The total inactivation of the enzyme in the dark would prevent the futile conversion of OAA to malate with associated consumption of available NADPH. There is now strong evidence that inactivation of NADP-MDH results from oxidation of dithiol groups on the enzyme forming a disulfide bond and that activation is due to the reversal of this reaction (Fig. 5). Earlier evidence for this view, and for this being a thioredoxin-m mediated reaction linked in turn to the redox state of the photosynthetic electron transport chain through ferredoxin, has been reviewed (Edwards et al., 1985).

Figure 5 outlines the mechanism of dark/light regulation of NADP-MDH as currently perceived. Sequence analysis has confirmed that the maize and sorghum enzymes contain a total of eight cysteine residues per subunit (Metzler et al., 1989; Issakidis et al., 1992). Evidence that dark/light regulation of the enzyme involves the oxidation-reduction of two pairs of these eight cysteine thiols (Jenkins et al., 1986; Hatch and Agostino, 1992) has been confirmed by site-directed mutagenesis studies (Issakidis et al., 1992, 1994). These latter studies provided evidence for which of the eight thiols are involved in the regulation of the enzyme; these are thiol pairs in short amino acid sequences situated at the N- and C-termini of the molecule that are unique to NADP-

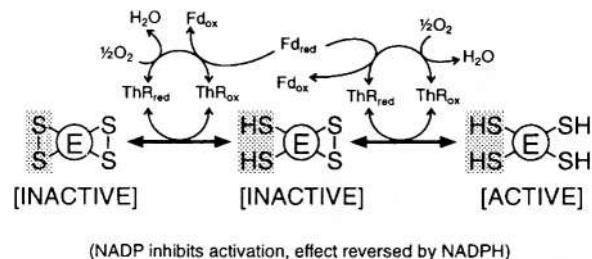


Fig. 5. Mechanism of dark/light regulation of NADP-MDH in C₄ leaves. The enzyme, E, is shown with two disulfide groups in its oxidized, inactive form. One disulfide group is more readily reduced (higher redox potential, indicated by shading) but both must be reduced to give the active form of the enzyme.

MDH. It seems likely that in the course of evolving from the NAD malate dehydrogenase gene these sequences were acquired specifically for regulation of activity. These thiol pairs apparently have different redox potentials, the one with the higher potential being readily reduced by mercaptoethanol (Hatch and Agostino, 1992). Whether this thiol pair is at the N or C-terminus remains uncertain (Issakidis et al., 1994). As inferred in Fig. 4, activation requires the reduction, or elimination by mutation, of both disulfide groups (Issakidis et al., 1994).

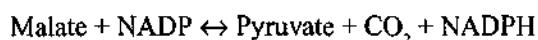
The equilibrium between active and inactive forms of NADP-MDH is under complex regulation determined by the ratio of NADPH to NADP. This ratio reflects the level of light interception, the consequent redox state of the components of non-cyclic electron flow, and the demand for NADPH. This control by the NADPH to NADP ratio depends on the extreme sensitivity of the thioredoxin-mediated activation of NADP-MDH to inhibition by NADP and the reversal of this effect by higher concentrations of NADPH (Ashton and Hatch, 1983). Similar inhibition by NADP has been shown for the activation of NADP-MDH from leaves (Scheibe and Jacquot, 1983). As a result, substantial activation of the enzyme only occurs under conditions where high ratios of NADPH to NADP prevail. From a quantitative model of this system it was predicted that a ratio of about 10 would be required to give about 25% of full activity, with steeply increasing activity as the ratio increases above 10. Later experiments confirmed these predictions (Rebeille and Hatch, 1986a). Inactivation of NADP-MDH through oxidation of thiols is also inhibited by NADP but the effect is much less since the Kd for NADP binding is much higher and inhibition is more readily reversed by NADPH (Ashton and Hatch, 1983). Another critical factor

influencing the balance between the active and inactive forms of NADP-MDH is the O₂ concentration (Edwards et al., 1985), primarily through the oxidation of reduced thioredoxin-m.

Several studies with leaves and isolated chloroplasts provide support for the mechanism outlined in Fig. 5, including the critical regulatory role of the NADPH to NADP ratio (Edwards et al., 1985). In leaves, the level of NADP-MDH activation increased under conditions favoring higher electron transport redox potential and NADPH/NADP ratio, for instance, higher light, low CO₂ and reduced O₂ levels. Likewise, with isolated mesophyll chloroplasts, treatments that would decrease the electron transport redox potential or NADPH/NADP ratio decreased the level of activation of NADP-MDH. For instance, the high NADP-MDH activities recorded with illuminated chloroplasts were very substantially reduced by the inclusion of DCMU to prevent reduction of electron transport intermediates or by adding reducible substrates such as PGA or OAA which oxidize NADPH (Hatch, 1977; Leegood and Walker, 1983; Rebeille and Hatch, 1986b).

D. NADP-Malic Enzyme

NADP-ME catalyses the oxidative decarboxylation of malate:



The enzyme is widely distributed in plants and animals and it appears that, like other C₄ enzymes, the C₄ NADP-ME evolved from an existing C₃ form (see Marshall et al., 1996). The C₄ isoform, exemplified by detailed studies on enzymes from maize, sugarcane, and *Flaveria* species (all of the NADP-ME subgroup of C₄ plants), occurs at higher activities and shows some unique properties compared with other plant NADP-MEs. Most importantly, the enzyme involved in C₄ photosynthesis is located in the chloroplasts of bundle sheath cells, in contrast to other plant NADP-MEs which are generally cytosolic. In addition to providing CO₂ to the bundle sheath, the enzyme also produces reducing equivalents, particularly important in monocot C₄ species of this subgroup which are deficient in bundle sheath Photosystem II (see Section II.A). Extracted activities are typically two- to three-fold greater than the rate of photosynthesis in NADP-ME type C₄ plants, and at least 25 times higher than the activities

in leaves of other subgroups of C₄ plants or in C₃ plants (Hatch, 1987). In addition to the C₄ isoform, which is specifically induced by light, plants of this C₄ subgroup also contain a 'constitutive' low activity cytosolic enzyme which can be measured in etiolated tissue and other non-green organs of the plant (Maurino et al., 1996). NADP-MEs of plants have been reviewed in detail (Edwards and Andreo, 1992).

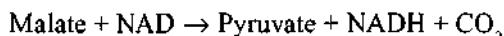
Responses of NADP-ME to varying pH, substrate, and metal ion concentrations are complex and have been implicated in regulation (Edwards and Andreo, 1992). Substrate concentration responses for the C₄ enzymes are generally hyperbolic, few metabolites have been found to affect activity, and no allosteric properties are evident. At high malate concentration (5–10 mM) activity is maximum at pH about 8.0–8.5, which may be the pH prevailing in bundle sheath chloroplasts in the light. At lower pH, 7.0–7.5, thought to be the range prevailing in darkened chloroplasts, affinity for malate is greater but activity is very low when measured at high malate concentration due to malate inhibition, also observed with enzymes from various other sources. For the maize enzyme activity declines about 10-fold between pH 8 and pH 7. Both Mg²⁺ and Mn²⁺ can serve as cofactors with the K_a for Mn²⁺ generally lower. While Mg²⁺ concentration is considered to increase in chloroplasts in the light, the situation for Mn²⁺ is uncertain. Therefore, from these properties of the enzyme it is possible that a degree of regulatory control in response to changes in light intensity may be afforded by the stromal environment. In addition, the oligomerization state of the enzyme is somewhat dependent on pH with a more active tetramer form occurring above pH 7.5 but a less active dimer form occurring at pH 7.0; buffer type, substrates and enzyme concentrations, and the presence of reductants also influence this interconversion (Edwards and Andreo, 1992). Although isolated NADP-MEs generally do not require the presence of reduced thiol compounds for activity or to maintain stability, a possible role for thiol/disulfide interchange in regulation of the maize enzyme has been suggested (Drincovich and Andreo, 1994).

While the maize enzyme is only inhibited weakly by the reaction products CO₂ and pyruvate, the enzyme mechanism indicates that NADPH should be a competitive inhibitor. Consequently, a major factor determining short-term regulation of C₄ NADP-MEs may be the NADP/NADPH ratio (Asami et al., 1979; Edwards and Andreo, 1992). In bundle sheath chloroplasts, malate decarboxylation by NADP-ME

is tightly coupled to PGA reduction, by NADP/NADPH cycling. Rapid control may be imposed on NADP-ME under any condition where turnover of the C₃ carbon reduction cycle is decreased. For example, on transfer from high to low light, any decrease in PGA (due to lowered ATP concentration and lower rate of RuBP carboxylation) and consequent transient increase in NADPH/NADP ratio, would decrease NADP-ME activity. This effect could be compounded by a large decline in activity if this change is accompanied by a shift of stromal pH from 8 to 7.5 or less (see above). It is possible that in the dark the poise of the NADPH/NADP ratio, combined with the lower pH, allows other biosynthetic reactions to occur but prevents malate decarboxylation.

E. NAD-Malic Enzyme

NAD-ME catalyses the decarboxylation of malate to produce pyruvate and CO₂:



This mitochondrial enzyme is present in all higher plants and functions in respiration, allowing the tricarboxylic acid cycle to operate when glycolytic input of pyruvate is low (see Wedding, 1989). In C₄ plants, NAD-ME is located in the bundle sheath mitochondria of both NAD-ME and PCK-types and serves to decarboxylate malate transported from the mesophyll cells, providing CO₂ for Rubisco (see Fig. 2). Activities of this enzyme in NAD-ME-type C₄ leaves range from 18 to 60 times that in C₃ leaf tissue, to cope with the high flux required in photosynthesis (Hatch, 1987). In PCK-types NAD-ME primarily provides NADH for the generation of ATP for the PEPCK reaction via mitochondrial electron transport (Hatch et al., 1988; Carnal et al., 1993). Many of the regulatory properties of the C₄ NAD-ME are common to the non-photosynthetic enzyme and the relevance of these to the C₄ pathway is discussed below.

Regulation of the C₄-acid decarboxylating enzymes could provide an effective control point for the coordination of the mesophyll C₄ cycle and the photosynthetic carbon reduction pathway in the bundle sheath. Since the NAD-ME reaction occurs in a non-photosynthetic compartment, i.e. the mitochondrion, some regulatory communication with other photosynthetic enzymes might be expected. Accordingly, the regulatory properties of NAD-ME

have been well characterized. NAD-ME fulfills most of the criteria for a classical ‘regulatory’ enzyme (Monod et al., 1965) and is allosterically activated by a range of metabolites including acetyl-CoA, CoA and FBP (reviewed in Artus and Edwards, 1985). Although never definitively shown in C₄ plants, the C₃ and CAM enzyme can be interconverted in vitro between a low activity dimer and higher activity tetramers and octamers (see Wedding, 1989). The proportions of these polymeric forms of the enzyme are affected by the protein concentration, the ionic strength and possibly the presence or absence of activators (Wedding, 1989). Demonstrations of the relevance of this to the in vivo regulation of the enzyme are technically difficult and often unconvincing. However there is some evidence that in C₄ plants the enzyme in vivo is an octamer (Murata et al., 1989a,b).

The C₄ NAD-ME from different species falls broadly into 3 groups (see Furbank et al 1991 and references therein). The enzyme from NAD-ME-type dicotyledonous plants generally show highly sigmoidal responses to the concentration of the substrate malate and a K-type (Monod et al., 1965) allosteric activation while the enzyme from NAD-ME -type monocotyledonous plants shows simple hyperbolic kinetics and mainly V-type activation (the V_{max} increases with activation rather than the K_m decreasing). NAD-ME from PCK-type species shows a mixed kinetic behavior. Potentially, in cases where extreme K-type kinetics are evident, sensitive regulation could be achieved by small changes in the levels of malate or FBP. The relevance of activation by acetyl CoA, CoA and FBP in vivo is, however, difficult to ascertain as very few studies have been made of metabolite levels in the relevant compartments of C₄ leaves. Under optimal conditions, CoA has little effect above 50 μM while mitochondrial pools could be as high as 200 μM (see Furbank et al., 1991) and FBP has not been measured in mitochondria.

Metabolites which may be of more physiological relevance in affecting NAD-ME activity are the adenylates. Furbank et al. (1991) showed that the enzyme from both NAD-ME monocots and dicots was inhibited by ATP and to a lesser degree ADP and AMP. This may be of particular relevance to the K-type enzymes where ATP at physiological levels could considerably increase the K_m for malate, essentially sensitizing the enzyme to falling malate levels when adenylate energy charge is low (low

light for example). The regulation of NAD-ME in PCK-type species is interesting as, although the enzyme acts to release CO₂ in the bundle sheath, its primary role is to provide NADH to the mitochondrion for generation of ATP (see above). NAD-ME from the PCK-type species *Urochloa panicoides* is strongly activated, not inhibited, by ATP. This may provide positive feedback to C₄ acid decarboxylation by NAD-ME in the following way. When PEP-carboxykinase is operating at full capacity under conditions of high photosynthetic flux (high light for example), both ATP and OAA concentrations may increase in the bundle sheath cells. This could increase the rate of OAA entry into the mitochondria and hence its rate of reduction by NAD malate dehydrogenase with associated generation of NAD and an increase in the malate pool. This, combined with ATP activation of NAD-ME could accelerate the flux of carbon through NAD-ME providing extra CO₂ (Furbank et al., 1991).

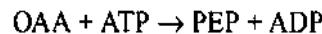
An added complication to the regulation of NAD-ME is the absolute requirement of the C₄ enzyme for Mn⁺⁺ (Hatch et al., 1974) and the observation that there is a strong allosteric interaction between Mn⁺⁺ levels and activators (Murata et al., 1989; Furbank et al., 1991). In vitro, in the absence of activator, millimolar concentrations of Mn⁺⁺ are required for full activity, reducing to micromolar levels in the presence of saturating CoA (Murata et al., 1989a,b). Levels of total Mn⁺⁺ in mitochondria are probably up to 400 μM but free concentrations are difficult to estimate and could be sub-micromolar (see Furbank et al., 1991) making extrapolation of the regulatory properties of this enzyme to the in vivo case very difficult.

An obvious potential regulatory mechanism for NAD-ME would be inhibition by CO₂ or bicarbonate. This would provide an effective negative feedback loop from CO₂ utilization by Rubisco. Evidence for CO₂ or HCO₃⁻ inhibition of NAD-ME is variable. The enzyme from *Atriplex spongiosa* and *Amaranthus edulis* is allosterically inhibited by HCO₃⁻ in the physiological range whereas the enzyme from *Panicum miliaceum* is largely unaffected (Chapman and Hatch, 1977). Murata et al., (1989a) found that the enzyme from *Amaranthus tricolor* showed no inhibition by HCO₃⁻ and inhibition by HCO₃⁻ of NAD-ME from the PCK-type species *Urochloa panicoides* was quite weak (Burnell, 1987). It may be that strong product inhibition by HCO₃⁻ is restricted to the NAD-ME-type species with a kinetically 'K-type' enzyme

(see above) but it should also be noted that the degree of inhibition is also pH dependent (Jenkins et al., 1987).

F. PEP-Carboxykinase

PEPCK catalyses the ATP-dependent decarboxylation of oxaloacetate producing PEP and ADP:



This enzyme is the predominant decarboxylating enzyme in the PCK-type C₄ subgroup (see Fig. 2) and is located in the cytosol of bundle sheath cells. PEPCK has been purified from a number of species (Burnell, 1986) and appears to be a hexameric protein in vivo with a subunit molecular mass of about 64 kDa. Like NAD-ME, this enzyme has several kinetic properties which flag it as a possible regulatory enzyme. Interestingly, PEPCK, like NAD-ME, has a strict requirement for Mn⁺⁺ as a divalent metal cofactor and shows allosteric control involving this cation (Burnell, 1986). PEPCK has a binding site for MnATP²⁻ in addition to a separate binding site for free Mn⁺⁺. The binding of free Mn⁺⁺ influences the interaction of the enzyme with several of its metabolite effectors. PEPCK is inhibited 50–60% by PGA and FBP at concentrations around 5 mM (Hatch and Mau 1977; Burnell 1986). PGA increases the K_m of the enzyme for MnATP²⁻. These levels of metabolites are in the physiological range for PGA but well above measured concentrations of FBP in leaves. Dihydroxyacetone phosphate also has an inhibitory effect at physiological levels (50% at 1 mM) and appears to inhibit binding of the regulatory Mn⁺⁺. Bicarbonate is only a weak inhibitor of this enzyme.

Once again, because of the lack of measurements of metabolite levels in defined compartments of C₄ leaves in response to environmental changes, it is difficult to put these potential regulatory mechanisms into a physiological perspective. It is worth noting, however, that due to the low K_m of PEPCK for OAA, it has been postulated that a mechanism must exist for inactivating the enzyme in the dark (Carnal et al., 1993), otherwise cellular pools of OAA would be depleted. One hypothesis is that cytosolic Mn⁺⁺ levels may follow the light and dark changes in other chloroplastic cations, providing an effective light regulation of both PEPCK and NAD-ME (see Furbank et al., 1991; Carnal et al., 1993).

Recently it has been demonstrated that if rigorous

precautions are taken to prevent proteolysis after extraction, the native molecular weight of PEPCK in many C₄ plants is about 68–72 kDa (Walker and Leegood 1996). PEPCK is rapidly cleaved by proteolysis, removing an N-terminal extension which results in a protein of molecular weight of 62–64 kDa, the form previously reported in the literature. This higher molecular weight form is consistent with the deduced amino acid sequence of the *Urochloa panicoides* enzyme (Finnegan and Burnell 1995). It appears that this longer form of PEPCK is phosphorylated on darkening of leaves from some but not all C₄ species (Walker and Leegood 1996; Walker et al 1997). This covalent modification may play an important role in regulation of the enzyme in vivo, although it is not yet clear how phosphorylation affects the kinetic properties of the enzyme.

G. Enzymes of the Photosynthetic Carbon Reduction Cycle

The enzymes of the PCR cycle in C₄ plants are largely regulated in a similar manner to their C₃ counterparts (Ashton et al., 1990). For example, the three PCR cycle enzymes activated by light through the thioredoxin mediated reduction of disulfide bridges presumably play a similar role in C₄ plants, regulating fluxes and pool-sizes in the bundle sheath chloroplast. However, from metabolite pool-size measurements in maize and *Amaranthus*, Leegood et al., (1989) suggest that thiol mediated regulation may be less important in regulating photosynthetic flux in C₄ species. For example, FBP levels in maize do not rise as light intensity is decreased, as they do in C₃ leaves (Leegood et al., 1989), suggesting that FBPase is not down-regulated in the former case.

Worthy of special mention in the context of C₄ plants, however, is Rubisco. Regulation of this enzyme might be expected to differ from that in C₃ plants due to the high CO₂ environment of the bundle sheath cell and the unique kinetic characteristics of the C₄ Rubisco (Andrews and Lorimer 1987). It has been shown that C₄ plants contain Rubisco activase (Salvucci et al., 1987) and the tight binding 'night-time inhibitor' of Rubisco, carboxyarabinitol 1-phosphate (Moore et al., 1991). There are, however, very few studies on Rubisco regulation in intact C₄ leaves. In one of these studies, Sage and Seemann (1993) showed that in most C₄ species rubisco is regulated by reversible carbamylation of catalytic sites but the degree of deactivation of rubisco seen at

low light varied considerably between species.

IV. Integrated Regulation of C₄ Photosynthesis

A. Rate Limiting Enzymes in the C₄ Pathway

Because of the complexity of the C₄photosynthetic pathway it has been difficult to determine which enzymes control photosynthetic flux under a particular set of environmental conditions. In C₃ plants, the relative pool sizes of PGA, triose phosphates and RuBP can be easily measured in leaves in response to CO₂ and light and there is a detailed knowledge of the kinetics and regulation of Rubisco. In addition, several laboratories have generated transgenic plants with reduced levels of key enzymes in photosynthesis and applied control theory to assess the role these enzymes play in determining photosynthetic rate (see Furbank and Taylor, 1995 for a review). Using these measurements and models ofphotosynthesis it has been determined that in C₃plants Rubisco is the major determinant of photosynthetic flux in air, at least in high light. In C₄ plants, Rubisco is not in direct communication with the atmosphere and less is known about its regulation. In addition, pools of metabolites are partitioned between organelles of the mesophyll and bundle sheath cells and there is the added complexity of the possible control of flux by the mesophyll localized enzymes of the C₄ cycle.

Until recently, the contribution of individual enzymes of the C₄ pathway to the control of photosynthetic flux has been estimated by correlating extracted enzyme activities with photosynthesis rates in comparisons between species or genotypes or within a species under different growth conditions (Furbank et al., 1997a). Experiments of this kind have provided evidence that Rubisco, PEPC, PPDK, NADP-MDH, NADP-ME and NAD-ME are all positively correlated with photosynthetic capacity (Furbank et al., 1997a). These results do not, however, provide definitive evidence that any ofthese enzymes limit or co-limit photosynthetic flux. Many other parameters may co-vary in these experiments and a strong positive correlation with photosynthetic performance and enzyme activity does not prove a causal relationship.

More recently, with the advent of efficient genetic transformation of the NADP-ME-type C₄ dicot

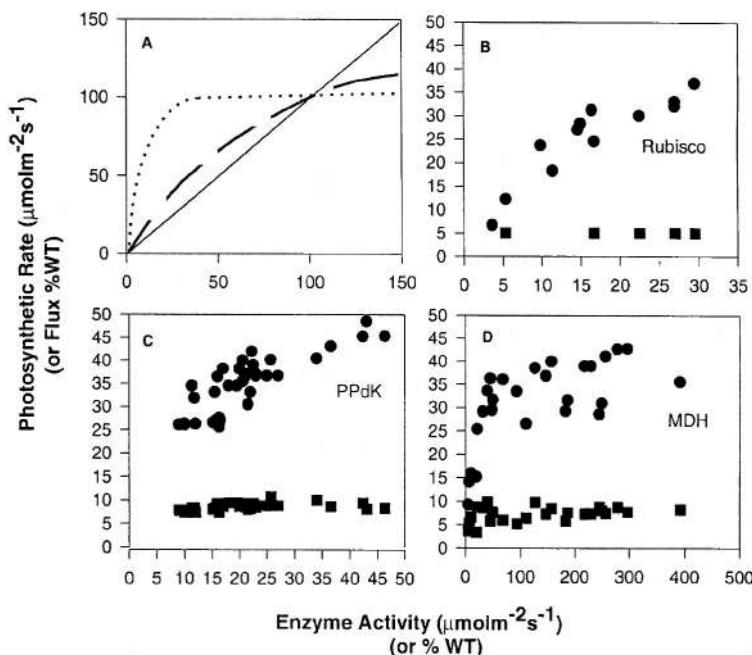


Fig. 6. Shows: A: The theoretical relationship between flux and enzyme activity in a series of transformed plants where a single enzyme in a pathway has been either reduced or increased in activity. The dotted line predicts the response for a non-limiting enzyme, the dashed line for a 'co-limiting' enzyme and the solid line a classically limiting enzyme ($C_j=1$); B, C and D show the relationship between photosynthetic flux at high light (●) and limiting light (■), in air, and extractable enzyme activity in leaves of *Flaveria* transformants with reduced levels of Rubisco, PPDK or NADP-MDH respectively (Furbank et al., 1997a).

Flaveria bidentis (Chitty et al., 1994), it has been possible to apply antisense RNA technology to address this question more precisely by altering levels of a single photosynthetic enzyme in the C₄ pathway. As discussed above, antisense RNA technology has proven very useful in combination with metabolic control theory, in determining the role of an enzyme in controlling flux through metabolic pathways in C₄ plants (recently reviewed in Furbank and Taylor 1995; Stitt 1995). Figure 6A shows the expected response of flux through a pathway when the activity of a single enzyme is decreased or increased. In the case of a classical 'rate limiting' enzyme, flux is linearly related to enzyme level, at least up to and slightly above wild-type activities. If the enzyme is 'non-limiting', transformants with greatly reduced levels of the enzyme will still support the same flux as wild-type individuals. In reality, enzymes tend to be 'co-limiting' (i.e. a curvilinear relationship between enzyme level and flux). In the case of co-limiting enzymes, their contribution to the control of flux is given by the tangent to the curve of flux versus enzyme activity at close to wild-type enzyme levels. This slope is the 'control coefficient,' C_j (Kacser and

Burns 1973). An enzyme with C_j = 1 has 100% control over flux while a C_j of zero indicates no control. Using the *Flaveria* transformation system, transgenic plants have been produced with reduced levels of Rubisco (Fig. 6B; Furbank et al., 1996), PPDK (Fig. 6C; Furbank et al., 1997a) and NADP-malate dehydrogenase (Fig. 6D; Trevanion et al., 1997). Using this analysis, we determined that under high light and atmospheric CO₂ levels, C_j for Rubisco was 0.6 (or greater) while for PPDK, C_j was between 0.2 and 0.4 and for NADP-MDH, C_j was zero (Furbank et al., 1997a). This control strength for Rubisco is similar to that determined for the C₃ plant tobacco under saturating illumination (Furbank and Taylor, 1995). As light intensity was decreased, the control of photosynthesis by both PPDK and Rubisco quickly fell to zero as the provision of light generated ATP and reductant presumably became limiting. These results indicate that at high light intensities, control of photosynthetic flux in C₄ plants is predominantly shared between Rubisco and PPDK with the bulk of control residing with the former enzyme. Recent evidence from mutants of PEPC and NAD-ME in *Amaranthus* suggests that the control

strength of PEPC may also be significant and of the same magnitude as PPDK under high light conditions (Dever et al., 1995, 1997).

It is interesting to note that NADP-MDH levels in *Flaveria* leaves are far in excess of those required for photosynthesis even when activation state in vivo is taken into account (Trevanion et al., 1997; Furbank et al., 1997a). Despite a complex covalent regulation mechanism which causes NADP-MDH activity to track photosynthetic rate, at no time is it 'rate limiting' (Furbank et al., 1997a). This observation shows the danger in using correlative evidence on enzyme levels and photosynthetic rate in wild-type plants to determine the role of an enzyme in controlling flux. It also poses the question of the necessity for regulation of these 'non-limiting' enzymes. We recently suggested (Furbank et al., 1997a) that conservation of metabolite levels may be the reason such complex regulation has evolved. For instance, the K_m of NADP-MDH for NADPH is quite low (around 50 μM) and without redox regulation of NADP-MDH and the complex regulation of activity by NADPH/NADP ratio (see Section III.C. and Fig. 5), chloroplast NADPH pools would be depleted to very low levels and the NADPH/NADP ratio would decline to around 0.05 (calculated in Furbank et al., 1997a). Such low levels of NADPH would effectively starve other biosynthetic processes and could cause instability during transients such as light-flecks, for example. A similar regulatory process has previously been proposed in C_3 plants for the enzymes of the PCR cycle (Woodrow et al., 1985) and dark inactivation of other high activity, low K_m enzymes of the C_4 pathway may be necessary (see above).

B. Coordination of the C_3 and C_4 Cycles

Although the mesophyll 'CO₂pump' cycle essentially operates independently of the PCR cycle in the bundle sheath, there are a number of biochemical links between the two cell types which could provide co-ordinate regulation of the two cycles. The most basic level of coordination is provided by the relative amounts of PEPC and Rubisco present in the leaf. These ratios can vary considerably between species (von Caemmerer and Furbank, 1999) but as the maximum extractable activity of PEPC in most C_4 leaves is far above the photosynthetic flux, the significance of this observation is uncertain. Changes in the levels of C_4 enzymes in response to

environmental conditions will be dealt with separately, but one would expect the co-ordinate regulation of expression of photosynthetic enzymes to be under strong genetic control (see Furbank and Taylor 1995). The following discussion will be confined to potential co-ordinate enzyme regulation and communication between the cell-types due to the levels and movement of metabolites.

Evidence for fine co-ordinate control of mesophyll and bundle sheath reactions is rare. Recent work using transgenic *Flaveria* with reduced levels of Rubisco (Furbank et al., 1996; von Caemmerer et al., 1997) suggests that unless Rubisco is reduced to quite low levels, no large-scale co-ordinate down-regulation of PEP carboxylation occurs. This is evidenced by the fact that with moderate reductions in the level of Rubisco, the initial slope of the A versus Ci response curve, determined by the kinetics and amount of PEPC, is unaffected (von Caemmerer et al., 1997). In these low Rubisco plants, if no down-regulation of the mesophyll reactions were to occur, CO₂ should build up in the bundle sheath compartment. This in fact does happen but to a lesser degree than that predicted by modeling of carbon isotope discrimination and photosynthesis (von Caemmerer et al., 1997), suggesting that some down-regulation of flux through the C_4 cycle does occur in the transformants. On balance, it appears that some regulatory coupling of the C_3 and C_4 cycles is occurring in these plants but the coupling is by no means absolute.

One way in which the C_3 and C_4 cycles could be in regulatory communication is through the traffic of metabolites and in particular through the interconversion of PEP and the 3-carbon sugar phosphates. In this case, it has been shown that during photosynthetic induction in maize leaves, large pools of triose phosphate are built up by carbon draining from the C_4 acid pool to PEP and then to PGA via phosphoglycerate mutase and enolase (Leegood and Furbank, 1984; Furbank and Leegood, 1984). There is also evidence for this pathway operating in sugarcane from randomization of label in the early ¹⁴C labeling experiments of Hatch and Slack (1966) and from metabolite measurements in leaves of *Amaranthus edulis* at varying CO₂ concentrations and irradiances (Leegood and von Caemmerer, 1988). This mechanism may be particularly important in NADP-ME type monocots. In many of these species there is little or no Photosystem II in the bundle sheath chloroplasts (Hatch, 1987), necessitating a

large traffic of PGA to the mesophyll chloroplasts for reduction. If this pool were to exchange freely with the mesophyll PEP pool, flux through the two pathways could be co-ordinated. In addition, this traffic of PGA and triose phosphate between the cell types could modulate activity of PEPC as triose phosphate (and hexose phosphates produced during sucrose synthesis in the mesophyll) are potent activators of this enzyme (Leegood et al., 1989). Additional coordination could operate at the level of reducing power in NADP-ME-type species because NADP-ME in the bundle sheath chloroplast generates part of the NADPH used in the PCR cycle (Fig. 2 and Hatch, 1987).

Another possible regulatory link between the C₃ and C₄ cycles is through regulation of C₄ acid decarboxylation. This could occur at two levels: firstly, by direct modulation of enzyme activity, largely dealt with in section III, and, secondly, through control of metabolite transport into the bundle sheath organelles. In the NAD-ME-type and NADP-ME-type species where C₄ acid decarboxylation occurs in the mitochondrion and the chloroplast, respectively, the transport of C₄ acids could be controlled by the levels of co-transported or antiported metabolites (Fig. 2 and Chapter 20, Dengler and Taylor). In the case of the NAD-ME types, this may be of particular relevance through the transport of aspartate and 2-oxoglutarate into the mitochondrion (Hatch, 1987). In experiments with both isolated mitochondria and bundle sheath cells from these species, malate and Pi must be added together with aspartate and 2-oxoglutarate to support high rates of pyruvate production (Furbank et al., 1990a). It has been proposed that the Pi and malate requirements are due to the counter exchange of 2-oxoglutarate and malate which in turn depends upon a Pi/malate antiporter (Furbank et al., 1990a). Although there is no net consumption of malate, this mechanism would provide a sensitive regulatory mechanism responsive both to cytosolic Pi (allowing positive feedback from sucrose biosynthesis) and malate levels. Malate is a significant component of the C₄ acid pool formed during photosynthesis in NAD-ME types (although aspartate is the predominant C₄ acid transported in this type) and the ratio of malate to aspartate formed would be responsive to chloroplast redox state through the activity of mesophyll NADP-MDH. Since malate levels in the bundle sheath would be in equilibrium with those in the mesophyll, malate level could provide both a feed-back and feed-forward regulatory

mechanism, indirectly responsive to light.

In summary, there is circumstantial evidence for coordinate regulation of the C₃ and C₄ cycles in vivo and, intuitively, one would expect some regulation of the flux in the two compartments. However, we are still some way from understanding how these processes operate in an intact leaf.

C. Regulation in Response to Light Intensity

A useful approach to understanding integrated regulation of C₄ photosynthesis has been to examine the response of photosynthetic rate, enzyme activity and metabolite levels to changes in light intensity (reviewed by Leegood et al., 1989). Using these techniques to examine the response of photosynthesis in maize to a step change in irradiance from 1700 to 140 $\mu\text{mol m}^{-2}\text{s}^{-1}$, Leegood et al. (1989) concluded that flux immediately following this transition was predominantly determined by ATP supply, not enzyme regulation. For example, they observed a rapid build up in pyruvate but only a slow reduction in PPDK activity to about 50% of high light values. Triose phosphate levels rapidly declined in low light, coincident with a fall in thylakoid energization, also supporting the hypothesis that ATP became limiting.

An examination of leaf metabolite levels at steady-state over the light response curve in C₄ plants in air shows a strong correlation between triose phosphate pools and photosynthetic flux (Leegood et al., 1989 and references therein). PEP levels on the other hand remain remarkably constant, prompting the authors to conclude that PEPC activity may be regulated to match photosynthetic flux through changes in levels of the activator triose phosphate.

Co-ordinate regulation of electron transport and carbon metabolism in C₄ plants appears to follow much the same pattern as in C₃ photosynthesis. Photosynthetic control of electron transport has been shown to operate in isolated maize mesophyll chloroplasts both at the level of proton back-pressure, reducing the rate of inter-system electron transfer and to a lesser degree via negative feedback on the quantum efficiency of Photosystem II, resulting from a high thylakoid ΔpH (Furbank 1988, Foyer et al., 1990). As quite high light intensities are required to saturate photosynthesis in C₄ plants, it is likely that regulation of photosynthesis by ATP supply and the interplay between electron transport and carbon metabolism could be of great importance in C₄ plants over a wide range of normal growth irradiances.

D. Adaptive Responses to Environmental Changes

Regulation of C₄ photosynthesis in terms of its longer term adaptive response to environmental variables such as light and temperature, as well as major nutrients such as nitrogen and phosphate will be considered briefly here. It is worth noting that when plants are exposed to suboptimal light, temperature, or nutrient levels during their whole growth period, photosynthesis is almost invariably reduced. However, many components of photosynthesis can be affected under such conditions and in C₄ plants effects are not generally specific to the C₄ processes. In such situations it is very difficult to dissect out any effects of the altered environmental parameter on C₄ processes specifically. A potentially more useful approach to assess these effects has been to examine changes in photosynthesis and the C₄ photosynthetic components in the short term (within the range of minutes to days) in response to an environmental change, after growth under an alternative condition. Relatively fewer such studies have been carried out.

An exception to this is the response of C₄ photosynthesis to low temperature, which has been extensively examined. In general, C₄ plants respond poorly to low temperature and photosynthetic efficiency becomes lower than that of C₃ species as temperature decreases (see Section II.B). The day and night minimum temperature during growth appears to be a major determinant of the distribution of C₄ plants, both in their natural ecosystems and in agricultural situations (Chapter 22, Rawsthorne and Monson). Because of this it has been tempting to consider that the poor growth at low temperature is closely related to C₄ physiology or biochemistry (reviewed by Long, 1983). However, despite many correlative studies of the low temperature sensitivity of a number of C₄ processes, no single causative factor or mechanism has clearly emerged. Physiological processes suggested to be involved in this low temperature sensitivity of C₄ plants have included the sensitivity of specific enzymes to low temperature, particularly PPDK and PEPC (Long, 1983; Usami et al., 1995; Krall and Edwards, 1993), translocation processes (Long, 1983), and, in earlier studies, carbohydrate loading in the bundle sheath chloroplasts leading to inhibition of photosynthesis (summarized by Ku et al., 1978). PEPCK, as well as PEPC has been implicated in a recent comparison of cold-sensitive and tolerant PCK-type species (Matsuba et

al., 1997). Whether there may be a more subtle indirect effect of low temperature on a C₄-specific process, for example, regulation of gene expression, or an enzyme regulatory mechanism has not yet been explored adequately. In relation to this, specific effects of low temperature on activation of C₄ enzymes, particularly PPDK, were observed in maize and sorghum (Taylor et al., 1974) and, recently, in *Echinochloa crusgalli* populations (Simon and Hatch, 1994). The low temperature effects on C₄ photosynthesis and growth of C₄ plants have been reviewed by several authors (Berry and Bjorkman, 1980; Miedema, 1982; Long, 1983).

Regarding regulation of C₄ photosynthesis in response to varying light intensity, there will be changes in the short term which presumably involve coordination between the C₄ and C₃ cycles, as discussed earlier in this section. The possible influence of light intensity in the regulation of activity of particular C₄ cycle enzymes, particularly PEPC, PPDK, and NADP-MDH, has been covered in Section III. In addition to these effects, adaptive changes of C₄ cycle enzyme activities have been shown to occur over several days, in response to changes in light intensity at which the plants are growing. In mature leaves, transfer from low to high light was accompanied by increases in PEPC and PPDK and on transfer from high to low light corresponding decreases occurred (Hatch et al., 1969). Interestingly, changes in RuBP carboxylase and other C₃ cycle enzymes were less marked. Later studies of acclimation during growth under various light intensities have also indicated a degree of regulation of the levels of C₄ enzymes (Usuda et al., 1985; Ward and Woolhouse, 1986). It is not established whether this control of enzyme level in mature leaves is primarily at the level of transcription or protein turnover. However, during greening of etiolated leaves, or in early leaf development, control of enzyme synthesis for most, if not all, C₄ enzymes appears to be transcriptional (Chapter 21, Pearcy and Sage). While irradiance itself may be the primary signaling factor controlling the level of C₄ cycle enzymes, expression of the genes for PEPC, PPDK, carbonic anhydrase and Rubisco are also affected by nutrient status and developmental cues, indicating that regulation is likely to be complex. In a C₄ *Flaveria* species, enzyme level appeared to respond to supplied carbohydrate rather than light directly (Furbank et al., 1997b).

The effects of changes in the supplied levels of the

nutrients, inorganic nitrogen and phosphate, on photosynthesis and enzymes in C₄ plants have also been examined. Phosphate deficiency dramatically decreased photosynthesis in maize, with most photosynthetic enzymes declining, including PPDK, PEPC and many C₃ cycle enzymes, in contrast to other cytosolic enzymes such as sucrose-phosphate synthase and UDP-glucose pyrophosphorylase (Usuda and Shimogawara, 1992). PEPC, PPDK and carbonic anhydrase protein levels also respond to increasing nitrate, with control at the transcriptional level since mRNA also increases (Sugiyama et al., 1984; Yamazaki et al., 1986; Burnell et al., 1990; Sugiharto et al., 1992). As in C₃ plants, photosynthetic capacity of C₄ plants is clearly related to the level of N-supply during growth (Wong, 1979).

References

- Agostino A, Heldt HW and Hatch MD (1996) Mitochondrial respiration in relation to photosynthetic C₄ acid decarboxylation in C₄ species. *Aust J Plant Physiol* 23: 1–7
- Andreo CS, Gonzalez DH, and Iglesias AA (1987) Higher plant phosphoenolpyruvate carboxylase: Structure and regulation. *FEBS Lett* 213: 1–8
- Andrews TJ and Lorimer GH (1987) Rubisco: Structure, mechanisms and prospects for improvement. In: Hatch MD and Boardman NK (eds) *The Biochemistry of Plants*, Vol 10, pp 132–211. Academic Press, London
- Arrio-Dupont M, Bakrim N, Echevarria C, Gadál P, Le Marechal P, and Vidal J (1992) Compared properties of phosphoenolpyruvate carboxylase from dark- and light-adapted *Sorghum* leaves: Use of rapid purification technique by immunochromatography. *Plant Sci* 81: 37–46
- Artus NN and Edwards GE (1985) NAD malic enzyme from plants. *FEBS Lett* 182: 225–233
- Asami S, Inoue K and Akazawa T (1979) NADP-malic enzyme from maize leaf: Regulatory properties. *Arch Biochem Biophys* 196: 581–587
- Ashton AR and Hatch MD (1983) Regulation of C₄ photosynthesis: Regulation of activation and inactivation of NADP malate dehydrogenase by NADP and NADPH. *Arch Biochem Biophys* 227: 416–24
- Ashton AR, Burnell JN, Furbank RT, Jenkins CLD and Hatch MD (1990) Enzymes of C₄ photosynthesis. In: Lea PJ (ed) *Methods in Plant Biochemistry*, Vol 3, Enzymes of Primary Metabolism, pp 39–72. Academic Press, London
- Bakrim N, Prioul J-L, Deleens E, Rocher J-P, Arrio-Dupont M, Vidal J, Gadál P and Chollet R (1993) Regulatory phosphorylation of C₄ phosphoenolpyruvate carboxylase: A cardinal event influencing the photosynthesis rate in *Sorghum* and Maize. *Plant Physiol* 101: 891–897
- Berry J and Björkman O (1980) Photosynthetic response and adaptation to temperature in higher plants. *Ann Rev Plant Physiol* 31: 491–543
- Budde RJA and Chollet R (1986) In vitro phosphorylation of maize leaf phosphoenolpyruvate carboxylase. *Plant Physiol* 82: 1107–1114
- Budde RJ, Holbrook GP and Chollet R (1985) Studies on the dark/light regulation of maize leaf pyruvate, Pi dikinase by reversible phosphorylation. *Arch Biochem Biophys* 242: 283–290
- Budde RJ, Ernst SM and Chollet R (1986) Substrate specificity and regulation of the maize (*Zea mays*) leaf ADP:protein phospho-transferase catalysing the inactivation of pyruvate, orthophosphate dikinase. *Biochem J* 236: 579–584
- Burnell JN (1986) Purification and properties of phosphoenolpyruvate carboxykinase from C₄ plants. *Aust J Plant Physiol* 13: 577–587
- Burnell JN (1987) Photosynthesis in phosphoenolpyruvate carboxykinase-type C₄ species: Properties of NAD-malic enzyme from *Urochloa panicoides*. *Aust J Plant Physiol* 14: 517–525
- Burnell JN and Hatch MD (1985a) Light-dark modulation of leaf pyruvate, Pi dikinase. *Trends Biochem Sci* 10: 288–291
- Burnell JN and Hatch MD (1985b) Regulation of C₄ photosynthesis: purification and properties of the protein catalysing ADP-mediated inactivation and P_i-mediated activation of pyruvate, P_i-dikinase. *Arch Biochem Biophys* 237: 490–503
- Burnell JN and Hatch MD (1986) Activation and inactivation of an enzyme catalysed by a single bifunctional protein: A new example and why. *Arch Biochem Biophys* 245: 297–304
- Burnell JN and Hatch MD (1988) Photosynthesis in phosphoenolpyruvate carboxykinase-type C₄ plants: Pathways of C₄ acid decarboxylation in bundle sheath cells of *Urochloa panicoides*. *Arch Biochem and Biophys* 260: 187–199
- Burnell JN, Jenkins CLD and Hatch MD (1986) Regulation of C₄ photosynthesis: The role of pyruvate in regulating pyruvate, Pi dikinase in vivo. *Aust J Plant Physiol* 13: 203–210
- Burnell JN, Suzuki I and Sugiyama T (1990) Light induction and the effect of nitrogen status upon the activity of carbonic anhydrase in maize leaves. *Plant Physiol* 94: 384–387
- Carnal NW, Agostino A and Hatch MD (1993) Photosynthesis in phosphoenolpyruvate carboxykinase-type C₄ plants: Mechanism and regulation of C₄ acid decarboxylation in bundle sheath cells. *Arch of Biochem and Biophys* 306: 360–367
- Chapman KSR and Hatch MD (1977) Regulation of mitochondrial NAD-malic enzyme involved in C₄ pathway photosynthesis. *Arch Biochem Biophys* 184: 298–306
- Chapman KSR, Berry JA and Hatch MD (1980) Photosynthetic metabolism in the bundle sheath cells of *Zea mays*: Sources of ATP and NADPH. *Arch Biochem Biophys* 202: 330–341
- Chastain CJ, Thompson BJ and Chollet R (1996) Maize recombinant C₄-pyruvate, orthophosphate dikinase: Expression in *Escherichia coli*, partial purification, and characterization of phosphorylatable protein. *Photosyn Res* 49: 83–89
- Chitty JA, Furbank RT, Marshall JS, Chen Z and Taylor WC (1994) Genetic transformation of the C₄ plant *Flaveria bidentis*. *Plant J* 6: 949–956
- Chollet R, Vidal J and O'Leary MH (1996) Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47:273–298
- Dever LV, Blackwell RD, Fullwood NJ, Lacuesta M, Leegood RC, Onek LA, Pearson M. and Lea PJ (1995) The isolation and characterization of mutants of the C₄ photosynthetic pathway. *J Exp Bot* 46: 1363–1376

- Dever LV, Leegood RC and Lea PJ (1997) Control of photosynthesis in *Amaranthus edulis* mutants with reduced amounts of PEP carboxylase. *Aust J Plant Physiol* 24: 469–476
- Doncaster HD and Leegood RC (1987) Regulation of phosphoenolpyruvate carboxylase activity in maize leaves. *Plant Physiol* 84: 82–87
- Drincovich MF and Andreo CS (1994) Redox regulation of maize NADP-malic enzyme by thiol/disulfide interchange: Effect of reduced thioredoxin on activity. *Biochim Biophys Acta* 1206: 10–16
- Duff SMG, Giglioli-Guivarc'h N, Pierre J-N, Vidal J, Condon SA and Chollet R (1996) In situ evidence for the involvement of calcium and bundle-sheath derived photosynthetic metabolites in the C₄ phosphoenolpyruvate carboxylase kinase signal-transduction chain. *Planta* 199: 467–474
- Edwards GE and Andreo CS (1992) NADP-malic enzyme from plants. *Phytochemistry* 31: 1845–1857
- Edwards GE and Walker DA (1983) C₃, C₄: Mechanisms and Cellular and Environmental Regulation of Photosynthesis. Blackwell Scientific Publications, Oxford
- Edwards GE, Huber SC, Ku SB, Rathnam CK, Gutierrez M and Mayne BC (1976) Variations in photochemical activities of C₄ plants in relation to CO₂ fixation. In: Burris RH and Black CC (eds) CO₂ Metabolism and Plant Productivity, pp. 83–112. University Park Press, Baltimore
- Edwards GE, Nakamoto H, Burnell JN and Hatch MD (1985) Pyruvate, Pi dikinase and NADP malate dehydrogenase in C₄ photosynthesis. *Annu Rev Plant Physiol* 36: 255–286
- Ehleringer J and Monson R (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. *Annu Rev Ecol Syst* 24: 411–439
- Ehleringer J and Pearcy RW (1983) Variations in quantum yield for CO₂ uptake in C₃ and C₄ plants. *Plant Physiol* 73: 555–559
- Farquhar GD (1983) On the nature of carbon isotope discrimination in C₄ species. *Aust J Plant Physiol* 10: 205–226
- Finnegan PM and Burnell JN (1995) Isolation and sequence analysis of cDNA's encoding phosphoenolpyruvate carboxykinase from the PCK-type C₄ grass *Urochloa panicoides*. *Plant Mol Biol* 27: 365–376
- Foyer C, Furbank RT, Harbinson J and Horton P (1990) The mechanisms contributing to photosynthetic control of electron transport by carbon assimilation in leaves. *Photosynth Res* 25: 83–100
- Furbank RT (1988) Regulation of electron transport in maize mesophyll chloroplasts: the relationship between chlorophyll a fluorescence quenching and O₂ evolution. *Planta* 176: 433–440
- Furbank RT and Leegood RC (1984) Carbon metabolism and gas exchange in leaves of *Zea mays* L. Interaction between the C₃ and C₄ pathways during photosynthetic induction. *Planta* 162: 457–462
- Furbank RT and Taylor WC (1995) Regulation of photosynthesis in C₃ and C₄ plants: A molecular approach. *Plant Cell* 7: 797–807
- Furbank RT, Agostino A and Hatch MD (1990a) C₄ acid decarboxylation and photosynthesis in bundle sheath cells of NAD-malic enzyme-type C₄ plants: Mechanism and the role of malate and orthophosphate. *Arch Biochem Biophys* 276: 374–381
- Furbank RT, Jenkins CLD and Hatch MD (1990b) C₄ photosynthesis: Quantum requirement, C₄ acid overcycling and Q cycle involvement. *Aust J Plant Physiol* 17: 1–7
- Furbank RT, Agostino A and Hatch MD (1991) Regulation of C₄ photosynthesis: Modulation of mitochondrial NAD-malic enzyme by adenylates. *Arch Biochem Biophys* 289: 376–381
- Furbank RT, Chitty JA, von Caemmerer S and Jenkins CLD (1996) Antisense RNA inhibition of *RbcS* gene expression reduces rubisco level and photosynthesis in the C₄ plant *Flaveria bidentis*. *Plant Physiol* 111: 725–734.
- Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S and Ashton AR (1997a) Genetic manipulation of key photosynthetic enzymes in the C₄ plant *Flaveria bidentis*. *Aust J Plant Physiol* 24: 477–485.
- Furbank RT, Pritchard J and Jenkins CLD (1997b) Effects of exogenous sucrose feeding on photosynthesis in the C₃ plant tobacco and the C₄ plant *Flaveria bidentis*. *Aust J Plant Physiol* 24: 291–299.
- Gadal P, Pacquit V, Giglioli N, Bui V-L, Pierre JN, Echevarria C and Vidal J (1996) The role of PEPC phosphorylation in the regulation of C₄ photosynthesis. In: Shewry PR, Halford ND and Hooley R (eds) Protein Phosphorylation in Plants, Proc of the Phytochemical Soc of Europe, No 39, pp 53–64. Clarendon Press, Oxford
- Gao Y and Woo KC (1996a) Regulation of phosphoenolpyruvate carboxylase in *Zea mays* by protein phosphorylation and metabolites and their roles in photosynthesis. *Aust J Plant Physiol* 23: 25–32
- Gao Y and Woo KC (1996b) Site-directed mutagenesis of *Flaveria trinervia* phosphoenolpyruvate carboxylase: Arg⁴⁵⁰ and Arg⁷⁶⁷ are essential for catalytic activity and Lys⁸²⁹ affects substrate binding. *FEBS Lett* 392: 285–288
- Giglioli-Guivarc'h N, Pierre J-N, Brown S, Chollet R, Vidal J and Gadal P (1996) The light-dependent transduction pathway controlling the regulatory phosphorylation of C₄ phosphoenolpyruvate carboxylase in protoplasts from *Digitaria sanguinalis*. *Plant Cell* 8: 573–586
- Hatch MD (1977) Light-dark mediated activation and inactivation of NADP malate dehydrogenase in isolated chloroplasts from *Zea mays*. *Plant Cell Physiol* 3: 311–314
- Hatch MD (1978) Regulation of enzymes in C₄ photosynthesis. In: Horecker BL and Stadtman ER (eds) Current Topic in Cellular Regulation, Vol 14, pp. 1–27. Academic Press, New York
- Hatch MD (1987) C₄ photosynthesis: A unique blend of modified biochemistry, anatomy and ultrastructure. *Biochem Biophys Acta* 895: 81–106
- Hatch MD (1992a) C₄ photosynthesis: An unlikely process full of surprises. *Plant Cell Physiol* 33: 333–342
- Hatch MD (1992b) The making of the C₄ pathway. In: Murata N (ed) Research in Photosynthesis, Vol. 3 pp. 746–756. Kluwer Academic Publishers, Dordrecht
- Hatch MD and Agostino A (1992) Bilevel disulphide group reduction in the activation of C₄ leaf NADP malate dehydrogenase. *Plant Physiol* 100: 360–366
- Hatch MD and Burnell JN (1990) Carbonic anhydrase activity in leaves and its role in the first step of C₄ photosynthesis. *Plant Physiol* 93: 825–828
- Hatch MD and Carnal NW (1992) The role of mitochondria in C₄ photosynthesis. In: Lambers H and van der Plas LHW (eds) Molecular, Biochemical and Physiological Aspects of Plant Respiration, pp. 135–148. SPB Academic Publishers, The Hague
- Hatch MD and Mau S-L (1977) Properties of phospho-

- enolpyruvate carboxykinase operative in C₄ pathway photosynthesis. Aust J Plant Physiol 4: 207–216
- Hatch MD and Slack CR (1968) A new enzyme for the interconversion of pyruvate and phosphoenolpyruvate and its role in the C₄ dicarboxylic acid pathway of photosynthesis. Biochem J 106: 141–146
- Hatch MD, Slack CR and Bull TA (1969) Light-induced changes in the content of some enzymes of the C₄ dicarboxylic acid pathway of photosynthesis and its effect on other characteristics of photosynthesis. Phytochemistry 8:697–706
- Hatch MD, Mau S-L and Kagawa T (1974) Properties of leaf NAD-malic enzyme from plants with C₄ pathway photosynthesis. Arch Biochem Biophys 165: 188–200
- Hatch MD, Agostino A and Burnell JN (1988) Photosynthesis in phosphoenolpyruvate carboxykinase-type C₄ plants: Activity and role of mitochondria in bundle sheath cells. Arch Biochem Biophys 261: 357–367
- Hatch MD, Agostino A and Jenkins CLD (1995) Measurement of the leakage of CO₂ from bundle sheath cells of leaves during C₄ photosynthesis. Plant Physiol 108: 173–181
- Hattersley PW (1992) C₄ photosynthetic pathway variation in grasses (Poaceae): Its significance for arid and semi-arid lands. In: Chapman GP (ed) Desertified Grasslands: Their Biology and Management, pp. 181–212. Academic Press, London
- Huber SC and Edwards GE (1975) Inhibition of phosphoenolpyruvate carboxylase from C₄ plants by malate and aspartate. Can J Bot 53: 1925–1933
- Huber SC and Sugiyama T (1986) Changes in sensitivity to effectors of maize leaf phosphoenolpyruvate carboxylase during light/dark transitions. Plant Physiol 81: 674–677
- Issakidis E, Miginiac-Maslow M, Decottignies P, Jacquot JP, Cretin C and Gadal P (1992) Site directed mutagenesis reveals an additional thioredoxin-dependent regulatory site in the activation of recombinant sorghum leaf NADP malate dehydrogenase. J Biol Chem 267: 21577–21583
- Issakidis E, Sacrin M, Decottignies P, Jacquot JP, Cretin C, Gadal P and Miginiac-Maslow M (1994) Identification and characterization of the second regulatory disulphide bridge of recombinant sorghum leaf NADP malate dehydrogenase. J Biol Chem 269: 3511–3517
- Jenkins CLD and Hatch MD (1985) Properties and reaction mechanism of leaf pyruvate, P_i dikinase. Arch Biochem Biophys 239: 53–62
- Jenkins CLD, Anderson LE and Hatch MD (1986) NADP malate dehydrogenase from Zea mays leaves: Amino acid composition and thiol content of active and inactive forms. Plant Sci 45:1–7
- Jenkins CLD, Burnell JN and Hatch MD (1987) Form of inorganic carbon involved as a product and an inhibitor of C₄ acid decarboxylases operating in C₄ photosynthesis. Plant Physiol 85: 952–957.
- Jiao JA and Chollet R (1991) Posttranslational regulation of phosphoenolpyruvate carboxylase in C₄ and crassulacean acid metabolism plants. Plant Physiol 95: 981–985
- Jiao JA, Podesta FE, Chollet R, O'Leary MH and Andreo CS (1990) Isolation and sequence of an active site peptide from maize leaf phosphoenolpyruvate carboxylase inactivated by pyridoxal 5'-phosphate. Biochim Biophys Acta 1041: 291–295
- Johnson HS and Hatch MD (1970) Properties and regulation of NADP malate dehydrogenase and malic enzyme in plants with the C₄ dicarboxylic acid pathway of photosynthesis. Biochem J 119: 273–280
- Jordan DB and Ogren WL (1984) The CO₂ specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Planta 161: 308–313
- Kacser H and Burns JA (1973) The control of flux. Symposia of the Society for Experimental Biol 27: 65–104
- Krall JP and Edwards GE (1993) PEP carboxylases from two C₄ species of *Panicum* with markedly different susceptibilities to cold inactivation. Plant Cell Physiol 34: 1–11
- Ku SB, Edwards GE and Smith D (1978) Photosynthesis and nonstructural carbohydrate concentration in leaf blades of *Panicum virgatum* as affected by night temperature. Can J Bot 56: 63–68
- Leegood RC and Furbank RT (1984) Carbon metabolism and gas exchange in leaves of *Zea mays* L. Changes in CO₂ fixation, chlorophyll a fluorescence and metabolite levels during photosynthetic induction. Planta 162: 450–456
- Leegood RC and von Caemmerer (1988) The relationship between contents of photosynthetic metabolites and the rate of photosynthetic carbon assimilation in leaves of *Amaranthus edulis* L. Planta 174: 253–262
- Leegood RC and Walker DA (1983) Modulation of NADP malate dehydrogenase in maize mesophyll chloroplasts. Plant Physiol 71: 513–518
- Leegood RC, Adcock MD and Doncaster HD (1989) Analysis of the control of photosynthesis in C₄ plants by changes in light and carbon dioxide. Phil Trans R Soc Lond B323: 339–355
- Lepiniec L, Vidal J, Chollet R, Gadal P and Cretin C (1994) Phosphoenolpyruvate carboxylase: Structure, regulation and evolution. Plant Science 99: 111–124
- Li B and Chollet R (1993) Resolution and Identification of C₄ phosphoenolpyruvate carboxylase protein kinase polypeptides and their reversible light activation in maize leaves. Arch Biochem Biophys 307: 416–419
- Li B, Zhang X-Q and Chollet R (1996) Phosphoenolpyruvate carboxylase kinase in tobacco leaves is activated by light in a similar but not identical way as in maize. Plant Physiol 111: 497–505
- Long SP (1993) C₄ photosynthesis at low temperatures. Plant Cell Environ 6: 345–363
- Marshall JS, Stubbs JD and Taylor WC (1996) Two genes encode highly similar chloroplastic NADP-malic enzymes in *Flaveria*: Implications for the evolution of C₄ photosynthesis. Plant Physiol 111: 1251–1261
- Matsuba K, Imaizumi N, Kaneko S, Samejima M and Ohsugi R (1997) Photosynthetic responses to temperature of phosphoenolpyruvate carboxykinase type C-4 species differing in cold sensitivity. Plant Cell Environ 20: 268–274
- Maurino VG, Drincovich MF and Andreo CS (1996) NADP-malic enzymes isoforms in maize leaves. Biochem Mol Biol Int 38: 239–250
- McNaughton GAL, Fewson CA, Wilkins MB and Nimmo HG (1989) Purification, oligomerisation state and malate sensitivity of maize leaf phosphoenolpyruvate carboxylase. Biochem J 261: 349–355
- Meister M, Agustino A and Hatch MD (1996) The roles of malate and aspartate in C₄ photosynthetic metabolism of *Flaveria bidentis* (L.). Planta 199: 262–269
- Metzler MC, Rothermel BA and Nelson T (1989) Maize NADP malate dehydrogenase: cDNA cloning, sequence, and mRNA characterization. Plant Mol Biol 12: 713–722

- Miedema P (1982) The effects of low temperature on *Zea mays*. *Adv Agron* 35: 93–128
- Monod J, Wyman J and Changeaux J-P (1965) On the nature of allosteric transitions: A plausible model. *J Mol Biol* 12: 88–95
- Moore Bd, Kobza J and Seemann JR (1991) Measurement of 2-carboxyarabinitol 1-phosphate in plant leaves by isotope dilution. *Plant Physiol* 96: 208–213
- Murata T, Ikeda J, Takana M and Ohsugi R (1989a) Comparative studies of NAD-malic enzyme from leaves of various C_4 plants. *Plant Cell Physiol* 30: 429–437
- Murata T, Ohsugi R, Matsuoka M and Nakamoto H (1989b) Purification and characterisation of NAD malic enzyme from leaves of *Eleusine coracana* and *Panicum dichotomiflorum*. *Plant Physiol* 89: 316–324
- Nakamoto H and Edwards GE (1987) Effect of adenine nucleotides on the reaction catalysed by pyruvate, P_i dikinase in maize. *Biochim Biophys Acta* 924: 360–368
- Nimmo GA, McNaughton GAL, Fewson CA, Wilkins MB and Nimmo HG (1987) Changes in the kinetic properties and phosphorylation state of phosphoenolpyruvate carboxylase in *Zea mays* leaves in response to light and dark. *FEBS Lett* 213: 18–22
- Nishido T and Takanashi H (1973) Glycine activation of PEP carboxylase from monocotyledonous C_4 plants. *Biochem Biophys Res Commun* 53: 126–133
- O'Leary MH (1982) Phosphoenolpyruvate carboxylase: An enzymologist's view. *Ann Rev Plant Physiol* 33: 297–315
- Rebeille F and Hatch MD (1986a) Regulation of NADP malate dehydrogenase in C_4 plants: Effect of varying NADPH to NADP ratios and thioredoxin redox state on enzyme activities in reconstituted systems. *Arch Biochem Biophys* 249: 164–170
- Rebeille F and Hatch MD (1986b) Regulation of NADP malate dehydrogenase in C_4 plants: Relationships among enzyme activity, NADPH to NADP ratios and thioredoxin redox states in intact maize mesophyll chloroplasts. *Arch Biochem Biophys* 249: 171–179
- Roeske CA and Chollet R (1989) Role of metabolites in the reversible light activation of pyruvate, P_i dikinase in *Zea mays* mesophyll cells in vivo. *Plant Physiol* 90: 330–337
- Sage R and Monson R (eds) (1999) C_4 Plant Biology. Academic Press, New York
- Sage RF and Seemann JR (1993) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in response to reduced light intensity in C_4 plants. *Plant Physiol* 102: 21–28
- Salvucci ME, Werneke JM, Ogren WL and Portis AR Jr (1987) Purification and species distribution of Rubisco activase. *Plant Physiol* 84: 930–936
- Scheibe R and Jacquot JP (1983) NADP regulates the light activation of NADP-malate dehydrogenase. *Planta* 157: 548–553
- Simon J-P and Hatch MD (1994) Temperature effects on the activation and inactivation of pyruvate, P_i dikinase in two populations of the C_4 weed *Echinochloa crusgalli* (barnyard grass) from sites of contrasting climates. *Aust J Plant Physiol* 21: 463–473
- Smith CM, Duff SM and Chollet R (1994) Partial purification of characterisation of maize leaf pyruvate, P_i dikinase regulatory protein: A low abundance, mesophyll chloroplast protein. *Arch Biochem Biophys* 308: 200–206
- Stiborova M (1988) Phosphoenolpyruvate carboxylase: The key enzyme of C_4 photosynthesis. *Photosynthetica* 22: 240–263
- Stitt M (1995) The use of transgenic plants to study the regulation of plant carbohydrate metabolism. *Aust J Plant Physiol* 22: 635–646
- Stowe LG and Teeri JA (1978) The geographic distribution of the C_4 species of the dicotyledonae in relation to climate. *American Naturalist* 112: 609–623
- Sugiharto B and Sugiyama T (1992) Effects of nitrate and ammonium on gene expression of phosphoenolpyruvate carboxylase and nitrogen metabolism in maize leaf tissue during recovery from nitrogen stress. *Plant Physiol* 98: 1403–1408
- Sugiyama T (1973) Purification, molecular and catalytic properties of pyruvate, P_i dikinase from maize leaf. *Biochemistry* 12: 2862–2867
- Sugiyama T, Mizuno M and Hayashi M (1984) Partitioning of nitrogen among ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, and pyruvate orthophosphate dikinase as related to biomass productivity in maize seedlings. *Plant Physiol* 75: 665–669
- Taylor AO, Slack RC and McPherson HG (1974) Plants under climatic stress VI. Chilling and light effects on photosynthetic enzymes of sorghum and maize. *Plant Physiol* 54: 696–701
- Trevanion SJ, Furbank RT and Ashton AR (1997) NADP malate dehydrogenase in the C_4 plant *Flaveria bidentis*: CoseNSE suppression of activity in mesophyll and in bundle sheath cells and consequences for photosynthesis. *Plant Physiol* 113: 1153–1165
- Uedan K and Sugiyama T (1976) Purification and characterisation of phosphoenolpyruvate carboxylase from maize leaves. *Plant Physiol* 57: 906–910
- Usami S, Ohta S, Komari T and Burnell JN (1995) Cold stability of pyruvate, orthophosphate dikinase of *Flaveria brownii*. *Plant Mol Biol* 27: 969–980
- Usuda H and Shimogawara K (1992) Phosphate deficiency in maize III. Changes in enzyme activities during the course of phosphate deprivation. *Plant Physiol* 99: 1680–1685
- Usuda H, Ku MSB and Edwards GE (1984) Activation of NADP malate dehydrogenase, pyruvate, P_i dikinase and fructose 1,6-bisphosphatase in relation to photosynthetic rate in maize. *Plant Physiol* 76: 238–243
- Usuda H, Ku MSB and Edwards GE (1985) Influence of light intensity during growth on photosynthesis and activity of several key photosynthetic enzymes in a C_4 plant (*Zea mays*). *Physiol Plant* 63: 65–70
- von Caemmerer S and Furbank RT (1999) Modelling C_4 photosynthesis. In: Sage RF and Monson RK (eds) C_4 Plant Biology, pp 173–211. Academic Press, New York
- von Caemmerer S, Millgate A, Farquhar GD and Furbank RT (1997) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase by antisense RNA in the C_4 plant *Flaveria bidentis* leads to reduced assimilation rates and increased carbon isotope discrimination. *Plant Physiol* 113: 469–477
- Walker RP and Leegood RC (1996) Phosphorylation of phosphoenolpyruvate carboxykinase in plants. Studies in plants with C_4 photosynthesis and crassulacean acid metabolism and in germinating seeds. *Biochem J* 317: 653–658
- Walker RP, Acheson RM, Tecsi LI and Leegood RC (1997) Phosphoenolpyruvate carboxykinase in C_4 plants: Its role and regulation. *Aust J Plant Physiol* 24: 459–468
- Wang Y-H and Chollet R (1993) Partial purification and

- characterisation of phosphoenolpyruvate carboxylase protein-serine kinase from illuminated maize leaves. *Arch Biochem Biophys* 304: 496–502
- Ward DA and Woolhouse HW (1986) Comparative effects of light during growth on the photosynthetic properties of NADP-ME type grasses from open and shaded habitats II. Photosynthetic activities and metabolism. *Plant Cell Environ* 9: 271–277
- Wedding RT (1989) Malic enzymes of higher plants. Characteristics, regulation and physiological function. *Plant Physiol* 90: 367–371
- Wong SC (1979) Elevated atmospheric partial pressure of CO₂ and plant growth: 1. Interactions of nitrogen nutrition and photosynthetic capacity in C₃ and C₄ plants. *Oecologia* 44: 68–74
- Woodrow IE, Furbank RT, Brooks A and Murphy DJ (1985) The requirements for a steady state in the C₃ reductive pentose phosphate pathway of photosynthesis. *Biochim Biophys Acta* 807: 263–271
- Yamazaki M, Watanabe A and Sugiyama T (1986) Nitrogen-regulated accumulation of mRNA and protein for photosynthetic carbon assimilating enzymes in maize. *Plant Cell Physiol* 27: 443–452

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Chapter 19

Transport During C₄ Photosynthesis

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Summary

C₄ photosynthesis involves intercellular metabolite transport, between the mesophyll and bundle-sheath, and intracellular shuttling of metabolites between the chloroplasts, cytosol and, in some cases, the mitochondria. In this chapter, the role of diffusion driven intercellular transport of metabolites via plasmodesmata is discussed, together with the impact on the ability of the bundle-sheath to concentrate CO₂. The chapter also considers the nature and role of organelle transporters for phosphorylated compounds, organic and amino acids in relation to C₄ photosynthesis in the mesophyll and bundle-sheath.

I. Introduction

Photosynthetic CO₂ fixation in C₄ plants depends crucially on metabolite transport, not only within cells, but also between mesophyll and bundle-sheath cells (hence C₄ photosynthesis has been termed ‘co-operative photosynthesis’ by Karpilov (1970)). This transport must occur at high rates which are equivalent to rates of photosynthesis. The operation of C₄ photosynthesis has far-reaching consequences for the structure of the leaves of C₄ plants. The necessity for metabolite transport between the mesophyll and bundle-sheath requires intimate contact between the cells and therefore limits the amount of mesophyll

tissue which can be functionally associated with bundle-sheath tissue. The leaf thickness is limited in C₄ plants and the interveinal distance (i.e. the number of mesophyll cells between adjacent bundle-sheaths) is characteristically smaller than in the leaves of C₃ plants, about 300 µm for C₃, and 100 µm for C₄, grasses (Kawamitsu et al., 1985), although there are appreciable differences in interveinal distance between the various C₄ decarboxylation types (Dengler and Nelson, 1999). Mesophyll cells are never more than one cell removed from bundle-sheath cells (Hattersley and Watson, 1975).

The intracellular metabolite transport between organelles is as important to the operation of C₄

photosynthesis as is intercellular metabolite exchange and it must also proceed at comparable fluxes. In the mesophyll, metabolite exchange occurs between chloroplasts and cytosol, while in the bundle-sheath, the mitochondria are also involved in the photosynthetic metabolism of NAD-ME and PCX-types (Chapter 18, Furbank et al.). In NAD-ME plants in particular, malate decarboxylation in the mitochondria will proceed at rates several-fold higher than the high rates of glycine decarboxylation which occur during photorespiration in C₃ plants (Chapter 5, Douce and Heldt).

II. Intercellular Metabolite Transport in C₄ Plants

The most important requirement for intercellular diffusion of metabolites is the presence of plasmodesmata which permit the rapid exchange of solutes between cells (Lucas et al., 1993; Chapter 11, Schobert et al.). Although it has been suggested that active transport of metabolites occurs (Oleson 1975; Raghavendra and Das 1978), the evidence strongly supports the view that metabolites diffuse freely between the mesophyll and bundle-sheath cells and, in any case, the bundle-sheath cell wall in many C₄ plants is heavily suberized, which would restrict apoplastic movement of solutes. Extensive pit-fields with plasmodesmata on the wall between mesophyll and bundle sheath cells provide symplastic connections between the different cells. The plasmodesmata are particularly frequent in primary pit-fields at the areas of contact between mesophyll and bundle sheath cells, and in two C₄ grasses, *Themeda triandra* and *Panicum maximum*, the majority of the plasmodesmata in vascular bundles are at this interface (56% and 77%, respectively), compared to only 32% in a C₃ grass (*Bromus unioloides*) (Botha and Evert, 1988; Botha, 1992). The involvement of plasmodesmata in transport of photo synthetic intermediates is also indicated by the fact that the plasmodesmatal frequency is related to the CO₂ assimilation rate (Botha, 1992). The cross-sectional area of the sphincter in plasmodesmata occupies 1.5–3% of the pit-field in the surface between mesophyll and bundle

sheath cells. The plasmodesmata exclude large molecules, such as cytosolic proteins, and the size exclusion limit in isolated bundle-sheath strands is about 900 Da (Burnell, 1988; Weiner et al., 1988; Valle et al., 1989), although there may be regulation of plasmodesmatal permeability in vivo (Chapter 11, Schobert et al.). Small molecules probably diffuse through micro-channels between the globular proteins in the appressed endoplasmic reticulum-protein complex (the desmotubule). The distances involved are small (1 μm or less) and intercellular metabolite exchange may be accomplished in 50 to 100 ms (Lucas et al., 1993).

Osmond (1971) used an analogy based on symplastic transport of solutes across the concentric cylinders of cortical and vascular tissue in roots and concluded that metabolite transport in C₄ photosynthesis could be sustained solely by diffusion, driven by gradients in the concentrations of metabolites. In the case of malate or aspartate, Hatch and Osmond (1976) estimated that a gradient with the concentration in mesophyll source cells 10 mM higher than in bundle sheath sink cells would be needed to sustain observed rates of photosynthesis in maize (which has centrifugally arranged bundle-sheath chloroplasts and thus a short diffusion path) and 30 mM in *Amaranthus* (which has centripetally arranged chloroplasts and, therefore, a longer diffusion path). Subsequent direct measurement of diffusion constants in isolated bundle-sheath strands for a range of small molecular mass compounds (values of ca. 3 μmol·min⁻¹·mg⁻¹ chlorophyll·mM⁻¹) has resulted in a revision of the required gradient down to 2 mM (Weiner et al., 1988). Another factor that could influence metabolite transport is the movement of water through the transpiration stream, which would aid movement from the bundle-sheath to the mesophyll and hinder movement in the opposite direction. Its magnitude, however, is likely to be less than 1% of the fluxes driven by metabolite concentration gradients (Stitt and Heldt 1985b).

Aside from the structural features which allow rapid intercellular communication, there is metabolic evidence for metabolite transport between the mesophyll and bundle-sheath. First, rapid metabolite movement may be inferred from the rapid transfer of ¹⁴C from C₄ acids (labeled in the mesophyll) to glycerate-3-P and products (which are labeled in the bundle sheath) following the supply of ¹⁴CO₂ to leaves of C₄ plants (Hatch and Osmond, 1976). Such a transfer can also be visualized by microauto-

Abbreviations: DHAP – dihydroxyacetone phosphate; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; PCK – phosphoenolpyruvate carboxykinase; PEP – phosphoenolpyruvate; Rubisco-ribulose-1,5-bisphosphate carboxylase/oxygenase

radiography of leaves from pulse-chase experiments. For example, after a 2 s pulse of ¹⁴CO₂ (when the majority of the label is in C₄ acids), the cytosol of the mesophyll cells is clearly labeled. However, a considerable amount of label is already found in bundle-sheath cells, and the majority of the label is transferred to the bundle-sheath during a 10 s chase (Osmond, 1971). Second, in all C₄ subtypes, the reduction of glycerate-3-P to triose-P is shared between the Benson-Calvin cycle in bundle sheath chloroplasts and the mesophyll chloroplasts. This requires intercellular transport of these metabolites in addition to the C₄ acids (malate and aspartate) and C3 acids (pyruvate, PEP and alanine) of the C₄ cycle. Metabolite measurements on intact leaves of maize show that the amounts of glycerate-3-P and triose-P are extremely high when compared with C₃ species (Leegood and Furbank 1984; Usuda 1987a,b; Leegood and von Caemmerer 1989) and amounts of triose-P are typically 20 times higher than in the leaves of C₃ plants. These large amounts reflect the concentration gradients of these metabolites within the leaf (Leegood and Furbank 1984; Tables 1 and 2).

Direct measurements of the gradients of metabolites in leaves of maize have been made by Leegood (1985), by Stitt and Heldt (1985a,b) and by Weiner and Heldt (1992). These are shown in Tables 1 and 2. These measured gradients are clearly higher than the predicted gradient of 2 mM (Weiner et al., 1988) and are sufficient to support metabolite transport at rates equal to the rate of photosynthesis. The most striking feature is the high contents of glycerate-3-P and triose-P and their asymmetric distribution, with the major portion of the triose-P within the mesophyll and the major part of the glycerate-3-P within the bundle-sheath, confirming the existence of metabolite gradients involving these compounds and the operation of a glycerate-3-P/triose-P shuttle between the mesophyll and bundle-sheath. Non-aqueous fractionation of maize leaves has subsequently shown

that these gradients of glycerate-3-P and triose-P exist between the cytosols of the mesophyll and bundle-sheath (Table 2)(Weiner and Heldt, 1992). The fact that the apparent gradient of pyruvate lies in the opposite direction to the expected flux originally suggested that this might be the result of the intracellular accumulation of pyruvate within mesophyll chloroplasts (a feature which was subsequently demonstrated using intact chloroplasts, Flügge et al., 1985, see below). There is, therefore, almost certainly a gradient of pyruvate between the bundle-sheath and the mesophyll cytosol *in vivo*. Efficient regulation of metabolism is required if these metabolite gradients are not to collapse. In maize, interchange of carbon between PEP and glycerate-3-P (Furbank and Leegood, 1984) must be curtailed in the bundle sheath in order to prevent the collapse of the gradient of glycerate-3-P, as must the overall conversion of pyruvate to triose phosphate and of glycerate-3-P to malate in the mesophyll.

Considerable technical difficulties still surround the measurement of the gradient of malate, since much (99%) of the malate pool is non-photosynthetic and is present in the vacuole or in non-photosynthetic leaf cells. These large malate pools readily contaminate the cellular or sub-cellular fractions obtained from intact leaves. No direct measurements have been made of metabolite gradients in leaves of C₄ plants other than maize. In leaves of *Amaranthus edulis*, an NAD-ME enzyme species, aspartate is transferred from the mesophyll to the bundle sheath and alanine is returned to the mesophyll cells. It has been shown that amounts of aspartate and alanine in leaves of *A. edulis* are sufficient to account for diffusion-driven transport of these compounds between the mesophyll and bundle sheath cells under many different flux conditions (Leegood and von Caemmerer, 1988). In PCK-type plants, transport may be rather more complex, as both malate and aspartate must be transferred from the mesophyll to

Table 1. Intercellular distribution of inorganic carbon and metabolites in leaves of maize. Mean values from Leegood (1985) and Stitt and Heldt (1985).

Metabolite	Concentration (mM)		Metabolite gradient Δ (mM)
	mesophyll	bundle-sheath	
glycerate-3-P	5.2	14.7	9.5
triose-P	11.9	2.8	9.1
malate	56.5	13.3	43.2
pyruvate	7.4	6.3	1.1
CO ₂ + HCO ₃ ⁻	0.03	0.6	0.57

Table 2. Contents of metabolites in non-aqueously prepared fractions of maize leaves. From Weiner and Heldt (1992). If the volume of the cytosol and chloroplasts is $20 \mu\text{l}\cdot\text{mg}^{-1}$ chlorophyll, then the following figures divided by 20 will give an approximate mM concentration. The volume of the cytosol is $45 \mu\text{l}\cdot\text{mg}^{-1}$ chlorophyll in *Panicum miliaceum* bundle-sheath cells (Valle et al., 1989)

Metabolite	Content ($\text{nmol}\cdot\text{mg}^{-1}$ chlorophyll)			
	bundle-sheath		mesophyll	
	chloroplast	extra-chloroplast	chloroplast	extra-chloroplast
malate	31	2970	78	821
glycerate-3-P	348	243	113	106
triose-P	45	29	86	250
alanine	896	928	480	896
glycine	60	80	44	46
serine	86	67	50	57
aspartate	308	220	308	264
glutamate	329	180	360	336
glutamine	123	82	110	95

the bundle sheath, and both PEP (and possibly some pyruvate) and alanine return to the mesophyll (Chapter 18, Furbank et al.; Fig. 2). Such a situation will also obtain in NADP-ME type plants which utilize PCK in the decarboxylation of aspartate, such as maize (Wingler et al., 1999). In both these C_4 types, if amino acids, such as aspartate, enter the bundle-sheath and phosphorylated compounds, such as PEP, return, this would also necessitate shuttles of Pi and glutamate/2-oxoglutarate between the two cell types. Gradients of 2-oxoglutarate and glutamate are entirely feasible (Leegood, 1985; Weiner and Heldt, 1992; Table 2). In addition, inter- and intra-cellular transport will be more complex in those NADP-ME species that show mixed formation of malate and aspartate, such as *Flaveria bidentis* (Meister et al., 1996) and in NADP-ME species that also contain NAD-ME, such as *Neostapfia colusana* (Keeley, 1998).

A. Implications of Intercellular Transport for Regulation of C_4 Photosynthesis

Coordination of the rate at which the Benson-Calvin and C_4 cycles fix CO_2 is necessary if photosynthesis is to proceed efficiently under different environmental conditions. The breakdown of such coordination during light flecks, for example, has been shown to result in inefficient CO_2 assimilation (Krall and Pearcy 1993). Reduction of glycerate-3-P, deriving from the bundle-sheath, in the mesophyll occurs in all C_4 species (Hatch and Osmond, 1976) and is probably a major component in this co-ordination. The triose-P which is formed from glycerate-3-P in the mesophyll

can either be utilized in carbohydrate synthesis or return to the bundle-sheath chloroplast.

An example of such co-ordination is to be found in the regulation of PEP carboxylase (Fig. 1). Products of glycerate-3-P reduction in the mesophyll, such as triose-P, and ultimately hexose-P, act as positive effectors of PEP carboxylase, and relieve inhibition by malate. They can be considered as metabolite 'messages' from the bundle-sheath. In leaves of maize, *Amaranthus edulis* and *Flaveria bidentis*, the amount of triose-P is always closely related to the assimilation rate whether the flux is changed by alterations in irradiance or CO_2 (Leegood and von Caemmerer 1988, 1989, 1994). Increasing concentrations of triose-P and hexose-P (indicating increased output from the Benson-Calvin cycle) would then increase the activity of PEP carboxylase which would increase the rate of CO_2 fixation in the mesophyll. On the other hand, if too much glycerate-3-P were diverted to PEP (Furbank and Leegood, 1984) and then to malate because of an excessive activity of PEP carboxylase, this would lead to accumulation of inhibitors of PEP carboxylase (malate and aspartate) if the CO_2 could not be fixed at similar rates by Rubisco. The decrease in glycerate-3-P would lead to decreases in the amounts of the activators of PEP carboxylase, triose-P and hexose-P, and thus decrease the rate at which glycerate-3-P is consumed. As another example, photorespiratory intermediates, such as glycine and serine, which accumulate in leaves when the intercellular concentration of CO_2 falls (Leegood and von Caemmerer 1994), can activate PEP carboxylase, particularly in dicots (Nishikido and Takanashi 1973; Doncaster and

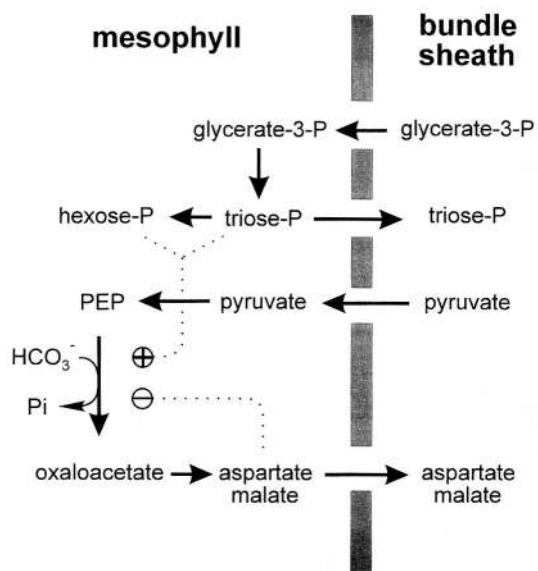


Fig. 1. Regulation of PEP carboxylase by metabolites, showing the inhibitory influence of the products (malate and aspartate), and activating metabolites (triose-P and hexose-P) deriving from the glycerate-3-P exported from the bundle-sheath. Most cofactors etc are omitted for clarity.

Leegood 1988), and probably again act as metabolite messages, transmitting information about the rate of CO₂ fixation in the bundle-sheath.

III. Gaseous Fluxes Between Bundle-Sheath and Mesophyll

An important feature of transport between the mesophyll and bundle-sheath is the extent to which the structural modifications which allow metabolite transport also allow leakage of CO₂ out of the bundle-sheath. If appreciable CO₂ leakage occurred, this would reduce the efficiency of C₄ photosynthesis. Thus a leak rate of 50% (equivalent to 100% overcycling of the C₄ acid cycle; Furbank et al., 1990a) would increase the quantum requirement for C₄ photosynthesis by about 4 mol quanta.mol⁻¹ CO₂ (Hatch et al., 1995). Modeling of the pool of HCO₃⁻ + CO₂ indicates that the pool of CO₂ in the bundle-sheath is about 70 μM, some 15 times ambient CO₂ (Jenkins et al., 1989b). Modeling of the inorganic carbon pool in mesophyll and bundle sheath cells has shown that the efflux of HCO₃⁻ via plasmodesmata is insignificant compared to the flux of C₄ acids (Furbank and Hatch 1987). Leakage of HCO₃⁻ via the plasmodesmata is not appreciable, nor is the leakage

of CO₂, because the diffusion coefficients of gases in solution are 10⁴ times less than in air.

Leakage of CO₂ from the bundle-sheath is likely to be influenced by the occurrence of a suberized lamella in the bundle-sheath cell wall. A suberized lamella is absent in dicotyledonous species, and in grasses is present only in species with either an uneven bundle sheath outline or with centrifugally located chloroplasts. In those species with uneven cell outlines the suberized lamella may be important in restricting CO₂ leakage through the high surface area of the bundle sheath/mesophyll interface (Hattersley, 1992). Hatch and Osmond (1976) and Hattersley and Browning (1981) have suggested that features of the leaf anatomy of NAD-ME monocots and dicots may compensate for the lack of a suberized lamella, so that the conductances to diffusion of CO₂ from the bundle-sheath need not be different. These include the location of the chloroplasts in a centripetal position in the bundle-sheath so that the diffusion pathway for CO₂ to the mesophyll is longer, and a higher bundle-sheath surface-to-volume ratio, especially for monocots (Hattersley and Browning, 1981). However, estimates made by Jenkins et al., (1989a) and by Furbank et al., (1989) suggest that, on average, those species with a suberized lamella do have a lower physical conductance to diffusion of CO₂ than those without it.

The leakiness (the fraction of CO₂ released by C₄-acid decarboxylation in the bundle-sheath which subsequently leaks out to the mesophyll) is determined not only by the physical conductance of the bundle-sheath but also by the relative capacities of the C₄ cycle to generate CO₂ and of the Benson-Calvin cycle to fix it and is thus a measure of the extent to which PEP carboxylations exceed carboxylations by Rubisco. Henderson et al., (1992) suggest that, once the bundle-sheath conductance to CO₂ is sufficiently low, leakiness may be largely determined by the relative activities of Rubisco and PEP carboxylase. Henderson et al., (1992) have employed short-term measurements of carbon isotope discrimination to estimate leakiness. For *Sorghum bicolor* and *Amaranthus edulis*, leakiness was estimated at 0.2 and was little affected by irradiance, temperature or CO₂ partial pressure, although it increased at low irradiance. For a range of monocots and dicots of various decarboxylation types, leakiness was also around 0.2, and was not correlated with the presence or absence of a suberized lamella which surrounds the bundle-sheath, but it was significantly

higher in two species: *Eleusine coracana* (NAD-ME; 0.3) and *Chloris gayana* (PCK; 0.25). Hatch et al., (1995) assessed leakiness by measuring the amount of $^{14}\text{CO}_2$ released from leaves during a chase in cold CO_2 . For nine species of grasses representing the different C_4 subgroups and two dicotyledonous NAD-ME species, the CO_2 leak rate ranged between 8 and 14%. However, very high CO_2 leak rates (averaging about 27%) were recorded for two NADP-malic enzyme type dicotyledonous species of *Flaveria* (*F. bidentis* and *F. trinervia*). This may be reflected in the observed low quantum yield in these *Flaveria* species (Monson et al., 1986). Jenkins et al., (1989a) also used a specific inhibitor of PEP carboxylase, DCDP (3,3-dichloro-2-(dihydroxyphosphinoyl-methyl)-propenoate) to inhibit photosynthesis in leaves of various C_4 plants. Photosynthesis could be restored by elevating the CO_2 concentration to about 0.8% and above. External CO_2 at a sufficiently high concentration will then lead, by diffusion through the bundle-sheath cell wall, to appreciable CO_2 concentrations in the bundle-sheath which can be fixed directly by Rubisco. A similar phenomenon is observed in mutants of *Amaranthus edulis* lacking PEP carboxylase (Dever et al., 1995). Permeabilities to CO_2 were approximately 100-fold lower than values for the permeabilities of mesophyll cells of C_3 plants (Jenkins et al., 1989a; Brown and Byrd 1993). Leakiness of the bundle-sheath to CO_2 was estimated at about 0.14.

Consideration of the reduced permeability of the bundle-sheath to CO_2 then raises questions about the O_2 concentration in the bundle-sheath. C_4 plants that do not evolve O_2 in the bundle-sheath because they lack photosystem II (i.e. NADP-ME species) present the simplest picture, but there have been no direct measurements of the concentration of O_2 in the bundle-sheath. Burnell and Hatch (1988a) have estimated that the O_2 concentration in the bundle-sheath of other C_4 plants may be as high as $1000 \mu\text{M}$ (four times ambient). However, modeling has shown that bundle-sheath O_2 concentrations can be several-fold greater than ambient O_2 concentration without large effects on CO_2 assimilation (von Caemmerer and Furnbank, 1999). Two features will tend to mitigate O_2 evolution in the bundle-sheath. First, reduction of glycerate-3-P in the mesophyll will result in a decrease in the requirement for reductant, and hence photosynthetic O_2 evolution, in the bundle-sheath. Second, the photosynthetic carbon fluxes through the mitochondria in NAD-malic enzyme

and PCK-types are 10- to 20-fold higher than rates of respiration in other tissues. Such high rates of respiration may lower the O_2 concentration in the bundle-sheath.

IV. Intracellular Metabolite Transport in C_4 Plants

Leaf organelles in C_4 plants share the translocators of organelles in leaves of C_3 plants (Chapter 6, Flügge), but also contain translocators with unique, or considerably altered, kinetic properties. The chloroplasts of C_4 plants also possess a peripheral reticulum, a membrane system of anastomosing tubules contiguous with the inner membrane of the chloroplast envelope. This is generally more highly developed in the mesophyll chloroplasts, particularly in C_4 dicots, although it is also prominent in the bundle-sheath chloroplasts of the C_4 Cyperaceae (Dengler and Nelson, 1999). Its function remains unexplored, although it may serve to increase the area of the chloroplast envelope so as to facilitate transport (Laetsch 1974; Hatch and Osmond 1976).

A. Mesophyll Chloroplasts

During photosynthesis in C_4 plants, the mesophyll chloroplasts import glycerate-3-P, deriving from the bundle-sheath, and export triose-P, and the bundle sheath chloroplasts export glycerate-3-P to the mesophyll and import triose-P that has been reduced by the mesophyll chloroplasts. This is quite different from chloroplasts of a C_3 plant, which export triose-P in exchange for Pi (Chapter 6, Flügge). In addition, the mesophyll chloroplasts also catalyze the export of PEP, formed in the chloroplast by the action of pyruvate P_i dikinase, in exchange for P_i to sustain PEP carboxylase in the cytosol (Fig. 2). Exchange of PEP, P_i , glycerate-3-P and triose-P occurs on a common Pi translocator in the chloroplast envelope of C_4 plants. The C_4 mesophyll Pi translocator is very similar to the C_3 -type phosphate translocator, with between 83 and 94% identity in amino acid residues. Minor changes in amino acid sequence have occurred to extend the substrate specificity of the C_3 phosphate translocator to recognize PEP in C_4 plants (Fischer et al., 1994). Ohnishi et al., (1989) studied the Pi translocator in mesophyll chloroplasts of *Panicum miliaceum* (NAD-ME type). The affinity of the translocator for glycerate-3-P and DHAP was lower

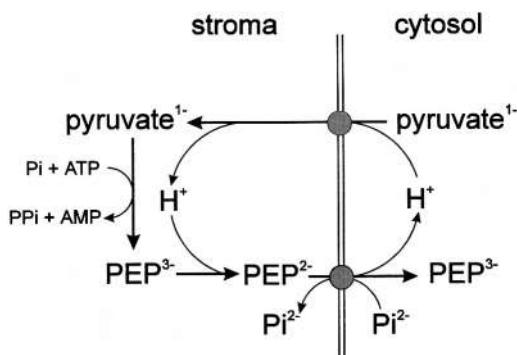


Fig. 2. Exchange of pyruvate and PEP across the envelope of a mesophyll chloroplast, catalysed by the pyruvate transporter (symport of pyruvate and H⁺) and the phosphate translocator (antiport of PEP and Pi) after conversion of pyruvate to PEP by pyruvate, Pi dikinase. After Aoki and Kanai (1995).

than for Pi, in contrast to C₃ chloroplasts, in which it is generally higher by a factor of two- to three-fold. Light stimulated the uptake of Pi in mesophyll chloroplasts, an effect not observed in bundle-sheath chloroplasts (and not previously reported for chloroplasts from C₃ plants). Light also increased the K_i for glycerate-3-P and DHAP in the mesophyll chloroplasts, further decreasing the affinity of the translocator for these compounds. The increased K_i values for these metabolites in the light would permit the exchange of PEP and Pi in the presence of the very high concentrations of glycerate-3-P and DHAP which are attained in the mesophyll cytosol and chloroplasts (Tables 1 and 2).

Chloroplasts transport pyruvate on a specific carrier in both C₃ and C₄ plants, but the translocator is much more active and is light-dependent in mesophyll chloroplasts of C₄ plants (Flügge et al., 1985). Pyruvate transport appears to be driven by an H⁺ gradient in NADP-malic enzyme grasses of the Arundineae and Andropogoneae and a Na⁺ gradient in other C₄ monocots and all dicots (Ohnishi and Kanai, 1990; Ohnishi et al., 1990; Aoki et al., 1992, 1994; Heldt and Flügge, 1992; Murata et al., 1992). It would appear that the H⁺ taken up by H⁺/pyruvate cotransport is released in vivo together with PEP (which is generated as PEP³⁻, but transported via the Pi translocator as PEP²⁻) (Aoki and Kanai, 1995; Fig. 2). In the Na⁺-type, pyruvate transport may occur by Na⁺-dependent acceleration of pyruvate-H⁺ cotransport (Aoki and Kanai, 1997). Interestingly, all C₄ plants require Na⁺ as an essential micronutrient (Brownell and Crossland 1972). The Na⁺-dependence of the pyruvate transporter in some C₄ species may

help explain this requirement in some species, although some other process in the conversion of pyruvate to PEP must also show Na⁺-dependence in NADP-ME species (Brownell et al., 1991).

The dicarboxylates (oxaloacetate, malate, 2-oxoglutarate, glutamate and aspartate) are transported across the chloroplast envelope in a carrier-mediated mode. These compounds undergo counter-exchange on the dicarboxylate translocator. The K_m for uptake of a particular dicarboxylic acid is similar to the K_i for the inhibition of uptake by other dicarboxylates. In maize, for example, the K_i (0.3 mM) for oxaloacetate inhibition of malate transport is comparable to the K_m (0.5 mM) for malate uptake (Day and Hatch, 1981). In NADP-ME species, and to some extent in PCK-type species, oxaloacetate generated by PEP carboxylase must be taken up into the chloroplasts for reduction to malate. Oxaloacetate uptake would clearly not occur when oxaloacetate concentrations are several orders of magnitude less than malate concentrations, as occurs in NADP-ME plants such as maize, in which OAA concentrations are probably less than 50 μM. There is, therefore, a quite separate and very active oxaloacetate carrier in maize mesophyll chloroplasts (K_m 45 μM) which is little affected by malate (K_i 7.5 mM) (Hatch et al., 1984).

B. Bundle Sheath Organelles

Although transport processes across the envelope of C₄ mesophyll chloroplasts are now adequately characterized, relatively little is known of transport into the bundle sheath chloroplasts, largely because of the difficulty of isolating these intact and in appreciable quantities from any C₄ plants. Bundle sheath chloroplasts have a phosphate translocator like that of C₃ and C₄ mesophyll chloroplasts (Chapter 6, Flügge). However, bundle-sheath chloroplasts are unusual because they must export glycerate-3-P and import triose-P at high rates. In C₃ plants, glycerate-3-P is not exported by chloroplasts to any great extent because it is transported by the translocator as the glycerate-3-P²⁻ ion, whereas glycerate-3-P³⁻ is the form that predominates at the pH which occurs in the illuminated stroma (Heldt and Flügge, 1992). It is possible that the stromal pH is lower in bundle sheath chloroplasts or that glycerate-3-P within the chloroplast reaches such high internal concentrations that transport of glycerate-3-P²⁻ from the chloroplast becomes inevitable. Ohnishi et al., (1989) studied

the Pi translocator in bundle-sheath chloroplasts of *Panicum miliaceum* (NAD-ME type). The chloroplast Pi translocator from the bundle-sheath had a surprisingly high affinity for PEP. Bundle-sheath chloroplasts had a higher K_m (Pi) (0.33 mM) compared with the mesophyll chloroplasts (0.14 mM), but the K_i (glycerate-3-P) was little different from that of the mesophyll chloroplasts.

Little is known about transport of organic and amino acids into bundle sheath chloroplasts. The bundle sheath chloroplasts of *Panicum miliaceum* (NAD-ME type) show a slow carrier-mediated uptake of pyruvate which has very similar characteristics to the carrier in wheat and pea chloroplasts and which is not light-stimulated, in contrast to mesophyll chloroplasts, but this could have a function unconnected to photosynthesis. In maize, malate decarboxylation by bundle-sheath chloroplasts is appreciably enhanced by aspartate and glutamate. As aspartate and glutamate are not required by NADP-ME, it has been suggested that a carrier specific for malate uptake is present which depends upon the presence of aspartate for maximum activity or that, if malate uptake is facilitated by an exchange carrier similar to that in C_3 plants or C_4 mesophyll chloroplasts, rapid aspartate or glutamate uptake via another carrier might allow malate to exchange with a concomitant efflux of aspartate or glutamate. In either case, the existence of specialized dicarboxylate carriers in maize bundle-sheath chloroplasts is implicated (Boag and Jenkins, 1985, 1986). Pyruvate has also been shown to have a stimulatory effect on malate transport into bundle-sheath chloroplasts of maize (Kanai and Edwards, 1999). Bundle-sheath chloroplasts also have a glycolate transporter (Ohnishi and Kanai, 1988), although the glycolate formed in the photorespiratory pathway is subsequently metabolized in the mesophyll.

An even greater area of uncertainty is metabolite transport into the mitochondria of bundle-sheath cells. In NAD-ME-type species, decarboxylation of C_4 acids by both isolated bundle-sheath cells and by mitochondria requires aspartate and 2-oxoglutarate. These act as a source of oxaloacetate, which is then endogenously reduced to malate, reductant being regenerated by the oxidation of malate by NAD-malic enzyme (Chapter 18, Furbank et al.). However, external malate and phosphate are also required (Furbank et al., 1990b). It has been proposed that the requirement for malate results from a counter-exchange of external malate for internal Pi (Day and

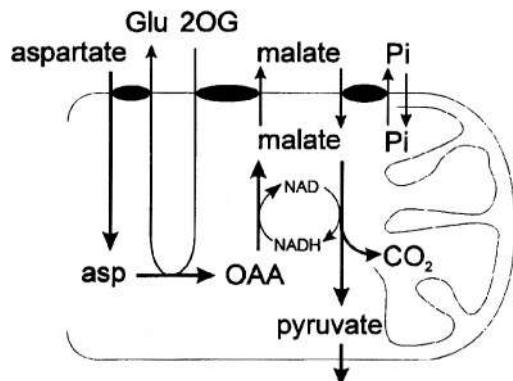


Fig. 3. Possible mitochondrial metabolite transport processes associated with decarboxylation of C_4 acids in bundle-sheath cells of NAD-ME-type C_4 species, including an aspartate-glutamate (Glu) exchange carrier, a 2-oxoglutarate (2OG)-malate exchange carrier (which can also transport oxaloacetate (OAA)(not shown)), a malate-Pi exchange carrier and Pi and pyruvate transport systems operating either in exchange for OH⁻ or cotransport with H⁺. The depiction of membranes is purely diagrammatic. See Furbank and Hatch (1990).

Wiskich, 1984), followed by exchange of external 2-oxoglutarate and internal malate. Pi would then be taken up again by a Pi/OH antiporter or Pi/H⁺ symporter (Furbank et al., 1990b; Douce et al., 1997; Fig. 3). This link between Pi and C_4 acid decarboxylation could provide feedback from sucrose synthesis (which releases Pi) to CO₂ fixation in the bundle-sheath. Similarly, sensitivity to malate could link CO₂ fixation in the mesophyll to rates of decarboxylation in the bundle-sheath, in a manner analogous to the regulation of malate decarboxylation by aspartate in NADP-ME plants (see above). In PCK-type species, in which malate is decarboxylated in the mitochondria and pyruvate and ATP exported to the cytosol, it is presumed that a similar dependence of malate transport on Pi obtains because malate decarboxylation is highly dependent on Pi, in addition to the requirement to generate ATP for the reaction catalysed by PCK (Carnal et al., 1993). Mitochondria are also likely to be involved in aspartate amino-transferase in NADP-ME type plants, as aspartate amino-transferase in the bundle-sheath of maize is mainly located in the mitochondria (Chapman et al., 1981; Wingler et al., 1999).

A mitochondrial 2-oxoglutarate/malate translocator has been cloned from leaves of *Panicum miliaceum*. It is highly homologous with the bovine 2-oxoglutarate/malate translocator and the recombinant protein transports malate, citrate and 2-oxogluta-

tarate when incorporated into liposomes (Taniguchi and Sugiyama, 1996).

V. Concluding Remarks

High rates of both intra- and inter-cellular transport of metabolites are crucial to the operation of CO₂ assimilation by C₄ plants. Both the chloroplasts and mitochondria are involved in intercellular metabolite transport during photosynthesis in most C₄ plants. Most of these transporters remain to be characterized at the molecular level and their interactions understood. This is particularly important in view of the many attempts to genetically modify photo-respiration and to introduce C₄ characteristics into C₃ plants. It is also evident that C₄ photosynthesis involves intercellular transport via plasmodesmata, which involves considerable structural modification of the mesophyll/bundle-sheath interface. There is still much to be understood about the factors which affect plasmodesmal development and permeability (Chapter 11, Schobert et al.). In addition, there are other anatomical differences between C₄ plants, for example, the centripetal versus centrifugal location of chloroplasts within the bundle-sheath and the presence of a double bundle-sheath in NAD-ME and PCK types (Dengler and Nelson, 1999) whose consequences for intra- and inter-cellular transport are likely to be profound but which have not been evaluated.

References

- Aoki N and Kanai R (1995) The role of phosphoenolpyruvate in proton/pyruvate cotransport into mesophyll chloroplasts of maize. *Plant Cell Physiol* 36: 187–189
- Aoki N and Kanai R (1997) Reappraisal of the role of sodium in the light-dependent active transport of pyruvate into mesophyll chloroplasts of C₄ plants. *Plant Cell Physiol* 38: 1217–1225
- Aoki N, Ohnishi J and Kanai R (1992) Two different mechanisms for transport of pyruvate into mesophyll chloroplasts of C₄ plants—a comparative study. *Plant Cell Physiol* 33: 805–809
- Aoki N, Ohnishi J and Kanai R (1994) Proton/pyruvate cotransport into mesophyll chloroplasts of C₄ plants. *Plant Cell Physiol* 35: 801–806
- Boag S and Jenkins CLD (1985) CO₂ assimilation and malate decarboxylation by isolated bundle sheath chloroplasts from *Zea mays*. *Plant Physiol* 79: 165–170
- Boag S and Jenkins CLD (1986) The involvement of aspartate and glutamate in the decarboxylation of malate by isolated bundle sheath chloroplasts of *Zea mays*. *Plant Physiol* 80: 115–119
- Botha CEJ (1992) Plasmodesmatal distribution, structure and frequency in relation to assimilation in C₃ and C₄ grasses in southern Africa. *Planta* 187: 348–358
- Botha CEJ and Evert RF (1988) Plasmodesmatal distribution and frequency in vascular bundles and contiguous tissues of the leaf of *Themeda triandra*. *Planta* 173: 433–441
- Brown RH and Byrd TG (1993) Estimation of bundle sheath cell conductance in C₄ species and O₂ sensitivity of photosynthesis. *Plant Physiol* 103: 1183–1188
- Brownell PF and Crossland CJ (1972) The requirement for sodium as a micronutrient by species having the C₄ dicarboxylic photosynthetic pathway. *Plant Physiol* 49: 794–797
- Brownell PF, Bielig LM and Grof CPL (1991) Increased carbonic anhydrase activity in leaves of sodium-deficient C₄ plants. *Aust J Plant Physiol* 18: 589–592
- Burnell JN (1988) An enzymic method for measuring the molecular exclusion limit of plasmodesmata of bundle sheath cells of C₄ plants. *J Exp Bot* 39: 1575–1580
- Carnal NW, Agostino A and Hatch MD (1993) Photosynthesis in phosphoenolpyruvate carboxykinase-type C₄ plants: Mechanism and regulation of C₄ acid decarboxylation in bundle sheath cells. *Arch Biochem Biophys* 306: 360–367
- Chapman KSR and Hatch MD (1981) Aspartate decarboxylation in bundle sheath cells of *Zea mays* and its possible contribution to C₄ photosynthesis. *Aust J Plant Physiol* 8: 237–248
- Day DA and Hatch MD (1981) Dicarboxylate transport in maize mesophyll chloroplasts. *Arch Biochem Biophys* 211: 738–742
- Day DA and Wiskich JT (1984) Transport processes of isolated mitochondria. *Physiol Veg* 22: 241–261
- Dengler NC and Nelson T (1999) Leaf structure and development in C₄ plants. In: Sage RF and Monson RK (eds) C₄ Plant Biology, pp 133–172. Academic Press, San Diego
- Dever LV, Bailey KJ, Leegood RC and Lea PJ (1995) Control of photosynthesis in *Amaranthus edulis* mutants with reduced amounts of PEP carboxylase. *Aust J Plant Physiol* 24: 469–476
- Doncaster HD and Leegood RC (1987) Regulation of phosphoenolpyruvate carboxylase activity in maize leaves. *Plant Physiol* 84: 82–87
- Douce R, Aubert S and Neuberger M (1997) Metabolite exchange between the mitochondrion and the cytosol. In: Dennis DT, Layzell DB, Lefebvre DD and Turpin DH (eds), Plant Metabolism, pp 234–251. Longman, London
- Fischer K, Arbinger B, Kammerer B, Busch C, Brink S, Wallmeier H, Sauer N, Eckerskorn C and Flügge U-I (1994) Cloning and in vivo expression of functional triose phosphate/phosphate translocators from C₃ and C₄ plants: Evidence for the putative participation of specific amino acid residues in the recognition of phosphoenolpyruvate. *Plant J* 5: 215–226
- Flügge UI, Stitt M and Heldt HW (1985) Light-driven uptake of pyruvate into mesophyll chloroplasts from maize. *FEBS Lett* 183: 335–339
- Furbank RT and Hatch MD (1987) Mechanism of C₄ photosynthesis. The size and composition of the inorganic carbon pool in bundle-sheath cells. *Plant Physiol* 85: 958–964
- Furbank RT and Leegood RC (1984) Carbon metabolism and gas exchange in leaves of *Zea mays* L. Interaction between the C₃ and C₄ pathways during photosynthetic induction. *Planta* 162: 457–462
- Furbank RT, Jenkins CLD and Hatch MD (1989) CO₂ concentrating mechanism of C₄ photosynthesis: permeability

- of isolated bundle sheath cells to inorganic carbon. *Plant Physiol* 91: 1364–1371
- Furbank RT, Jenkins CLD and Hatch MD (1990a) C_4 photosynthesis: quantum requirements, C_4 acid overcycling and Q-cycle involvement. *Aust J Plant Physiol* 17: 1–7
- Furbank RT, Agostino A and Hatch MD (1990b) C_4 acid decarboxylation and photosynthesis in bundle-sheath cells of NAD-malic enzyme-type C_4 plants: Mechanism and the role of malate and orthophosphate. *Arch Biochem Biophys* 276: 374–381
- Hatch MD and Osmond CB (1976) Compartmentation and transport in C_4 photosynthesis. In: Stocking CR and Heber U (eds) *Encyclopedia of Plant Physiology*, New Series Vol 3, pp. 144–184, Springer-Verlag, Berlin
- Hatch MD, Dröscher L, Flügge UI and Heldt HW (1984) A specific translocator for oxaloacetate transport in chloroplasts. *FEBS Lett* 178: 15–19
- Hatch MD, Agostino A and Jenkins CLD (1995) Measurement of the leakage of CO_2 from bundle-sheath cells of leaves during C_4 photosynthesis. *Plant Physiol* 108: 173–181
- Hattersley PW (1992) C_4 photosynthetic pathway variation in grasses (Poaceae): Its significance for arid and semi arid lands. In: Chapman GP (ed) *Desertified Grassland: Their Biology and Managements*, Linnean Society Symposium Series, Vol 13, pp 181–212. Academic Press, London
- Hattersley PW and Browning AJ (1981) Occurrence of the suberised lamella in leaves of grasses of different photosynthetic type. I. In parenchymatous bundle sheath and PCR ('Kranz') sheaths. *Protoplasma* 109: 371–401
- Hattersley PW and Watson L (1992) Diversification of photosynthesis. In: Chapman GP (ed) *Grass Evolution and Domestication*, pp 38–116. Cambridge University Press, Cambridge
- Heldt, HW and Flügge, UI (1992) Metabolite transport in plant cells. In: Tobin AK(ed) *Plant Organelles*, pp 21–47. Cambridge University Press, Cambridge
- Henderson SA, von Caemmerer S and Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C_4 species. *Aust J Plant Physiol* 19: 263–285
- Jenkins CLD, Furbank RT and Hatch MD (1989a) Inorganic carbon diffusion between C_4 mesophyll and bundle sheath cells: Direct bundle sheath CO_2 assimilation in intact leaves in the presence of an inhibitor of the C_4 pathway. *Plant Physiol* 91: 1356–1363
- Jenkins CLD, Furbank RT and Hatch MD (1989b) Mechanism of C_4 photosynthesis. A model describing the inorganic carbon pool in the bundle sheath cells. *Plant Physiol* 91: 1372–1381
- Kanai R and Edwards GE (1999) The biochemistry of C_4 photosynthesis. In: Sage RF and Monson RK (eds) C_4 Plant Biology, pp 49–87. Academic Press, San Diego
- Karpilov YS (1970) Cooperative photosynthesis in xerophytes. *Proc Moldavian Inst Irrigation Agric Res* 11: 3–66 (in Russian)
- Kawamitsu Y, Hakoyama S, Agata W and Takeda T (1985) Leaf interveinal distances corresponding to anatomical types in grasses. *Plant Cell Physiol* 26: 589–593
- Keely JE (1998) C_4 photosynthetic modifications in the evolutionary transition from land to water in aquatic grasses. *Oecologia* 116: 85–97
- Krall JK and Pearcey RW (1993) Concurrent measurements of oxygen and carbon dioxide exchange during lightflecks in maize (*Zea mays* L.). *Plant Physiol* 103: 823–828
- Laetsch WM (1974) The C_4 syndrome: A structural analysis. *Ann Rev Plant Physiol* 25: 27–52
- Leegood RC (1985) The intercellular compartmentation of metabolites in leaves of *Zea mays*. *Planta* 164: 163–171
- Leegood RC and Furbank RT (1984) Carbon metabolism and gas exchange in leaves of *Zea mays* L. Changes in CO_2 fixation, chlorophyll fluorescence and metabolite levels during photosynthetic induction. *Planta* 162: 450–156
- Leegood RC and von Caemmerer S (1988) The relationship between contents of photosynthetic intermediates and the rate of photosynthetic carbon assimilation in leaves of *Amaranthus edulis* L. *Planta* 174: 253–262
- Leegood RC and von Caemmerer S (1989) Some relationships between the contents of photosynthetic intermediates and the rate of photosynthetic carbon assimilation in leaves of *Zea mays* L. *Planta* 178: 258–266
- Leegood RC and von Caemmerer S (1994) Regulation of photosynthetic carbon assimilation in leaves of C_3 - C_4 intermediate species of *Moricandia* and *Flaveria*. *Planta* 192: 232–238
- Lucas WJ, Ding B and van der Schoot C (1993) Plasmodesmata and the supracellular nature of plants. *New Phytol* 125:435–476
- Meister M, Agostino A and Hatch MD (1996) The roles of malate and aspartate in C_4 photosynthetic metabolism of *Flaveria bidentis* (L.). *Planta* 199: 262–269
- Monson RK, Moore B, Ku MSB and Edwards GE (1986) Cofunction of C_3 and C_4 photosynthetic pathways in C_3 , C_4 and C_3 - C_4 intermediate *Flaveria* species. *Planta* 168: 493–502
- Murata S, Kobayashi M, Matoh T and Sekiya J (1992) Sodium stimulates regeneration of phosphoenolpyruvate in mesophyll chloroplasts of *Amaranthus tricolor*. *Plant Cell Physiol* 33: 1247–1250
- Nishikido T and Takanashi H (1973) Glycine activation of PEP carboxylase from monocotyledonous C_4 plants. *Biochem Biophys Res Comm* 53: 126–133
- Ohnishi J and Kanai R (1988) Glycerate uptake into mesophyll and bundle sheath chloroplasts of a C_4 plant, *Panicum miliaceum*. *J Plant Physiol* 133: 119–121
- Ohnishi J and Kanai R (1990) Pyruvate uptake induced by a pH jump in mesophyll chloroplasts of maize and sorghum, NADP-malic enzyme type C_4 species. *FEBS Lett* 269: 122–124
- Ohnishi J, Flügge UI and Heldt HW (1989) Phosphate translocator of mesophyll and bundle sheath chloroplasts of the C_4 plant, *Panicum miliaceum*. Identification and kinetic characterization. *Plant Physiol* 91: 1507–1511
- Ohnishi J, Flügge UI, Heldt HW and Kanai R (1990) Involvement of Na^+ in active uptake of pyruvate in mesophyll chloroplasts of some C_4 plants: Na^+ /pyruvate transport. *Plant Physiol* 94: 950–959
- Oleson P (1975) Plasmodesmata between mesophyll and bundle sheath cells in relation to the exchange of C_4 -acids. *Planta* 123: 199–202
- Osmond CB (1971). Metabolite transport in C_4 photosynthesis. *Aust J Biol Sci* 24: 159–163
- Raghavendra AS and Das VSR (1978) (Na^+ - K^+)-stimulated ATPase in leaves of C_4 plants: Possible involvement in active transport of C_4 acids. *J Exp Bot* 29: 39–47
- Stitt M and Heldt HW (1985a) Control of photosynthetic sucrose synthesis by fructose-2,6-bisphosphate. Intercellular metabolite distribution and properties of the cytosolic fructose

- bisphosphatase in leaves of *Zea mays* L. *Planta* 164: 179–188
- Stitt M and Heldt HW (1985b) Generation and maintenance of concentration gradients between the mesophyll and bundle-sheath in maize leaves. *Biochim Biophys Acta* 808: 400–414
- Taniguchi M and Sugiyama T (1996) Isolation, characterization and expression of cDNA clones encoding a mitochondrial malate translocator from *Panicum miliaceum* L. *Plant Mol Biol* 30: 51–64
- Usuda H (1986) Non-autocatalytic build-up of ribulose 1,5-bisphosphate during the initial phase of photosynthetic induction in maize leaves. *Plant Cell Physiol* 27: 745–749
- Usuda H (1987a) Changes in levels of intermediates of the C₄ cycle and reductive pentose phosphate pathway under various concentrations of CO₂ in maize leaves. *Plant Physiol* 83: 29–32
- Usuda H (1987b) Changes in levels of intermediates of the C₄ cycle and reductive pentose phosphate pathway under various light intensities in maize leaves. *Plant Physiol* 84: 549–554
- Valle EM, Craig S, Hatch MD and Heldt HW (1989) Permeability and ultrastructure of bundle sheath cells isolated from C₄ plants: Structure-function studies and the role of plasmodesmata. *Bot Acta* 102: 276–282
- von Caemmerer S and Furbank RT (1999) Modeling C₄ photosynthesis. In: Sage RF and Monson RK (eds) C₄ Plant Biology, pp 173–211. Academic Press, San Diego
- Weiner H and Heldt HW (1992) Inter- and intracellular distribution of amino acids and other metabolites in maize (*Zea mays* L.) leaves. *Planta* 187: 342–246
- Weiner H, Burnell JN, Woodrow IE, Heldt HW and Hatch MD (1988) Metabolite diffusion into bundle sheath cells from C₄ plants. Relation to C₄ photosynthesis and plasmodesmatal function. *Plant Physiol* 88: 815–822
- Wingler A, Walker RP, Chen Z-H and Leegood RC (1999) Phosphoenolpyruvate carboxykinase is involved in the decarboxylation of aspartate in the bundle sheath of maize. *Plant Physiol* 120:539–545

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Chapter 20

Developmental Aspects of C₄ Photosynthesis

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Summary

Development of a fully functional C₄ leaf requires modification of the C₃ ground state at several hierarchical levels, including vein spacing in whole leaves, cell division and cell enlargement patterns within the photosynthetic tissue precursors, ultrastructural changes in bundle sheath (BS) and mesophyll (M) cells, and regulation of gene expression through differential transcription, translation, and protein turnover. Both spatial and temporal patterns of BS and M cell differentiation indicate that cell position is the primary determinate of photosynthetic cell fate and that cell-to-cell communication is essential for interpretation of position. The nature of signaling pathways in C₄ development is unknown, but recent demonstrations of macromolecular trafficking through plasmodesmata and of the role of receptor-like kinases in tissue pattern formation provide clues to the mechanisms co-opted by C₄ plants. The regulation of photosynthetic gene expression occurs at two

levels (which may coincide temporally) in C_4 plants: gene expression occurs first in an organ- and cell-specific way only in those cells where chloroplasts develop and replicate to high numbers; the positional signals involved are likely common to both C_3 and C_4 plants. Second, gene expression patterns that are restricted to either BS or M cells occur; these patterns are limited to C_4 plants, but may use some of the same positional signals to achieve differentiation of BS and M cells. Experimental approaches that are providing insights into the developmental regulation of these genes include reporter gene fusions to identify *cis*-acting sequences and screens to identify mutations that disrupt C_4 developmental patterns. The evidence to date indicates that each C_4 gene uses its own regulatory mechanism to achieve cell specificity; even if all cell-specific genes respond to the same signal, these signals appear to activate different mechanisms which in turn activate or repress the expression of C_4 genes.

I. Introduction

C_4 photosynthesis requires developmental regulation at several levels of leaf organization. In all plants, regardless of photosynthetic pathway, the internal architecture of the leaf is determined in a series of hierarchical processes. When leaves are formed by the shoot apical meristem, the surface layer, precursor to dermal tissue system, is distinct from internal layers, based on differential planes of cell division (Esau, 1965). Delimitation of the vascular tissue system from other internal tissues occurs during very early stages of leaf expansion. Formation of individual tissue types within each of the three tissue systems (dermal, ground, and vascular) involves distinctive planes of cell division and enlargement to give rise to each tissue in its appropriate position. Cell differentiation requires differential enlargement and the expression of cell-specific biochemical and structural characteristics, including cell wall and organelle components. Although these processes occur at different levels of organization (leaf, tissue system, tissue, cell, organelle), they are coordinated during development so that a characteristic temporal sequence is followed and elements are formed in characteristic spatial patterns. Formation of a fully functional C_4 leaf requires modification of the C_3 ground state developmental programs that determine

all of these processes, specifically: 1) overall tissue pattern within the leaf, 2) cell pattern within tissues, and 3) biochemical compartmentation and structural cell differentiation (Fig. 1). Although far more is known about cell specific enzyme expression on a molecular and genetic level than any other aspect of C_4 photosynthesis, this chapter reviews alterations to all three levels of leaf organization that are required for development of the C_4 syndrome, discusses the interactions between these levels, and identifies experimental approaches that will advance our understanding of the developmental basis of C_4 photosynthesis.

A. Relationship between C_4 Biochemistry and Structure

In C_3 plants, all chloroplast-containing cells, including leaf M and BS cells, are equivalent in terms of photosynthetic pathway, although BS cells typically have fewer and smaller chloroplasts (Fig. 1). In both cell types atmospheric CO_2 is assimilated by carboxylase activity of the chloroplast enzyme RuBPCase and reduced by the activities of other Calvin cycle enzymes, using ATP and NADPH produced by the light reactions to form three carbon triose phosphate molecules and, ultimately, sucrose and starch. As a consequence of the oxygenase activity of RuBPCase, C_3 photosynthetic tissues also experience the energetically wasteful process of photorespiration. C_4 plants effectively suppress photorespiration by a spatial separation of the C_3 Calvin cycle activity in BS cells and of the C_4 cycle in M cells. The C_4 cycle acts as a biochemical CO_2 pump, concentrating CO_2 ten-fold over atmospheric concentrations and thus greatly reducing the oxygenase activity of RuBPCase (Edwards and Walker, 1983; Hatch, 1987; Furbank and Taylor, 1995). CO_2 is initially fixed within M cells by

Abbreviations: BS – bundle sheath; CA – carbonic anhydrase; Cab – chlorophyll *a/b* binding protein of Photosystem II light harvesting complex; chp – chloroplastic; cyt – cytoplasmic; E – epidermis; GDCST – T subunit of glycine carboxylase complex; LSu – RuBPCase large subunit; M – mesophyll; NAD-ME – NAD-malic enzyme; NADP-MDH – NADP-dependent malic dehydrogenase; NADP-ME – NADP-malic enzyme; P – parenchyma; PCK – PEP carboxykinase; PEP – phosphoenolpyruvate; PEPCase – phosphoenolpyruvate carboxylase; PPDK–pyruvate, orthophosphate dikinase; PS II – Photosystem II; RuBPCase – ribulose-1,5-bisphosphate carboxylase/oxygenase; V – vein

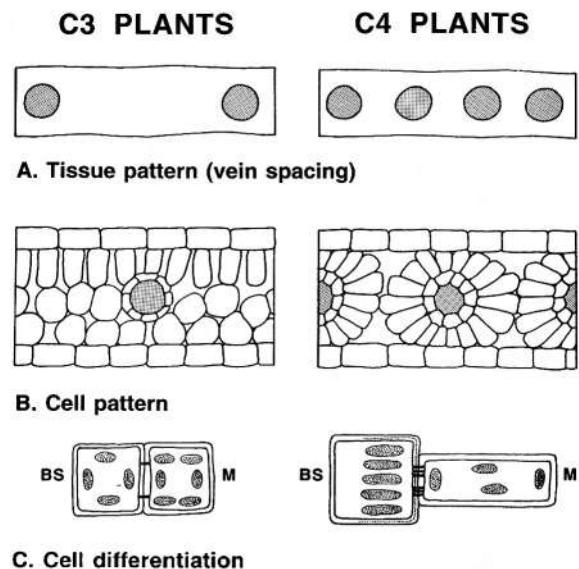


Fig. 1. C₄ developmental modifications of C₃ ground state. A. Altered tissue pattern results in closer vein spacing in C₄ plants. B. Altered cellular patterns of division and enlargement within bundle sheath and mesophyll tissues results in enlarged BS and radial arrangement of elongate M cells in C₄ plants. C. Cell differentiation in C₄ plants involves replication and enlargement of BS chloroplasts, asymmetric placement of organelles, and deposition of a suberin lamella (dotted line) in BS cell walls.

PEPCase, using PEP as a substrate and forming the C₄ acid oxaloacetate. Oxaloacetate is then either reduced to malate by NADP-MDH within the M cell chloroplasts or transaminated to aspartate in the cytosol. The C₄ acids diffuse from the M cells to the BS cells through connecting cytoplasmic channels, the plasmodesmata, and are decarboxylated within the BS cells, thus maintaining higher CO₂ concentrations at the site of RuBPCase. The released CO₂ is reassimilated by the carboxylase activity of RuBPCase, and three carbon pyruvate diffuses back through the plasmodesmata to M cell chloroplasts where it is phosphorylated to PEP by the activity of PPDK.

Three variants of this basic scheme that differ in C₄ acid and decarboxylating enzyme are known: 1) the NADP-ME type, in which malate is decarboxylated by NADP-ME in BS chloroplasts, 2) the NAD-ME type in which decarboxylation is accomplished by NAD-ME in BS mitochondria, and 3) the PCK type in which decarboxylation is accomplished by PCK in the BS cytosol (as well as NAD-ME activity in the mitochondria) (Gutierrez et al., 1974; Hatch et al., 1975, 1987). As a result of the compart-

mentation of initial CO₂ fixation steps in M cells and the subsequent carbon reduction steps in BS cells, the two photosynthetic cell types of C₄ plants differ strikingly in enzyme complement. Since all of these enzymes are either components of the Calvin cycle or participate in other nonphotosynthetic functions within the plant, C₄ photosynthesis depends on regulatory mechanisms that produce these cell-specific expression patterns (Moore, 1982; Hermans and Westhoff, 1990; Ku et al., 1996). In addition, C₄ forms of these enzymes may differ from their C₃ counterparts by having much higher activities and levels of expression, different kinetic properties, and different patterns of light-regulated expression and intracellular localization (Bauwe and Chollet, 1986; Harpster and Taylor, 1986; Hatch, 1987; Hatch and Burnell, 1990; Schäffner and Sheen, 1992; Kubicki et al., 1994).

C₄ photosynthetic biochemistry is accompanied by a distinctive pattern of leaf anatomy historically referred to as 'Kranz anatomy' (Fig. 1; Brown, 1975; Hatch, 1987). Despite the great diversity in basic leaf architecture in the plant families in which C₄ photosynthesis occurs, certain anatomical features are invariably associated with the pathway and are regarded as being essential for its operation. These are: 1) structural specialization of the two types of photosynthetic cells, 2) spatial configuration of these tissues that maximizes contact between M and BS cells, and 3) features that limit the rate of CO₂ leakage from the bundle sheath cells (Hattersley and Browning, 1981; Hattersley, 1984; Hattersley and Watson, 1992). Although unusual anatomical types occur (Dengler and Nelson, 1998), typical C₄ BS cells are large in comparison to their C₃ counterparts, have more numerous chloroplasts, and an asymmetric arrangement of cytoplasmic components. M cells are similar in size to those of C₃ species and, like them, have peripherally arranged chloroplasts (Fig. 1C). In C₄ plants, BS and M cells are intimately associated; M cells are usually elongate in a radial direction, an arrangement that permits each cell to be in contact with a BS cell (Fig. 1B). BS cells are typically associated with leaf veins and thus veins are closer together in C₄ species (Fig. 1A). Numerous plasmodesmata extend across the shared BS/M cell wall, providing an extensive pathway for the diffusion of metabolites (Fig. 1C). The cell wall at the BS/M cell interface is often highly modified: a suberin lamella, thought to be impermeable to CO₂, is deposited in the BS portion of the wall of many C₄

species. The volume of intercellular airspace is lower in C_4 species than in related C_3 species, and both the shape of BS cells and the arrangement of M cells reduce exposure of the BS cell surface to intercellular airspace, thus reducing the pathway for CO_2 leakage (Dengler et al., 1994).

The C_4 pathway is an adjunct to the universal C_3 pathway and depends not only on novel patterns of photosynthetic enzyme expression, but also on cell-specific changes in patterns of cell enlargement, cell wall modifications, chloroplast division and growth, and placement of organelles within the cell. The genetic programs that guide these developmental processes are all present in some form in C_3 plants, so that the primary requirement for the development of Kranz anatomy is their position-specific regulation. In addition, the planes of cell division and of directional cell enlargement are modified during formation of C_4 mesophyll tissue, and formation of leaf vascular pattern is altered to result in the close vein spacing of C_4 plants. These diverse developmental events must be regulated in a coordinated way as each leaf expands and matures. Thus the development of C_4 photosynthesis offers an opportunity to address questions not only of the mechanisms of cellular differentiation, but also how developmental pathways and regulatory mechanisms are coupled across organizational levels.

B. Multiple Evolutionary Origins

The evolutionary pattern of C_4 photosynthesis makes questions about its developmental regulation particularly interesting and challenging. The taxonomic distribution of C_4 plants indicates that this photosynthetic pathway evolved numerous times during the diversification of flowering plants. C_4 photosynthesis occurs in genera of at least 16 dicotyledon families, including the Amaranthaceae, Asteraceae, Chenopodiaceae, Euphorbiaceae, and Portulacaceae (Downton, 1975; Ragavendra and Das, 1978). Although C_4 species are relatively numerous in the Chenopodiaceae and Euphorbiaceae, each of the 16 dicot families contains only a few genera (in some cases only a single genus) with C_4 species, while the remaining genera of the family are wholly C_3 . The predominance of C_3 photosynthesis in each family and the lack of a common ancestor having the C_4 photosynthetic pathway is indicative of a series of independent evolutionary origins for C_4 photosynthesis. Only two families of monocotyledons, the

grasses (Poaceae) and sedges (Cyperaceae) have C_4 species, but these are more numerous. It is estimated that one-half (5,000 species) of all grass species are C_4 , and phylogenetic analyses of the family indicate at least four different origins for subgroups with C_4 photosynthesis (Hattersley and Watson, 1992; Kellogg, 1996; Sinha and Kellogg, 1996). In addition, the complex pattern of photosynthetic pathway variation within the grasses also indicates that reversals from C_4 to C_3 photosynthesis have occurred more than once (Hattersley, 1987). The phylogeny of the sedge family is less well understood, but it is estimated that about 40% of a total 4,000 species are C_4 and that the pathway has arisen three to four times (Bruhl, 1995).

These evolutionary patterns raise important questions for understanding some aspects of the developmental regulation of the C_4 syndrome: if there have been 20 or more separate evolutionary origins of C_4 photosynthesis, are there as many different molecular mechanisms that regulate each aspect of the C_4 syndrome or is the spectrum of possibilities limited? Do genes co-opted for C_4 photosynthesis share common regulatory mechanisms so that a suite of genes is expressed under the control of a 'master switch'? Are the biochemical variants of C_4 photosynthesis always equivalent; for example, is the same mechanism used for the regulation of BS-specific NAD-ME expression in a grass and a dicotyledon? The answers to these questions require the methodological approaches of comparative biology where developmental regulation of biochemical and anatomical traits is compared between closely related groups of C_3 and C_4 species (Hattersley and Watson, 1992; Kellogg, 1996; Monson, 1996). While the primary goal of comparative studies is to understand evolutionary diversification, their findings may also be useful for understanding some aspects of developmental regulation. Our goals in this review are to identify both the structural and biochemical features that are essential for the operation of the C_4 pathway, to review our current understanding of their developmental regulation, and to discuss future prospects for resolving outstanding questions. Although we focus on certain model organisms, we also speculate on whether different evolutionary events arrived at common developmental mechanisms or whether substantial diversity exists in the developmental regulation of C_4 photosynthesis.

II. Formation of Tissue Pattern

A. Vein Spacing

Compartmentation of the primary carbon assimilation and photosynthetic carbon reduction steps of the C₄ pathway imposes a physiological requirement for the rapid flux of metabolites between BS and M cells. In C₃ grass species, ten or more M cells may intervene between the BS cells of adjacent veins, while in C₄ grasses no M cell is more than one cell removed from the bundle sheath and, in fact, most M cells are in direct contact with a BS cell (Fig. 1A,B; Hattersley and Watson, 1975). This requirement results in the reduction of total mesophyll tissue volume in the leaves of C₄ species in comparison to their C₃ relatives (Hattersley, 1984; Dengler et al., 1994) and, because BS tissue is typically (but not universally) associated with leaf veins, in the close spacing of adjacent leaf veins (Crookston and Moss, 1974; Kawamitsu et al., 1985; Dengler et al., 1994). Sinha and Kellogg (1996) concluded that, given the diversity in positional relationships between BS and M cells and in accumulation patterns of photosynthetic enzymes other than RuBPCase and PEPCase, interveinal distance was one of the few consistent characteristics that consistently distinguishes C₄ from C₃ species. Thus, evolution of C₄ photosynthesis has universally required modification of the regulatory pathways that determine vein pattern early in leaf development.

B. Vein Pattern Ontogeny

Precursors of dermal and ground tissues are present from leaf inception, but precursors of vascular tissues become established during early stages of leaf development. It is only after the establishment of vascular pattern that BS and M cells become delimited in relation to the veins (Langdale and Nelson, 1991; Nelson and Dengler, 1992). In dicotyledons, the most common leaf vascular pattern becomes established in three discrete phases: 1) the midvein provascular strand develops in continuity with the stem vasculature, extending from the stem into a new leaf primordium, 2) secondary vein provascular strands grow progressively from the midvein toward the margin concurrent with the formation of the leaf lamina, and 3) minor vein provascular strands form a network of small veins between the secondary veins, usually in a basipetal direction (Fig. 2A-C). Minor vein formation coincides with the early stages of leaf

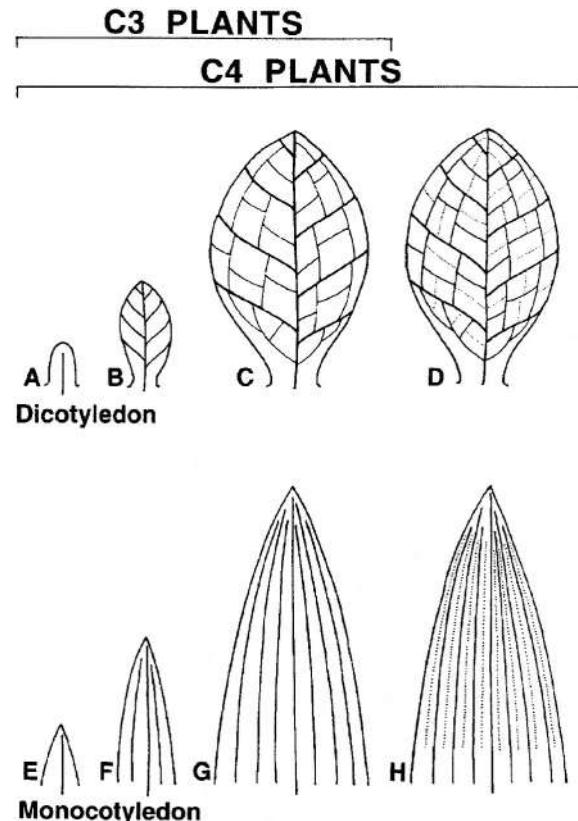


Fig. 2. Vein pattern ontogeny in C₃ dicotyledon (A-C) and C₃ monocotyledon (E-G). C₄ patterns require an additional iteration of late stages, resulting in reduced interveinal distances in C₄ dicotyledons (D) and C₄ monocotyledons (H)

expansion and is coordinate with it so that as new veins are intercalated between older ones, the mean distance between adjacent veins tends to remain constant. These last stages of vascular pattern formation are those that must be modified during leaf development in a C₄ dicotyledonous species: the developmental programs that determine vein spacing patterns must be altered to result in the short interveinal distance in the leaves of C₄ species (Fig. 2D). Development of the striate venation pattern of grass leaves such as maize differs in several respects from dicotyledons (Evert et al., 1996; Dengler et al., 1997). The largest longitudinal veins initially appear in isolation from stem vasculature and connections between these veins and the stem vascular bundles occur only secondarily. As the grass leaf grows in width, new longitudinal veins are intercalated between previously formed veins, maintaining a constant mean distance between adjacent veins (Fig. 2E-G). This

process is modified in C_4 species so that formation of small longitudinal veins is prolonged or accelerated, resulting in a greater number of more closely spaced veins (Fig. 2H).

C. Regulation of Vein Pattern Formation

Since C_4 species are distinguished from C_3 species by closer vein spacing, a key step in the evolution of a C_4 species is an alteration in the regulatory pathways that determine vascular pattern. At present, very little is known about the genetic and molecular control of C_4 vein spacing. A screen of the M2 generation from EMS-mutagenized seed of the C_4 grass *Panicum maximum* produced several putative mutants with an increase in both interveinal distance and number of M cells intervening between adjacent veins (Fladung, 1994). One of these, the *lis1* mutation, had lower photosynthetic rates and more C_3 -like CO_2 compensation points, indicating that vein spacing is critical for the efficient operation of the C_4 pathway. Unfortunately, it was not possible to carry these putative mutants to advanced generations, so the molecular and physiological roles of *lis1* in determining C_4 vein spacing is unknown.

Other experimental and genetic evidence points to the importance of the polar transport of auxin in the formation of vascular strands. Based on careful observations of regeneration of vascular tissue around mechanically induced wounds in stems and leaves in response to auxin application, Sachs (1981, 1989, 1991) proposed that canalization of auxin flow occurred through the following steps: 1) initially all cells in a developmental field are equivalent transporters of auxin, 2) gradually certain cells become better auxin transporters and, through positive feedback, their capacity to transport auxin increases with auxin flux, and 3) the cells transporting auxin are induced to differentiate as provascular tissue, thus becoming determined as the preferred transport channels. Surrounding cells would be drained of auxin and therefore would be inhibited from forming vascular tissue.

Several auxin transport mutations in *Arabidopsis thaliana* lend support to Sach's hypothesis. Among other functions, the *MONOPTEROS* (*MP*) gene appears to regulate leaf vascular pattern formation (Przemack et al., 1996). Vascular strands in mutant leaves show discontinuities and improper alignment of component tissues. These mutants have impaired auxin transport capacity, indicating that normal

vascular pattern formation is dependent on the polar flow of auxin. Similarly, mutations in the *LOPPED* (*LOP*) gene result in a greatly reduced leaf lamina that lacks minor venation, but has an oversized or twinned midvein (Carland and McHale, 1996). Mutant plants have normal levels of free auxin, but reduced capacity for auxin transport, also pointing to a role for the polar transport of auxin in normal vascular pattern formation. If auxin transport characteristics are indeed crucial for vascular pattern formation, it is likely that mechanisms associated with the positive feedback loop postulated by Sachs became modified during the evolution of C_4 from C_3 species. If perception of auxin level or response to a specific auxin signal is altered, then canalized pathways for auxin transport might arise at closer spacing, resulting in characteristic C_4 vein densities in mature leaves.

III. Cell Pattern within Tissues

The physiological requirement for contact between M and BS cells has resulted in an alteration of the layered arrangement of C_3 mesophyll tissue to the typical C_4 arrangement in which M cells are arrayed in a radial pattern in relation to the leaf veins (Fig. 1B). The radial arrangement of C_4 mesophyll is acquired relatively late during leaf development (Dengler et al., 1995) and requires a change in the pattern of enlargement of ground meristem cells as they differentiate as M cells, as well as a possible change in cell division pattern (Fig. 3B). Such alterations in plant cell division and cell expansion are generally anticipated by changes in cytoskeleton components: cell division plane reflects the orientation of the preprophase band of microtubules and microfilaments, and the direction of cell elongation is perpendicular to the predominant orientation of cytoskeleton elements (Lloyd et al., 1992; Cyr, 1994). The *fass* and *ton* mutations of *Arabidopsis* affect both aspects of the cytoskeleton: cortical microtubules are disorganized and preprophase bands are not formed (Torres-Ruiz and Jurgens, 1994; Traas et al., 1995). Although tissues of mutant plants occur in the correct positions, they show defective planes of cell division and cell enlargement, indicating that these genes are necessary for normal formation of cell patterns within tissues. These mutant phenotypes are expressed throughout the plant, indicating that they are not regulated in an organ- or

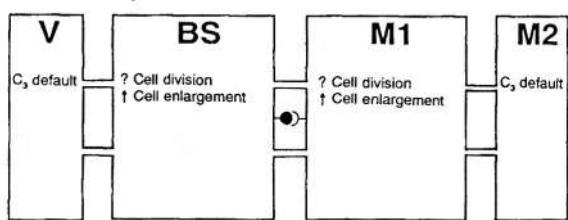
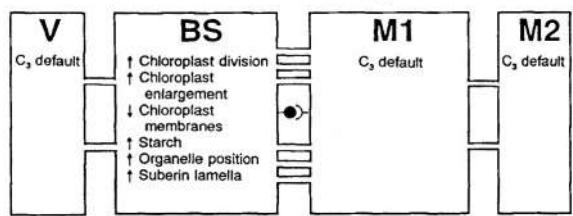
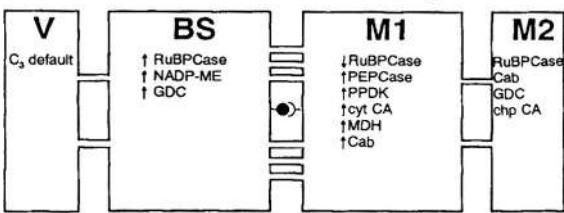
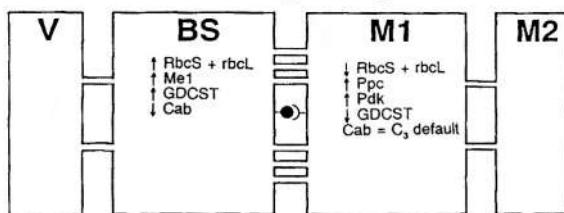
A. Vein spacing**B. Tissue pattern****C. Cell differentiation - structure****D. Cell differentiation - biochemistry****E. Cell differentiation - gene regulation**

Fig. 3. Temporal sequence (and presumed hierarchy of genetic control) of C₄ development. A. Vein spacing. B. Cell pattern within tissue, involving patterns of cell division and enlargement. C. Structural aspects of cell differentiation. D. Biochemical aspects of cell differentiation. E. Differential gene expression. Cell-cell signaling pathways through plasmodesmata and/or putative membrane-bound receptor-like kinases may be involved at each level. Note differing patterns between M cells adjacent to BS (M1) and more distant M (M2) cells.

cell-specific manner.

Several genes that have a role in chloroplast development also affect cell elongation in a tissue-specific manner. For instance, *Arabidopsis* plants with a mutation in the *PALE CRESS (PAC)* gene have

defective chloroplasts, and normal elongation of palisade mesophyll during leaf development is disrupted (Reiter et al., 1994). In mutant plants mesophyll layers form normally, but the distinctive elongate palisade cell shape is never fully developed, indicating genetic regulation over initial cell elongation and maintenance of elongate shape during development. Similarly, the *differentiation and greening (dag)* mutation of *Antirrhinum* and *defective chloroplasts and leaves-mutable (dcl-m)* mutation of tomato block elaboration of internal chloroplast membranes and also repress normal elongation of palisade mesophyll (Chatterjee et al., 1996; Keddie et al., 1996). These mutant phenotypes indicate that normal palisade cell elongation requires a plastid-derived signal and, while there is no direct evidence, such a signaling pathway presumably would interact with the cytoskeleton to produce differential cell elongation. Although these genes have been identified in C₃ species, these studies may have identified signaling pathways that could be involved in C₄ mesophyll-specific elongation patterns. In C₄ plants any regulatory mechanism that links chloroplast development, cytoskeletal organization, and cell enlargement must act downstream of the positional signals that identify M cells. Such genes must be among the first to be specifically activated in the development of a C₄ leaf.

IV. Bundle Sheath and Mesophyll Cell Structural Differentiation

A. Chloroplast Dimorphism

The chloroplast complements of mature BS and M cells differ strikingly: BS cells typically have greater numbers and larger chloroplasts, leading to a higher proportion of cell volume occupied by the plastome (Laetsch, 1974; Kirchanski, 1975; Dengler et al., 1986, 1996; Liu and Dengler, 1994; Ueno, 1996). Individual BS and M cell chloroplasts differ in shape as well as size: M cell chloroplasts tend to be elliptical in section, while BS chloroplasts are more likely to have a flattened profile. Also, starch grains are usually present in mature BS chloroplasts, but reduced or absent in M chloroplasts. The most dramatic differences between BS and M cell chloroplasts lie in the development of internal chloroplast membranes. In NADP-ME species, BS cell chloroplast thylakoids form only a small number of rudimentary grana,

while M cell chloroplasts have numerous large grana (Brangeon, 1973; Laetsch, 1974; Kirchanski, 1975; Ueno et al., 1988; Dengler et al., 1996; Nishioka et al., 1996). Reduced granal stacking in BS chloroplasts is correlated with reduced abundance of PS II polypeptides in NADP-ME type grasses (Schuster et al., 1985; Meierhoff and Westhoff, 1993; Kubicki et al., 1994). In NAD-ME type species, grana development is similar in BS and M cell chloroplasts, but chloroplasts are still dimorphic, large in BS and small in M cells (Laetsch 1974; Dengler et al., 1986; Liu and Dengler, 1994).

Another conspicuous difference between BS and M cell chloroplasts is the degree of development of a peripheral reticulum from the inner chloroplast envelope, although the pattern of variation is not consistent among C_4 types. In a number of C_4 dicotyledons, peripheral reticulum is better developed in M than BS cells (Laetsch, 1974; Chapman et al., 1975; Sprey and Laetsch, 1978; Liu and Dengler, 1994), but in C_4 species of the Cyperaceae, peripheral reticulum is more prevalent in BS cells, forming extensive arrays of vesicles or anastomosing tubules at the periphery of the chloroplasts (Ueno et al., 1988). Thus, C_4 developmental programs must involve regulation of chloroplast number per cell, chloroplast enlargement, elaboration of internal membranes to form grana and peripheral reticulum, and expression and activity of starch synthetic pathways (Fig. 3C).

Chloroplast number per cell typically increases about ten-fold as cells enlarge during leaf development (Ellis and Leech, 1985; Boffey, 1992). Proplastids divide as cells divide, but with the cessation of mitosis, enlarging plastids may continue to divide until the mature number of chloroplasts per cell is reached. Regulation of plastid replication and subsequent enlargement is cell-specific, since mesophyll, parenchymatous bundle sheath, vascular parenchyma, and epidermal guard cells all differ in chloroplast number and size in mature leaves (Pyke and Leech, 1994). Several nuclear genes that affect this process have been identified in *Arabidopsis* (Pyke and Leech, 1992, 1994; Pyke et al., 1994). Mutations in *ACCUMULATION AND REPLICATION OF CHLOROPLASTS* (*ARC*) genes result in a reduction in number of chloroplasts, but there is a compensating increase in chloroplast size so that total complement per cell is similar in mutants and wildtype. Chloroplast ultrastructure is affected only slightly, indicating that *arc* mutations are primarily defects in the plastid division process (Pyke et al., 1994). *ARC* genes also

appear to have cell-specific effects: in wildtype leaves, chloroplasts of the parenchymatous bundle sheath (equivalent of C₃ BS cells) are smaller than chloroplasts of M cells, but in *arc1* mutants they are larger (Pyke and Leech, 1994). *ARC* genes may be among those which must be regulated differently in C₄ compared with C₃ plants. In C₄ species, chloroplast division must be upregulated during development of C₄ BS cells, but, since BS cells typically have both more and larger chloroplasts, chloroplast enlargement must also be regulated, resulting in a total chloroplast complement that is dramatically larger than that of C₃ BS and M (or C₄ M) cells.

Development of the dimorphic chloroplast ultrastructure of BS and M cells is best known for maize and other C₄ grass species where there is a longitudinal gradient of cell maturation stages in cell files produced by the basal elongation zone (Laetsch and Price, 1969; Brangeon, 1973; Kirchanski, 1975; Dengler et al., 1996). At the youngest stages, BS and M cell proplastids are morphologically identical, with numerous starch grains and rudimentary grana. As plastids in both cell types increase in size, stroma thylakoids become more extensive and appressed regions become more frequent; starch grains disappear during this growth phase. When BS chloroplasts reach about 50% of their mature size, appressed thylakoids become disassociated, reducing the number and extent of grana, despite the continued growth of stroma thylakoids. Loss of grana is correlated with reduced activity of PS II as indicated by photoreduction of tetrazolium blue (Wrischer, 1989) and by chlorophyll a fluorescence measurements (J. A. Langdale, personal communication). At late developmental stages, conspicuous starch grains appear in BS cell chloroplasts, but are less prevalent in M cell chloroplasts. Mutations in *Bundle sheath defective-mutable* genes (*Bsd1-m1* and *Bsd2-m1*) affect chloroplast ultrastructure in BS and M cells differentially, essentially arresting BS chloroplast development at an early stage and/or retarding growth patterns (Langdale and Kidner, 1994; Roth et al., 1996). Although *Bsd1-m1* and *Bsd2-m1* appear to play a primary role in *rbcL* transcript accumulation, these genes also appear to have at least an indirect role in regulating the divergent developmental pathways of BS and M cell chloroplasts.

B. Asymmetric Placement of Organelles

BS cell organelles typically have an asymmetric

placement within the cell, while M cell organelles are symmetrically distributed, as is characteristic for other vacuolate parenchyma cells. In NADP-ME type species, BS cell chloroplasts, mitochondria, microbodies and nuclei are located in a centrifugal position, toward the mesophyll side of the cell, while in NAD-ME type species, organelles are located in a centripetal position, adjacent to the vascular tissue (Gutierrez et al., 1974; Hatch et al., 1975). The organelles of immature BS cells in the basal extension zone of grass leaves have a symmetrical distribution around a central vacuole; as cells cease elongation at the distal end of the extension zone, organelles migrate toward their final position (Miyake and Yamamoto, 1987; Dengler et al., 1996).

Both microtubules and microfilaments are known to be involved in some types of chloroplast movements (Williamson, 1986); however, chloroplast-associated microtubules were not observed in leaf blades of the NAD-ME grass *Eleusine corocana* although they were present in the cortical cytoplasm of the same cells (Miyake and Yamamoto, 1987). Evidence for the involvement of actin microfilaments in chloroplast migration in developing *Eleusine* leaf blades was obtained from a series of experimental treatments of isolated elongating leaf blades: cytochalasin B, an inhibitor of microfilaments, almost completely blocked chloroplast migration, while colchicine, an inhibitor of microtubule assembly, had no effect (Miyake and Nakamura, 1993). Chloroplast movement was also inhibited by cycloheximide, an inhibitor of cyt protein synthesis, indicating that production of actin and possibly force-generating ATPases may be required for migration. Most growth hormone treatments had no effect on this process, but chloroplast movement was inhibited by auxin treatment, indicating that auxin itself or the physiological status of elongating tissues might prevent migration. These processes do not appear to be specific for chloroplasts themselves because nuclei, mitochondria and microbodies always moved with the chloroplasts. Light is likely an important part of the signaling pathway for the asymmetric placement of organelles, since dark treatment prevented chloroplast migration in *Eleusine* and is required for organellar movement in other systems (Haupt, 1982). Organelle position appears to be fixed once BS cells have differentiated, although the striking centrifugal position of BS cell chloroplasts was lost in response to severe water stress in maize (Lal and Edwards, 1996).

C. Suberin Lamella

The vascular bundles of all grasses, both C₃ and C₄, are surrounded by an inner sheath layer, the mestome sheath (Esau, 1965). Mestome sheath cells are non-chlorenchymatous and have thickened cell walls that are modified by deposition of a layer of suberin (O'Brien and Carr, 1970). In NADP-ME type grasses such as maize, the outer chlorenchymatous bundle sheath layer is absent and mestome sheath cells are modified as BS cells (Dengler et al., 1985). In these species, the suberin lamella is thought to carry out the additional function of restriction of apoplastic leakage of CO₂ from C₄ BS cells to the intercellular airspace, thus contributing to the maintenance of high CO₂ concentration and suppression of photo-respiration (Hattersley and Browning, 1981). In maize, deposition of suberin in BS walls coincides with maturation of the last-formed xylem elements in the vein: the suberin lamella first appears at isolated sites within the BS cell walls and becomes continuous in each cell as the last xylem elements mature (Evert et al., 1996). Just before maturation, the suberin lamellae become thickened and polylamellate in regions of the BS/M cell wall with conspicuous aggregations of plasmodesmata. Positional regulation of suberin lamella deposition is presumably unaltered during leaf development in NADP-ME grasses, but, in PCK type C₄ grasses, suberin lamellae are deposited in both the inner mestome sheath cell walls and in the outer bundle sheath layer (the C₄ BS cells) (Hattersley and Browning, 1981; Eastman et al., 1988). Thus the evolution of Kranz anatomy in PCK grasses requires novel spatial control of suberin lamella deposition and associated modification of the suberin lamella at the site of plasmodesmatal aggregations (Fig. 1C, 3C).

D. Plasmodesmata and Cell-Cell Communication in C₄ Development

Both spatial and temporal patterns of BS and M cell differentiation indicate that cell position determines cell fate and that cell-cell communication is essential for interpretation of position (Langdale and Nelson, 1991; Nelson and Langdale, 1992). For instance, ground tissue cells differentiate as C₄ M cells only when adjacent to BS cells, indicating that a BS-derived signal is required for determination of cell identity (Langdale et al., 1988b). In maize, the modified husk leaves that surround the female

inflorescence have wide vein spacing, with up to 20 M cells between adjacent veins, in contrast to typical foliage leaf blades with only two M cells between veins. In husk leaves, M cells next to the veins express C_4 enzymes, but more distant M cells accumulate RuBPCase instead. Similarly, in *Atriplex rosea*, M cells directly adjacent to BS cells accumulate PEPCase, but this enzyme is not detected in M cells more than one cell removed from the bundle sheath (Dengler et al., 1995). In *Flaveria*, M cells that are more than one cell away from BS cells have significantly fewer chloroplasts and show very little GUS expression with *Ppc* or *Pdk* promoters (Stockhaus et al., 1997).

The sequence of events during leaf development indicates that cell identity is determined or expressed earlier in BS than M cells. During maize leaf ontogeny, LSu transcripts accumulate in BS cell precursors well before the appearance of PEPCase transcripts in adjacent M cells (Langdale et al., 1988a). In *Atriplex rosea*, RuBPCase is detected in BS cell precursors two to four days before PEPCase is detected in M cell precursors, and ultrastructural evidence of cell differentiation such as growth of chloroplasts begins earlier in BS than M cells (Liu and Dengler, 1994; Dengler et al., 1995). Based on observations of these developmental patterns in maize, Nelson and Langdale proposed that the positional control over C_4 -type tissue cell differentiation is established by a developmental signal that is produced in the vascular tissue and diffuses outward, first to BS and then to M cells (Langdale and Nelson, 1991; Nelson and Langdale, 1992). Interaction of this putative signal with a BS-specific factor induces BS-specific gene expression, while interaction with an M cell-specific factor would reduce RuBPCase expression and induce C_4 enzyme expression. The outwardly diffusing developmental signal would not reach more distant M cells which would continue to express the C_3 ground state. This hypothesis best fits the observational data, but as yet there is no direct evidence for a signal molecule functioning in communication between BS and M cells. Other systems, however, provide a model for understanding how developmental signals might be communicated between cells.

Plasmodesmata form cytoplasmic channels between adjacent cells and provide a potential route for the intercellular movement of signaling molecules. High densities of plasmodesmata are present at the BS-M cell interface in the mature leaves of C_4 plants and provide an extensive symplastic pathway for the

diffusion of C_4 acids and pyruvate between BS and M cells (Botha and Evert, 1988; Robinson-Beers and Evert, 1991a; Evert et al., 1996). It is possible that this pathway also functions in the exchange of informational molecules that coordinate metabolism, as suggested for communication between the M cells and phloem companion cells that function in sieve tube loading in C_3 leaves (Kragler et al., 1998). Recent evidence indicates that such a pathway may also function in the regulation of developmental processes. Analysis of genetic mosaics has shown that a number of genes with important developmental roles act non-cell autonomously. For instance, mutations in the maize *KNOTTED1* (*Kn1*) homeobox gene result in the formation of ectopic knots and ligule fragments on the surface of the maize leaf blade (Smith et al., 1992). These structures are derived from epidermal tissues, yet are formed in wildtype tissue when the middle mesophyll layer carries a mutant allele (Hake and Freeling, 1986). Thus internal layers must be able to communicate with surface tissues to induce the alteration in cell division planes that produces these structures. Microinjection experiments have shown that fluorescein-labeled *Kn1* protein can move symplastically between M cells, effectively mediating its own transport through plasmodesmata by increasing the size exclusion limit (Lucas et al., 1995).

Developmental changes in the plasmodesmata that interconnect vascular tissue, BS cells, and M cells may affect the potential pathway for the movement of macromolecular developmental signals. Primary plasmodesmata are formed during cytokinesis and thus provide cytoplasmic continuity among cells that share a cell lineage. During leaf development in C_4 NADP-ME type grasses, the cell lineage that gives rise to provascular tissue (and ultimately xylem, phloem and bundle sheath) is distinct from the surrounding ground meristem lineage that gives rise to mesophyll (Dengler et al., 1985). In addition, the layer at the vein periphery that gives rise to the bundle sheath layer forms a distinct lineage from inner provascular layers at early stages of vein development. Thus, initially, plasmodesmatal connections would provide only a limited pathway for the movement of signal molecules between BS and M cell precursors (Fig. 3B). The original patterns of primary plasmodesmatal connections can become highly modified during development, however, by the formation of secondary plasmodesmata (Fig. 3C-E); this modification essentially overrides the

potential limitation of cell lineage on development by establishing new pathways for cell-cell communication (Lucas, 1995; Kragler et al., 1998).

At present, there is only limited evidence for the formation of secondary plasmodesmata at the BS-M cell interface in C₄ plants. In developing leaves of the NADP-ME type grass *Arundinella hirta*, only a few isolated plasmodesmata are present in the shared BS-M cell wall at the provascular stage (Dengler et al., 1996), but as BS and M cells differentiate, new aggregations of plasmodesmata appear and the mean density of plasmodesmata remains constant, despite a many-fold increase in cell surface area. Detailed ultrastructural observations of plasmodesmata development in the C₄ NADP-ME type grasses maize and sugar cane did not detect secondary plasmodesmata formation, however, possibly because these studies focussed on later developmental stages (Robinson-Beers and Evert, 1991a; Evert et al., 1996).

In maize, plasmodesmata at the BS-M cell interface mature shortly before sink to source transition when BS and M cells are fully differentiated. Thus, while there is clear evidence for a substantial pathway for cell-cell communication in almost-mature and mature leaves, this pathway appears to be limited at early stages of development when differences in BS and M cell structure and cell-specific expression of photosynthetic enzymes and their mRNAs begin to be expressed. Although the experiments showing that *KNI* protein effectively altered the size exclusion limit of plasmodesmata were conducted using mature tobacco leaves, *KNI* protein occurs in the outer layer of the maize shoot apical meristem where *KNI* mRNA is undetected, suggesting that macromolecules can move through the plasmodesmata of undifferentiated tissues (Jackson et al., 1994; Lucas et al., 1995). Therefore it is possible that a putative signal, required for the induction of the C₄ pattern of BS and M cell differentiation, moves between cell layers even at a stage when the interconnecting plasmodesmata are few and possibly immature.

While plasmodesmata may play an important role in cell-cell communication during development of C₄ photosynthetic tissues, there may be additional or alternative mechanisms involved in the interpretation of positional information. In common with other eukaryotic organisms, plants are known to possess receptor-like molecules with receptor kinase activity that are located in the plasma membrane (Walker, 1994). Plants possess a large family of receptor-like

kinase genes, and each gene likely encodes a distinct protein kinase that may play a unique role in cellular signaling. For instance, mutant *erecta* (*er*) phenotypes in *Arabidopsis* indicate that this gene plays a role in the coordination of cell growth patterns during shoot development (Torii et al., 1996). *ERECTA* encodes a putative transmembrane receptor kinase with an extracellular ligand binding domain, indicating that it influences development through its role in intercellular signal transduction. Similarly, the *CRINKLY4* (*CR4*) gene encodes a receptor-like kinase and is required for normal epidermal cell patterning in the leaves of maize (Becraft et al., 1996). Genes with a similar function are likely involved in signaling between provascular tissue and surrounding cells early during C₄ leaf development (Fig. 3), but have not yet been identified from mutant screens.

V. Compartmentation of Photosynthesis

A. Genes Coding for C₄ Enzymes

The efficiency of C₄ photosynthesis is dependent on the strict compartmentation of enzyme activities responsible for the fixation and subsequent reduction of atmospheric CO₂. The compartmentation of C₄ cycle enzymes is due to cell-specific expression of their corresponding genes (Fig. 3E). In principle, this could be accomplished by a simple mechanism in which a factor specific to each cell type is produced during the differentiation of M and BS cells. Each factor would interact with a common cell-specific DNA sequence to induce and/or repress the expression of a set of genes in that cell type. A simple model for the evolution of genes coding for C₄ enzymes would involve gene duplications to create new copies of genes already coding for non-photosynthetic functions followed by the acquisition of cell-specific sequences as well as regulatory sequences for high-level, light-induced expression. However, analyses of gene structures and programs of expression has failed to find any evidence of a simple master switch mechanism; rather, the evidence points to a surprising diversity in gene structure, composition of gene families and mechanisms of regulation. We will limit our discussion to the most studied genes, especially those for which regulatory mechanisms can be inferred. Although gene names may differ between species and labs, we will use a single name for each gene or family to avoid confusion.

PEPCase is encoded by a gene family in all plants studied to date (Ku et al., 1996). The dicot genus *Flaveria* has been particularly useful in making evolutionary comparisons between genes because some species show full development of the C_4 pathway, others show uniform C_3 characteristics, while another group are C_3 - C_4 intermediates, showing a range of characteristics from C_3 -like to C_4 -like. Hermans and Westhoff (1990, 1992) compared *Ppc* genes in *Flaveria trinervia*, with full development of C_4 characteristics, and *F. pringlei*, a C_3 species. Both species have similar numbers of genes which were divided into four subfamilies based on sequence relatedness. In *F. trinervia*, one member of the *PpcA* subfamily was shown to encode a C_4 isoform of PEPCase, whereas *PpcB* and *PpcC* genes encode non-photosynthetic forms (Ernst and Westhoff, 1997). The *F. trinervia* *PpcA1* gene is more similar to the *F. pringlei* *PpcA1* gene than it is to any other *F. trinervia* *Ppc* genes. *PpcA* genes from the two species were therefore defined as being orthologous even though their patterns of expression are strikingly different. In *F. pringlei*, *PpcA* genes are expressed at low levels in leaves, roots and stems while *PpcA* expression is high-level and M cell-specific in *F. trinervia*. The identification of orthologous genes helps to define those whose comparison will best show evolutionary relationships of gene structures and regulatory mechanisms. We will use the term 'non-photosynthetic isoform' to describe gene family members encoding isoforms not involved in C_4 photosynthesis, rather than C_3 isoform. The latter term can be confusing, sometimes referring to non-photosynthetic forms in C_4 plants and other times referring to orthologous forms in C_3 plants.

In maize the different isozymic forms of PEPCase are encoded by a small gene family of about five members (Harpster and Taylor, 1986; Hudspeth et al., 1986) while sorghum has only three genes (Lepiniec et al., 1993) and amaranth has 10–20 genes (Rydzik et al., 1996). In each case, a subfamily of one or more members encodes the C_4 isozymic form. Our current understanding of *Ppc* gene families is consistent with the evolution of genes encoding the C_4 isoform through gene duplications.

Another case of likely gene duplication has been found with the small gene family encoding NADP-ME in *Flaveria* species. Marshall et al. (1996) identified two genes coding for chloroplastic NADP-ME, one of which, *Me1*, was shown to encode the C_4 isoform located in BS cells of *F. bidentis*, a C_4 species. The *Me2* gene was shown to be expressed at

low levels throughout the plant. *Me1* was expressed at low levels in C_3 species and its level of expression in C_3 - C_4 intermediate species was correlated with the degree of C_4 characteristics in each species.

In contrast, PPDK has been shown to be encoded by a single gene in *Flaveria* species (Rosche and Westhoff, 1995). However, two transcripts result from this single *Pdk* gene. One codes for the mature form of the enzyme plus a chloroplast transit sequence. This 3.4 kb mRNA is present at high levels in M cells and at lower levels in stems. A second, shorter RNA codes for only the mature form of the enzyme and is presumed to be cytoplasmically localized. This mRNA is present only at very low levels and is found in roots and stems. Maize has a dual function *Pdk* gene that has a similar expression program to the *Flaveria* one, but it also has a second *Pdk* gene which codes only for the mature form (Glackin and Grula, 1990; Sheen, 1991; Matsuoka, 1995) and is expressed at very low levels.

B. Genes for C_3 Photosynthesis Showing Differential Expression in C_4 Plants

The restriction of the photosynthetic carbon reduction cycle (Calvin cycle) to BS cells in C_4 plants is due to altered expression of the genes encoding component enzymes. Most studies have focused on RuBPCase, whose LSu is encoded by the plastid gene *rbcL* and SSu by a small family of nuclear *RbcS* genes. Although the overall expression of genes from two different genomes is coordinated, recent work has shown that there are some differences within the *RbcS* gene family. Ewing et al. (1998) used gene specific probes to measure the expression of two *RbcS* genes in leaf blades, where all cells are C_4 , and husk leaves, where M cells distant from veins are C_3 . Both genes were expressed at high levels in leaf blades but the *RbcS1* gene was preferentially expressed in husk leaves.

The glycine cleavage system of the photo-respiratory pathway is another example of enzymes active in C_3 plants that are restricted to BS cells in C_4 plants (Ohnishi and Kanai, 1983). The activity of GDCST is also BS-specific in C_3 - C_4 intermediate species (Bauwe et al., 1987; Hylton et al., 1988). The complex has four subunits and is located in the mitochondrion. Each subunit is encoded by a single nuclear gene in *Flaveria* and the gene for the H subunit is differentially spliced in an organ preferential manner (Kopriva et al., 1995).

C_4 plants belonging to the NADP-ME group exhibit

dimorphic chloroplasts with those in the BS having very few granal regions and little to no PS II activity (Woo et al., 1970). The relative absence of PS II activity is not due to the complete absence of component polypeptides, rather three nuclear encoded extrinsic polypeptides of the oxygen evolving complex are deficient (Oswald et al., 1990). Chloroplast encoded PS II subunits were found to differ in quantity depending on the species and the stage of development. The light harvesting complex of PS II is also absent or present at very low levels in BS chloroplasts, caused by the absence of transcripts of the nuclear *Cab* gene family which codes for the chlorophyll *a/b* apoproteins (Broglie et al., 1984; Schuster et al., 1985).

VI. Gene Regulation Mechanisms

The differential expression of photosynthetic genes is a key component of the differentiation process of M and BS cells. To understand the mechanisms regulating differential expression, the first step has been to define the programs of expression of the genes described in the previous section. Most studies have been conducted in maize, amaranth and *Flaveria* species using RNA northern blots and protein western blots to measure mRNA and protein accumulation and *in situ* hybridization and immunolocalization techniques to identify expression patterns of mRNAs and proteins in tissue sections. These techniques have identified the steps of gene expression at which regulatory control is active and have provided hints as to the signals responsible for regulation.

The second experimental step has been to dissect the mechanisms of regulatory control. Genetic transformation techniques, both transient and stable, are being used to identify regulatory DNA sequences and the protein factors which interact with these sequences. These protein factors become important molecular probes for the third experimental step which is to bridge the gap between morphological differentiation at the organ and cell level and regulation of gene expression. Analysis of mutants affecting C₄ development and the growing field of signal transduction pathways also will contribute to this objective.

A. Temporal Regulation

Because significant amounts of C₄ enzymes, including RuBPCase, are not found prior to the differentiation

of M and BS cells, temporal and spatial regulation of gene expression could be entirely controlled by the differentiation process. But detailed analyses have uncovered a more complex story, with differences found between genes and between species. Berry and colleagues used fluorescent *in situ* techniques to determine the patterns of accumulation of mRNAs and their protein products in amaranth. The mRNAs for PEPCase and PPDK were detected in apical meristems and leaf primordia, but the onset of accumulation of the two proteins was coincident with cellular differentiation in young leaves (Ramsperger et al., 1996). The mRNAs for both subunits of RuBPCase showed a similar pattern of accumulation in meristems and leaf primordia, but the LSu and SSu polypeptides were also detected in leaf primordia prior to cell differentiation. Both polypeptides became BS cell-specific in a basipetal pattern which correlated well with the transition of leaf metabolism from sink to source (Wang et al., 1993b). The α -subunit of NADP-ME, although located in BS cells, shows a pattern of accumulation similar to M cell-specific PEPCase and PPDK (Long and Berry, 1996). The α -subunit mRNA, however, is always found in BS cells and does not accumulate prior to cell differentiation.

In maize, the BS cell-specific mRNAs for SSu and NADP-ME were found to accumulate in leaf primordia prior to cell differentiation (Langdale et al., 1988a), while protein accumulation was concurrent with the development of Kranz anatomy. The accumulation of *Ppc* and *Pdk* mRNAs was coincident with cell differentiation (Langdale and Nelson, 1991; Nelson and Langdale, 1992).

Recent experiments with stably transformed *Flaveria bidentis* (C₄ species) provide additional evidence of C₄ gene activity prior to cell differentiation. The promoter of the *Me1* gene, which codes for the BS cell-specific C₄ isoform of NADP-ME, directs *gus* gene expression at high levels in apical and axillary meristem regions (Marshall et al., 1997). Although it is possible that this promoter activity does not represent the activity of the intact gene, it is none the less interesting that the promoter does have a meristem active element. In contrast, the promoter from the *Pdk* gene did not direct *gus* gene expression in meristem regions (Rosche et al., 1998).

B. Hierarchy of Developmental Controls

The discoordinated accumulation of mRNAs and their proteins described in the previous section

indicates that the differential expression of C_4 genes is regulated at post-transcriptional as well as transcriptional levels. Although *Ppc* and *Pdk* mRNAs were detected in amaranth leaf primordia, they became cell-specific with the differentiation of M cells and greatly increased in abundance (Ramsperger et al., 1996). Transcriptional regulation could account for the M cell-specific increase in mRNA abundance and its disappearance from BS cells could be due to selective mRNA degradation or normal mRNA turnover in the absence of BS cell transcription. Of course, an increase in mRNA could also involve a regulated increase in its stability, not just its rate of synthesis. Regulation at the level of translation or protein turnover must be responsible for the delayed accumulation of PEPCase and PPDK polypeptides. Their initial accumulation occurs in cells destined to become M cells as judged by their position relative to developing veins in amaranth leaves (Ramsperger et al., 1996). With further growth and development both polypeptides become M cell-specific.

Both *rbcL* and *RbcS* mRNAs accumulate in amaranth meristems and leaf primordia (Ramsperger et al., 1996). The levels of both mRNAs are relatively high in young leaves and are not BS cell-specific until a later stage. Both SSu and LSu polypeptides were first detected in the ground meristem of leaf primordia and then became progressively localized in pre-BS cells even though their mRNAs were still present at high levels in pre-M cells. Berry and colleagues have described the first stages of *rbcL* and *RbcS* gene expression as a default C_3 -like pattern. The transition to the BS cell-specific C_4 pattern is correlated with the transition from sink to source metabolism as the C_4 pathway becomes functional in fully differentiated M and BS cells (Wang et al., 1993b).

Maize leaves grown in the dark exhibit a default C_3 -like pattern of gene expression. RuBPCase is found in both cell types and C_4 mRNAs and proteins are not detectable. Transferring etiolated leaves to the light suppresses RuBPCase expression in M cells and induces the accumulation of C_4 enzymes in their appropriate cell type (Sheen and Bogorad, 1987; Langdale et al., 1988b).

A number of strategies have been used to identify the *cis*-acting DNA sequences responsible for regulating the expression of C_4 genes. These include comparisons of DNA sequences in the flanking non-coding regions of closely related C_4 and non- C_4 genes and the identification of binding sites for

nuclear proteins which are presumed to have regulatory function by virtue of their sequence-specific binding. The most powerful strategy is to identify key regulatory sequences by loss or gain of function when fused to a reporter gene and assayed in transformed cells or whole plants. A major difficulty with this approach has been the lack of a reliable and efficient transformation system for any C_4 plant. To overcome this limitation, reporter gene activity has been assayed in transiently transformed cells or organs of C_4 plants or in stably transformed C_3 plants. Both approaches have provided useful information and, in some cases, have fueled interesting speculation about the evolution of C_4 gene regulation. Recently, stable transformation systems have been developed for the C_4 species *Flaveria bidentis* (Chitty et al., 1994) and the C_3 - C_4 intermediate species *F. pubescens* (Chu et al., 1997). These systems have proven to be efficient and reasonably rapid so that a small research laboratory is able to generate an adequate number of transformed plants to make progress in the identification of *cis*-acting DNA sequences. Transformation of maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), sugarcane (Bower and Birch, 1992), and sorghum (Casas et al., 1993) have been reported but these systems have not so far provided a useful level of efficiency, speed, or accessibility. However, recent improvements in maize and sugarcane transformation may change the picture (Ishida et al., 1996).

Sheen and coworkers developed a transient expression system using protoplasts isolated from maize tissues which include dark- and light-grown leaves. Although it must be kept in mind that the process of making protoplasts may induce cellular dedifferentiation, Sheen's method has been shown to retain cell specialization and to reflect light-induced changes in gene expression (Sheen, 1990, 1991). Leaf protoplasts are primarily M cells. This approach was used to identify the promoter region of the complex, dual function maize *Pdk* gene (Sheen, 1991) and to identify sequences from the 5' regions of the C_4 *Ppc* gene (Schäffner and Sheen, 1992) and the *RbcS* genes (Schäffner and Sheen, 1991) which are involved in high level leaf expression. Positive and negative acting promoter elements responsible for regulation of transcriptional activity were identified. In addition, evidence for post-transcriptional regulation of maize *RbcS* genes was also presented (Sheen, 1990). *RbcS* promoter constructs were shown to be active in M cell protoplasts.

Matsuoka and Numazawa (1991) performed a similar analysis of the maize *Pdk* gene using biolistic delivery of constructs to maize leaves to measure transient reporter gene activity. They identified an element that conferred high level M cell expression and showed specific binding of a nuclear protein, presumably, a *trans*-acting regulatory factor.

While these transient expression assays have been useful in identifying DNA sequences controlling high-level, leaf-specific expression, the analysis of sequences controlling cell specificity is more readily accomplished in stably transformed plants. As was expected, transformation of C₄ gene constructs into C₃ plants has helped in the analysis of light regulation and high-level expression in leaves. But this approach also has provided some insights into the mechanisms of cell-specific gene regulation and their evolutionary origins.

Maize *Pdk*, *Ppc* and *RbcS* genes have been transformed into rice, which has the advantage of a well differentiated bundle sheath as is true of all grasses. The *Pdk* and *Ppc* promoters conferred high-level reporter gene expression preferentially in M cells (Matsuoka et al., 1993, 1994). The authors concluded that rice, although a C₃ plant, has *trans*-acting factors capable of recognizing M cell-specific sequences in both C₄ genes. Nuclear proteins with similar binding properties as found in maize were isolated from rice for both promoters (Matsuoka et al., 1993, 1994). The major change in the evolution of both C₄ genes must have been the acquisition of *cis*-acting sequences controlling M cell-specific expression. These results, and those from the maize leaf protoplast experiments (Sheen, 1991; Schäffner and Sheen, 1992), suggest that M cell-specificity is regulated primarily by differential transcription of C₄ *Ppc* genes. However, a maize *RbcS* promoter also directed high-level expression in M cells in rice (Matsuoka et al., 1994). The authors conclude that the *trans*-acting factors that suppress *RbcS* expression in C₄ plants must be absent from rice where *RbcS* genes are expressed at high levels in M cells.

Stockhaus et al. (1994) compared the activities of two *Ppc* promoters in tobacco. One promoter was from the *PpcA1* gene of *Flaveria trinervia*, a C₄ species, which encodes the C₄ isoform, and the other was from the orthologous *PpcA1* gene of *F. pringlei*, a C₃ species. The *F. trinervia* promoter directed high-level, leaf-specific expression in tobacco, primarily in the palisade mesophyll. The *F. pringlei* promoter directed low-level expression in leaves, stems and

roots, a pattern similar to that of the *PpcA1* gene in *F. pringlei*. These authors also conclude that new *cis*-acting sequences played a major role in the evolutionary origin of the *F. trinervia PpcA1* gene and that *trans*-acting factors necessary for high-level, M cell expression are present in C₃ plants. Both promoters were recently transformed into *F. bidentis*, a C₄ species closely related to *F. trinervia* (Stockhaus et al., 1997). The *F. trinervia PpcA1* promoter directed high-level M cell-specific reporter gene expression whereas the promoter from *F. pringlei* was active at a low level in leaves and stems but not roots. These results confirmed the importance of *cis*-acting sequences in the C₄ gene and provide clear evidence that transcriptional regulation may be the primary level of M cell-specific control of the C₄ *Ppc* gene in *Flaveria*.

Transcriptional regulation also appears to be responsible for M cell-specific expression of the *F. trinervia Pdk* gene (Rosche et al., 1998). Sequences flanking the coding region for the chloroplast form of the enzyme were found to direct high-level M cell expression in transgenic *F. bidentis* plants. Sequences from this same region were also shown to direct lower-level expression in BS cells and in stems and roots, suggesting that M-specific *cis*-acting sequences were added to a non-photosynthetic gene without disrupting the original expression program of that gene. Photosynthetic and non-photosynthetic functions are thereby provided by one gene without duplication.

A more complex story has emerged from studies of the *F. bidentis Me1* gene, which codes for the C₄ isoform of NADP-ME. Sequences from the 5' end of the gene were found to direct only very low levels of *gus* gene expression in leaves. By using cell separation techniques and a very sensitive fluorescence assay for GUS enzyme activity, Marshall et al. (1997) were able to show that this low level of GUS was primarily in BS cells. When sequences from the 3' end were added to the *gus* gene construct with *Me1-5'* sequences, high-level GUS activity was found preferentially in BS cells. In contrast with other C₄ gene promoters, none of the *Me1* constructs showed any significant expression in transformed tobacco. Marshall et al. (1997) concluded that sequences at the 5' end of the gene controlled BS specificity and sequences at the 3' end are responsible for high level expression, but the interaction of 5' and 3' sequences were dependent upon a *trans*-acting factor only present in C₄ plants. Experiments are in progress to

determine if the *MeI-3'* sequences act as a transcriptional enhancer or if they affect post-transcriptional processes. Because reporter gene expression was detected in M cells, the authors concluded that additional, post-transcriptional mechanisms were required to make NADP-ME accumulation strictly BS cell-specific. The 5' region of the *MeI* gene was also shown to direct high-level expression in the meristematic regions of the shoot apex and axillary buds, a pattern similar to that described for *Pdk* and *Ppc* mRNA accumulation in amaranth (Ramsperger et al., 1996). High-level GUS activity was detected in stems which was primarily localized in cells of the vascular cambium and xylem parenchyma. These cells showed the highest chloroplast numbers but no evidence of C₄-type cellular differentiation, suggesting that promoter activity can be induced by a photosynthetic developmental program without Kranz-type differentiation.

Sequences at the 3' end of the maize *RbcS* gene are also involved in cell specificity. Viret et al. (1994) introduced reporter gene constructs into maize leaves by microprojectile bombardment and showed that 3' sequences were responsible for suppressing *RbcS* promoter activity in M cells but had little effect on quantitative levels. It is not known whether the *RbcS* 3' sequences affect transcription or a post-transcriptional process.

The glycine cleavage system is restricted to BS cells in C₄ and C₃-C₄ intermediate plants. The promoter activity of the gene encoding the T protein, *GDCST*, has been measured in stably transformed *F. pubescens*, a C₃-C₄ intermediate species, and in tobacco (H. Bauwe, personal communication). Sequences from the 5' end of the gene confer BS cell-specific GUS expression in *F. pubescens*. This same construct directs GUS expression in a BS-like pattern in tobacco; GUS is detected in cells adjacent to veins but is relatively absent in mesophyll cells that are more than one or two cells distant from a vein. These authors conclude that *trans*-acting factors responsible for BS cell-specific expression are present in the bundle sheath of C₃ plants and that the evolutionary acquisition of BS-specific *cis*-acting sequences has been primarily responsible for the changed localization of GDC proteins in C₄ and C₃-C₄ intermediate species. These data and conclusions contrast with those of Marshall et al. (1997) on the *MeI* gene, whose expression appears to be dependent on a *trans*-acting factor only present in the C₄ plant.

The compartmentalized expression of the genes coding for C₄ enzymes is accomplished by complex mechanisms which activate high level expression of each gene in its appropriate cell type and, in some cases, repress the expression of that gene in the other cell type (Fig. 3E). Cell-specific repression may be particularly important for genes encoding components of C₃ photosynthesis whose expression must be redirected in C₄ plants.

C. Role of Environmental Cues

Light is the principle environmental signal that influences plant development. A great deal of current experimental effort is devoted to understanding light perception and the transduction of light signals which induces or represses gene expression and alters programs of development (Chory, 1993; von Arnim and Deng, 1996). Light affects the expression of C₄ genes in at least two ways. Light has been shown to act directly on the transcription of a number of nuclear genes, the best studied cases being *RbcS* and *Cab* in C₃ plants (Tobin and Silverthorne, 1985; Thompson and White, 1991). But light also has an indirect effect on gene expression by first activating further development of leaf cells and of the chloroplasts in them. In this case, the role of light may be to remove a developmental block so that leaf cell development can proceed far enough for the gene to become competent to respond to direct light activation. Just as C₄ genes have different programs of regulation, so are their responses to light different. When constructs with the maize *Pdk* or *Ppc* promoters were electroporated into etiolated maize leaf protoplasts, light was able to directly activate the *Pdk* (Sheen, 1991) but not the *Ppc* promoter (Schaffner and Sheen, 1992). The *Ppc* promoter was activated in protoplasts from greening leaves.

Light also has a major role in regulating cell-specific gene expression. Langdale and Nelson (1991) showed that *RbcS* and *rbcL* transcripts accumulated in both cell types in etiolated maize leaves, but at relatively low levels. Light increased the levels of both mRNAs in BS cells and caused them to decrease in M cells. Langdale and Nelson (1991) have concluded that light was responsible for the induction of a regulatory signal emanating from veins, which induces C₄ cell type differentiation. As described previously, a significant aspect of C₄ cell differentiation is the induction of cell-specific expression of C₄ genes.

Although this chapter is focused on the assembly of the C₄ pathway during leaf development, it must be remembered that light cues can affect gene expression at all stages. The products of photosynthesis affect the expression of genes coding for components of the photosynthetic apparatus through a sensing mechanism involving simple sugars (Koch, 1996; Jang and Sheen, 1997). Environmental variables such as light will have a major effect on the rates of photosynthesis and sugar production. Light cues also can induce leaf senescence which in turn will reduce gene expression.

D. Possible Mechanisms of Hierarchical Control

Although differences are seen in the patterns of expression among genes coding for components of the C₄ pathway and in the apparent mechanisms controlling their expression, there are some common principles governing the regulation of these genes. One is that expression of C₄ genes is controlled not only by the differentiation of BS and M cells but also by the developmental program of the leaf. In some cases this organ-specific regulation is evident as gene transcription commences in the leaf primordium or in leaf parenchyma cells prior to cell differentiation. The fact that promoters of some C₄ genes also show leaf-specific expression in transgenic C₃ plants indicates that these promoters respond to developmental signals controlling leaf development. Another indication of regulation at the organ level in C₄ plants is the observation that, prior to M and BS cell differentiation, genes, such as *RbcS*, show a 'default' C₃ pattern of expression in leaf parenchyma cells with transcripts accumulating in pre-M cells as well as pre-BS cells.

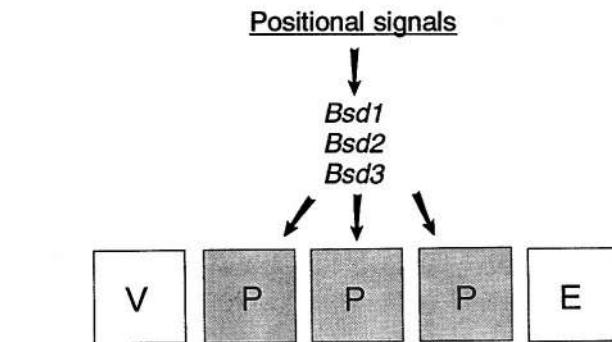
High-level expression of photosynthesis genes occurs only in those cells where chloroplasts develop and replicate to high numbers. In a C₃ plant, positional signals in the developing leaf are thought to be responsible for activating plastid differentiation and replication and for inducing the expression of photosynthesis genes in both genomes (Taylor, 1989). That early transcriptional activity of C₄ genes occurs primarily in regions giving rise to M and BS cells suggests that this same positional signaling also regulates the expression of C₄ genes.

The developmental status of the chloroplast has been shown to influence the expression of several nuclear photosynthesis genes through an hypothesized chloroplast signal (Oelmuller, 1989; Taylor,

1989). The functional role of this signal is not clear nor is its mechanism of action. It has been proposed to provide feedback-like information required for high-level expression of photosynthesis genes in the nucleus (Taylor, 1989). Whether this chloroplast signal also affects the expression of C₄ genes is not clear. Burgess and Taylor (1987) provided evidence that maize *Pdk* gene expression could be at least partially controlled by the chloroplast signal, but expression of the C₄ *Ppc* gene, which encodes a cytoplasmic enzyme, was not. However, the maize mutation, *bsd3*, which blocks chloroplast development at a very early stage in both cell types, affects only the accumulation of C₃ proteins, not C₄ pathway enzymes (J. A. Langdale, personal communication). A correlation between C₄ gene expression and the developmental status and/or photosynthetic competence of the chloroplast can also be seen in *Amaranthus tricolor*. Some leaves have non-photosynthetic red and yellow sectors where the synthesis of C₄ enzymes is significantly reduced (McCormac et al., 1997). Yellow sectors have normal Kranz anatomy and vein patterns, however, there is a loss of cell-specificity of *rbcL* and *RbcS* transcripts, along with decreased rates of transcription of several plastid genes. It is possible that in some of these examples the developmental status of the chloroplast is merely one indicator of the developmental status of the cell, which is the primary regulator of gene expression.

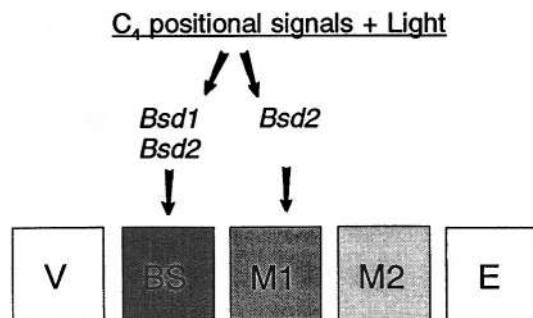
As illustrated in Fig. 4, we divide the hierarchy of gene regulation in C₄ leaves into two major levels. The first level involves the establishment of the potential for high-level gene expression in those cells in the leaf primordium that are determined to become photosynthetically active. This determination is accomplished by positional signals yet to be identified. It is possible that the same positional signals that activate chloroplast development and replication also establish the potential for high-level expression. In some cases the potential is evident in the accumulation of transcripts in the leaf primordium prior to differentiation. In other cases the potential is not realized until cell differentiation occurs and high level gene expression occurs in a cell-specific fashion. This level of regulation will result in the C₃ 'default' pattern of expression that is seen for some C₄ genes in the early stages of leaf development. It probably uses the same positional signals as in the development of a C₃ leaf, the only difference being the establishment of high expression potential in pre-BS

Level 1: Establishment of potential for high-level gene expression in cells determined to become photosynthetic parenchyma [C₃ default pattern -- leaf specific]



- activation of chloroplast replication and development
- partial activation of chloroplast genes
- set potential of nuclear genes

Level 2: Differentiation of BS and M cells [C₄ pattern -- leaf specific, cell specific]



- full activation of chloroplast genes
- full activation of nuclear genes
- repress genes activated at Level 1 in wrong cell type

Fig. 4. Two levels of hierarchical control operate in the development of the C₄ pattern of photosynthetic gene expression.

cells of C₄ leaves. It should be emphasized that 'C₄ default' refers only to the pattern of gene expression and does not mean that C₃ photosynthesis occurs prior to the differentiation of M and BS cells.

The appearance of *RbcS* and *rbcL* transcripts in the apical meristem of amaranth (Ramsperger et al., 1996) indicates that this first level of regulation will turn out to be more complex than the simple version presented here. Just as cell fate is determined by progressive interpretation of positional signals, the activation of the potential for high-level photo-

synthetic gene expression most likely will occur in a progressive fashion.

The second level of regulation in leaves of C₄ plants involves the differentiation of M and BS cells (Fig. 4). As discussed above, Langdale and Nelson (1991) have presented evidence in maize for a positional signal arising in veins that promotes the differentiation of the adjacent ring of parenchyma cells into BS and the cells adjacent to them into M. This differentiation process has two consequences for gene expression. One is the full activation of

high-level expression of photosynthesis genes and the other is the restriction of the expression of some genes to one cell type. Even with genes whose transcripts accumulate prior to the differentiation of Kranz anatomy, high-level expression is only evident in differentiated cells.

It is not clear if the same developmental signal is responsible for activating high-level expression and restricting expression to one cell type. Histochemical analyses of mRNA and protein accumulation suggest that the two processes occur simultaneously. However, regulation at multiple levels is involved in controlling the expression of some genes, indicating that mechanisms may be complex. Cell-specific gene expression is, in some cases, due to a combination of activation in one cell type and repression in the other. The evidence to date also indicates that each C₄ gene uses its own regulatory mechanism to achieve cell specificity. There is no obvious similarity between *cis*-acting DNA sequences within promoters nor is there any obvious similarity in the protein factors binding to these sequences. The recently discovered Dof1 factor in maize has been shown to transactivate expression of only the C₄ Ppc promoter and have no effect on the activities of Pdk, RbcS or Cab promoters (Yanagisawa and Sheen, 1998). Even if all M cell-specific genes respond to the same M cell signal, this signal seems to activate different mechanisms which in turn activate or repress the expression of C₄ genes.

Leaf-specific expression of C₄ transgenes in C₃ plants could be said to provide additional evidence for the first level of regulation by the developmental program of the leaf. Promoters from C₄ genes are recognized by transcription factors in the C₃ leaf so that expression is activated in photosynthetic cells. However, the fact that C₄ promoter activity shows some cell preference in C₃ leaves has also led to the conclusion that there are cell positional signals in a C₃ leaf which can interact with the cell-specific *cis*-acting sequences of the C₄ transgene. Maize Ppc and Pdk promoters are expressed in M but not BS cells of rice (Matsuoka et al., 1993, 1994) as is the RbcS promoter. It must be kept in mind that the rice BS cells do not show full development of photosynthetic characteristics, such as large numbers of chloroplasts. Therefore, the lack of expression of C₄ promoters in rice bundle sheath cells could be due to the lack of full activation of photosynthetic gene expression.

What are the signals responsible for developmental regulation of C₄ genes? Several experimental approaches are providing insights into these

regulatory signals. One uses reporter gene fusions to define the functional *cis*-acting sequences of promoters. Proteins binding to these sequences are then good candidates for some components of the regulatory hierarchy. Once gene clones are isolated for these DNA binding proteins, their function can be tested in transgenic plants. Although this approach is extremely laborious, some progress is being made. Yanagisawa and Sheen (1998) exploited the maize protoplast transient expression system to show that a previously identified DNA binding protein, Dof1, activates transcription of the C₄ Ppc promoter but not other C₄ promoters. They were also able to show that although Dof1 was present in roots and stems, a related protein, Dof2, was responsible for repressing transcription in roots and stems. Furthermore, Dof1 activity was shown to be light dependent; binding to the Ppc promoter was significantly different in greening compared to etiolated protoplasts. The protoplast system may prove to be an efficient way of identifying nuclear proteins with regulatory activities.

An understanding of patterns of accumulation of these transcriptional regulators should tell us much more about mechanisms of developmental control. Because each C₄ gene appears to be controlled by a different set of DNA binding proteins it has been difficult to find common regulatory signals by examining the promoters of genes expressed in one or the other cell type. But studies of the expression programs of the genes coding for the regulatory proteins may reveal common control mechanisms. Similar approaches can be used to identify the regulators of post-transcriptional processes which are particularly important components of regulation at the second stage in the developmental hierarchy.

Another approach in the study of developmental regulation is to identify mutations that disrupt C₄ differentiation. A difficulty with this approach is the lack of an obvious phenotype. Langdale and colleagues screened pale-green maize mutants for those with alterations in amounts of C₄ enzymes or with cell-specific chloroplast defects. Although most mutants showed defects in both cell types a few have been identified that are specific to BS cells. These *bundle sheath defective* mutants appear to be defective in the differentiation of BS cells after the delineation of M and BS cells has occurred. Characterization of mutant phenotypes and, in one case, the isolation of the gene involved, has provided some insights into the control of Kranz differentiation and the relationships between the C₃ default level of cell

development and C_4 differentiation.

The *bsd1* mutation causes aberrant development of BS chloroplasts and a major reduction in BS-specific C_4 enzymes (Langdale and Kidner, 1994). However, in dark-grown maize leaves *bsd1* affects the accumulation of photosynthetic enzymes in both cell types. The authors propose that the *Bsd1* gene acts in early stages of leaf cell development at the C_3 default level but its activity becomes restricted to BS cells once C_4 differentiation occurs. The *Bsd1* gene has recently been isolated and shown to encode a nuclear-localized protein with similarities to transcription factors (Hall et al., 1998). *Bsd1* gene expression is primarily restricted to BS cells. Another maize mutation, *bsd2*, also disrupts BS chloroplast development and blocks the accumulation of RuBPCase (Roth et al., 1996). However, both *RbcS* and *rbcL* transcripts are present in mutant leaves and *rbcL* transcripts accumulate ectopically in M chloroplasts as well as in the BS. Roth et al. (1996) speculate that the *Bsd2* gene is active in both cell types where its primary function is to control the accumulation of *rbcL* mRNA and its translational activity. The defect in BS chloroplasts appears to be pleiotropic, due to the loss of photosynthetic activity.

A third mutation, *bsd3*, affects chloroplast biogenesis in both cell types, but C_4 enzymes accumulate normally (J. A. Langdale, personal communication). However, the C_3 proteins, Cab and RuBPCase, are present in reduced amounts due to reduced levels of *rbcL* and *Cab* mRNAs. Because the mutant blocks chloroplast biogenesis at a very early stage, Langdale and colleagues (J. A. Langdale, personal communication) speculate that the early block in chloroplast development interrupts the signaling between chloroplast and nucleus necessary for normal *Cab* gene expression, perhaps disrupting the hypothetical chloroplast signal (Oelmuller, 1989; Taylor, 1989).

The *bundle sheath defective* mutations have provided unique insights into genes that control or facilitate the full development of photosynthetic capacity in BS cells and their interactions with the C_4 differentiation program. These mutations show that many genes function at all stages of leaf cell development, but the C_4 differentiation program may restrict their activity to one cell type. Gene function may also change, as is the case with *Bsd2* which prevents *rbcL* accumulation in M cells and promotes its accumulation and translation in BS cells.

To date mutations have not been identified in

genes controlling C_4 differentiation. Perhaps these mutations would have more deleterious phenotypes and their identification will require screening for different phenotypes than previously employed. It is also noteworthy that no mesophyll defective mutations have been identified by Langdale and colleagues despite analyzing more than 150 pale-green and yellow maize mutants (J. A. Langdale, personal communication). A mesophyll defective mutation was created using antisense technology in transgenic *Flaveria bidentis*: plants showing major reductions in the level of PPDK were severely retarded in growth and extremely sensitive to photoinhibition even when grown heterotrophically (Furbank et al., 1997), indicating that screening under the appropriate conditions may be necessary.

VII. Future Directions and Model Experimental Systems

One of the most consistent and striking features of C_4 leaf development is the alteration of vein pattern, yielding the short interveinal distances characteristic of mature C_4 leaves. In one sense, the developmental phenomenon is comparable to a heterochronic shift where one process is prolonged in relation to another; in this case the successive formation of minor veins is extended in relation to leaf expansion, so that mature vein spacing is altered. It is possible that genes involved in other heterochronic shifts such as those controlling the transition from juvenile to adult leaves in maize and *Arabidopsis* (Moose and Sisco, 1996; Telfer et al., 1997) might participate in the developmental regulation of this important early step in C_4 leaf development. Alternatively, and perhaps more likely, genes regulating the spacing of elements in a repeated pattern, such as stomata or trichomes on the leaf surface (Yang and Sack, 1995; Larkin et al., 1996), might also function in vein spacing. Regardless of the actual mechanism involved in C_4 vein spacing and its parallels in other developmental processes, identification of genes regulating vein spacing through mutagenesis screens will be an essential first step.

Gene expression resulting in both C_4 tissue pattern and biochemical compartmentation appears to be regulated by the interpretation of positional information. The organization of tissues around veins involves oriented planes of cell division and cell enlargement in bundle sheath and mesophyll

precursors, while maturation of BS and M cells requires differential patterns of chloroplast (and mitochondrion) replication and development, of organelle placement within the cell, of cell wall modification, and of photosynthetic gene expression. The spatial relationships between veins, BS and M cells, as well as the temporal sequence of developmental events, indicates that a vein-derived signal may be crucial for these developmental events. Currently, very little is understood about how such signals might be sent and perceived, although clonal analysis of genetic mosaics of mutant and wildtype tissue sectors clearly indicates that gene expression in one tissue layer may influence developmental pattern in a quite different layer (Hake and Freeling, 1986; Fowler and Freeling, 1996). Phenotypic analysis of mutants at the *rough sheath1*, *hairy sheath frayed1*, *Knotted1*, and four *Liguleless* loci indicate that these genes function in the interpretation of positional information by leaf cells (Fowler and Freeling, 1996). A clearer understanding of genes such as these that have major morphological effects when mutated might also shed light on the interpretation of positional information at a more subtle anatomical level.

Other approaches will also be important for dissecting C₄ developmental pathways. Genes such as *ERECTA* in *Arabidopsis* and *CRINKLY4* in maize indicate that receptor-like kinases are involved in signal transduction pathways required for the proper formation of cell pattern (Torii et al., 1996; Becroft et al., 1996). Similar genes may be involved in communication between vein, bundle sheath and mesophyll tissues in C₄ leaves. Parallel pathways might involve the symplastic movement of informational macromolecules through modified plasmodesmata as demonstrated for the *Kn1* protein in tobacco mesophyll cells (Lucas et al., 1995). Solving the mystery of cell-cell signalling during C₄ leaf development will require a combination of mutant gene identification, understanding the modes of gene action and regulation, and investigating the consequences of mutant gene defects at the level of cell biology. As an example of the approach that needs to be taken, mutants in the *KNOLLE* gene of *Arabidopsis* disrupt cell pattern within tissues, but not overall tissue pattern. *KNOLLE* encodes for a syntaxin-like protein and affects plane of cell plate formation (Lukowitz et al., 1996). Genes with functions similar to *KNOLLE* must act in response to signals that specify tissue position during C₄ leaf

development.

Maize will likely play an important role in future research as a C₄ model system, especially if maize transformation efficiency improves to the point that it becomes accessible to academic research laboratories. *Flaveria bidentis* already fulfils the transformation role, but does not have much possibility for genetic analysis. Transformation approaches will be crucial to demonstrate the function of proteins potentially involved in generating and interpreting positional signals in C₄ leaf development, especially for candidate proteins identified in other plants, such as *Arabidopsis*. But the research community must not lose sight of the extensive developmental diversity among C₄ plants and become overly focused on one or two model systems. We must know enough about morphogenesis and developmental gene regulation in other C₄ plants to be confident of the generalizations from our model systems.

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References

- Bauwe H and Chollet R (1986) Kinetic properties of phosphoenolpyruvate carboxylase from C₃, C₄, and C₃-C₄ intermediate species of *Flaveria* (Asteraceae). *Plant Physiol* 82: 695–699
- Bauwe H, Keerberg O, Bassner R, Pärnik T and Bassner B (1987) Reassimilation of carbon dioxide by *Flaveria* (Asteraceae) species representing different types of photosynthesis. *Planta* 172: 214–218
- Becraft PW, Stinard PS and McCarty DR (1996) *CRINKLY4*: a TNFR-like receptor kinase involved in maize epidermal differentiation. *Science* 273: 1406–1409
- Boffey SA (1992) Chloroplast replication. In Baker N and Thomas H (eds) *Crop Photosynthesis: Spatial and Temporal Determinants*, pp 361–379. Elsevier, Amsterdam
- Botha CEJ and Evert RF (1988) Plasmodesmatal distribution and frequency in vascular bundles and contiguous tissues of the leaf of *Themeda triandra*. *Planta* 173: 433–441
- Bower and Birch R (1992) Transgenic sugarcane plants via microprojectile bombardment. *Plant J* 2: 409–416
- Brangeon J (1973) Compared ontogeny of the two types of chloroplasts of *Zea mays*. *J Microsc* 16: 233–242

- Broglie R, Coruzzi G, Keith B and Chua N-H (1984) Molecular biology of C₄ photosynthesis in *Zea mays*: Differential localization of proteins and mRNAs in the two leafcell types. *Plant Molec Biol* 3: 431–444
- Brown WV (1975) Variations in anatomy, associations, and origins of Kranz tissue. *Amer J Bot* 62: 395–402
- Bruhl JJ (1995) Sedge genera of the world: Relationships and a new classification of the Cyperaceae. *Aust J Syst Bot* 8: 125–305
- Burgess DG and Taylor WC (1987) Chloroplast photooxidation affects the accumulation of cytosolic mRNAs encoding chloroplast proteins in maize. *Planta* 170: 520–527
- Carland FM and McHale MA (1996) *LOP1*: A gene involved in auxin transport and vascular patterning in *Arabidopsis*. *Development* 122: 1811–1819
- Casas AM, Kononowicz AK, Zehr UB, Tomes DT, Axtell JD, Butler LG, Bressan RA and Hasegawa PM (1993) Transgenic sorghum plants via microprojectile bombardment. *Proc Natl Acad Sci USA* 90: 11212–11216
- Chapman EA, Bain JM and Gove DW (1975) Mitochondria and chloroplast peripheral reticulum in the C₄ plants. *Aust J Plant Physiol* 2: 207–223
- Chatterjee M, Sparvoli S, Edmunds C, Garosi P, Findlay K and Martin C (1996) *DAG*, a gene required for chloroplast differentiation and palisade development in *Antirrhinum majus*. *EMBO J* 15: 4194–4207
- Chitty JA, Furbank RT, Marshall JS, Chen Z and Taylor WC (1994) Genetic transformation of the C₄ plant *Flaveria bidentis*. *Plant J* 6: 949–956
- Chory J (1993) Out of darkness: Mutants reveal pathways controlling light-regulated development in plants. *Trends Genet* 9: 167–172
- Chu C-C, Qu N, Bassuner B and Bauwe H (1997) Genetic transformation of the C₃-C₄ intermediate species, *Flaveria pubescens* (Asteraceae). *Plant Cell Rep* 16: 715–718
- Crookston RK and Moss DN (1974) Interveinal distance for carbohydrate transport in leaves of C₃ and C₄ grasses. *Crop Sci* 14: 123–125
- Cyr RJ (1994) Microtubules in plant morphogenesis: Role of the cortical array. *Annu Rev Cell Biol* 10: 153–180
- Dengler NG and Nelson T (1999) Leaf structure and development in C₄ plants. In: Sage RF and Monsom RK (eds) *C₄ Plant Biology*, pp 133–172. Academic Press, New York
- Dengler NG, Dengler RE and Hattersley PW (1985) Differing ontogenetic origins of PCR ('Kranz') sheaths in leafblades of C₄ grasses (Poaceae). *Amer J Bot* 72: 284–302
- Dengler NG, Dengler RE and Hattersley PW (1986) Comparative bundle sheath and mesophyll differentiation in the leaves of the C₄ grasses *Panicum effusum* and *P. bulbosum*. *Amer J Bot* 73: 1431–1442
- Dengler NG, Dengler RE, Donnelly PM and Hattersley PW (1994) Quantitative leaf anatomy of C₃ and C₄ grasses (Poaceae): Bundle sheath and mesophyll surface area relationships. *Ann Bot* 73: 241–255
- Dengler NG, Dengler RE, Donnelly PM and Filosa M (1995) Expression of the C₄ pattern of photosynthetic enzyme accumulation during leaf development in *Atriplex rosea* (Chenopodiaceae). *Amer J Bot* 82: 318–327
- Dengler NG, Donnelly PM and Dengler RE (1996) Differentiation of bundle sheath, mesophyll and distinctive cells in the C₄ grass *Arundinella hirta* (Poaceae). *Amer J Bot* 83: 1391–1405
- Dengler NG, Woodvine MA, Donnelly PM and Dengler RE (1997) Formation of vascular pattern in developing leaves of the C₄ grass *Arundinella hirta*. *Int J Plant Sci.* 158: 1–12
- Downton WJS (1975) The occurrence of C₄ photosynthesis among plants. *Photosynthetica* 9: 96–105
- Eastman PAK, Dengler MG and Peterson CA (1988) Suberized bundle sheaths in grasses (Poaceae) of different photosynthetic types. I. Anatomy, ultrastructure and histochemistry. *Protoplasma* 142: 92–111
- Edwards G and Walker D (1983) *C₃, C₄*: Mechanism, and Cellular and Environmental Regulation of Photosynthesis. Blackwell Scientific Publications, Oxford.
- Ellis JR and Leech RM (1985) Cell size and chloroplast size in relation to chloroplast replication in light-grown wheat leaves. *Planta* 165: 120–125
- Ernst K and Westhoff P (1997) The phosphoenolpyruvate carboxylase (ppc) gene family of *Flaveria trinervia* (C₄) and *F. pringlei* (C₃): Molecular characterisation and expression analysis of the *ppcB* and *ppcC* genes. *Plant Molec Biol* 34: 427–443
- Esau K (1965) *Plant Anatomy*. John Wiley and Sons, New York
- Evert RF, Russin WA and Bosabalidis AM (1996) Anatomical and ultrastructural changes associated with sink-to-source transition in developing maize leaves. *Int J Plant Sci* 157: 247–261
- Ewing RM, Jenkins GI and Langdale JA (1998) Transcripts of maize *RbcS* genes accumulate differentially in C₃- and C₄-tissues. *Plant Molec Biol* 36: 593–599
- Fladung M (1994) Genetic variants of *Panicum maximum* (Jacq.) in C₄photosynthetic traits. *J Plant Physiol* 143: 165–172
- Fowler JE and Freeling M (1996) Genetic analysis of mutations that alter cell fates in maize leaves: Dominant *liguleless* mutations. *Devel Genet* 18: 198–222
- Fromm ME, Morrise F, Armstrong C, Williams R, Thomas J and Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Bio/Technology* 8: 833–839
- Furbank RT and Taylor WC (1995) Regulation of photosynthesis in C₃ and C₄plants: A molecular approach. *Plant Cell* 7: 797–807
- Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanian SJ, von Caemmerer S and Ashton AR (1997) Genetic manipulation of key photosynthetic enzymes in the C₄ plant *Flaveria bidentis*. *Aust J Plant Physiol* 24: 477–485
- Glackin CA and Grula JW (1990) Organ-specific transcripts of different size and abundance derive from the same pyruvate, orthophosphate dikinase gene in maize. *Proc Natl Acad Sci USA* 87: 3004–3008
- Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willets NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP and Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* 2: 603–618
- Gutierrez M, Gracen VE and Edwards GE (1974) Biochemical and cytological relationships in C₄ plants. *Planta* 119: 279–300
- Hake S and Freeling M (1986) Analysis of genetic mosaics shows that the extra epidermal cell division in Knotted mutant maize plants are induced by adjacent mesophyll cells. *Nature* 320: 621–623
- Hall LN, Rossini L, Cribb L and Langdale JA (1998) *GOLDEN 2*,

- a novel transcriptional regulator of cellular differentiation in the maize leaf. *Plant Cell* 10: 925–936
- Harpster M and Taylor WC (1986) Maize phosphoenolpyruvate carboxylase: Cloning and characterization of mRNAs encoding isozymic forms. *J Biol Chem* 261: 6132–6136
- Hatch MD (1987) C₄ photosynthesis: A unique blend of modified biochemistry, anatomy and ultrastructure. *Biochim Biophys Acta* 895: 81–106
- Hatch MD and Burnell JN (1990) Carbonic anhydrase activity in leaves and its role in the first step in C₄ photosynthesis. *Plant Physiol* 93: 380–383
- Hatch MD, Kagawa T and Craig S (1975) Subdivision of C₄ pathway species based on differing C₄ acid decarboxylating systems and ultrastructural features. *Aust J Plant Physiol* 2: 111–128
- Hattersley PW (1984) Characterization of C₄ leaf anatomy in grasses (Poaceae). Mesophyll:bundle sheath ratios. *Ann Bot* 53: 163–179
- Hattersley, PW (1987) Variation in photosynthetic pathway. In: Soderstrom TR, Campbell CS and Barkworth ME (eds) *Grass Systematics and Evolution*, pp 49–64. Smithsonian Institution Press, Washington DC
- Hattersley PW and Browning AJ (1981) Occurrence of the suberized lamella in leaves of grasses of different photosynthetic types. I. In parenchymatous bundle sheaths and PCR ('Kranz') sheaths. *Protoplasma* 109: 371–401
- Hattersley PW and Watson L (1975) Anatomical parameters for predicting photosynthetic pathways of grass leaves: The 'maximum lateral cell count' and the 'maximum cells distant count.' *Phytomorphology* 25: 325–333
- Hattersley PW and Watson L (1992) Diversification of photosynthesis. In: Chapman GP (ed) *Grass Evolution and Domestication*, pp 38–116. Cambridge University Press, Cambridge
- Haupt W (1982) Light-mediated movement of chloroplasts. *Annu Rev Plant Physiol* 33: 205–233
- Hermans J and Westhoff P (1990) Analysis of expression and evolutionary relationships of phosphoenolpyruvate carboxylase genes in *Flaveria trinervia* (C₄) and *F. pringlei* (C₃). *Molec Gen Genet* 224: 459–468
- Hermans J and Westhoff P (1992) Homologous genes for the C₄ isoform of phosphoenolpyruvate carboxylase in a C₃ and a C₄ *Flaveria* species. *Molec Gen Genet* 234: 275–284
- Hudspeth RL, Glackin CA, Bonner J and Grula JW (1986) Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate, orthophosphate dikinase: Expression of different gene family members in leaves and roots. *Proc Natl Acad Sci USA* 83: 2884–2888
- Hylton CM, Rawsthorne S, Smith AM and Jones DA (1988) Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C₃-C₄ intermediate species. *Planta* 175: 452–459
- Ishida Y, Saito H, Ohta S, Hiei Y, Komari T and Kumashiro T (1996) High efficiency transformation of maize mediated by *Agrobacterium tumefaciens*. *Natur Biotech* 14: 745–750
- Jackson E, Veit B and Hake S (1994) Expression of maize KNOTTED-related homeobox genes in the shoot apical meristem predicts pattern of morphogenesis in the vegetative shoot. *Development* 120: 405–413
- Jang J-C and Sheen J (1997) Sugar sensing in higher plants. *Trends Plant Sci* 2: 208–214
- Kawamitsu Y, Hakoyama S, Agata W and Takeda T (1985) Leaf interveinal distances corresponding to anatomical types in grasses. *Plant Cell Physiol* 26: 589–593
- Keddie J, Carroll B, Jones JDG and Gruissem W (1996) The *DCL* gene of tomato is required for chloroplast development and palisade cell morphogenesis in leaves. *EMBO J* 15: 4208–4217
- Kellogg EA (1996) Integrating genetics, phylogenetics, and developmental biology. In: Sobral BWS (ed) *The Impact of Plant Molecular Genetics*, pp 159–172. Birhauser, Boston
- Kirchanski SJ (1975) The ultrastructural development of the dimorphic plastids of *Zea mays*. *Amer J Bot* 62: 695–705
- Koch KE (1996) Carbohydrate-modulated gene expression in plants. *Annu Rev Plant Physiol Plant Molec Biol* 47: 509–540
- Kopriva S, Cossu R and Bauwe H (1995) Alternative splicing results in two different transcripts for the H-protein of the glycine cleavage system in the C₄ species *Flaveria trinervia*. *Plant J* 8: 435–441
- Kragler F, Lucas WJ and Monzer J (1998) Plasmodesmata: Dynamics, domains and patterning. *Ann Bot* 81: 1–10
- Ku MSB, Kano-Murakami Y and Matsuoka M (1996) Evolution and expression of C₄ photosynthesis genes. *Plant Physiol* 111: 949–957
- Kubicki A, Steinmuller K and Westhoff P (1994) Differential transcription of plastome-encoded genes in the mesophyll and bundle-sheath chloroplasts of the monocotyledonous NADP-malic enzyme-type C₄ plants maize and *Sorghum*. *Plant Mol Biol* 25: 669–679
- Laetsch WM (1974) The C₄ syndrome: A structural analysis. *Ann Rev Plant Physiol* 25: 27–52
- Laetsch WM and Price I (1969) Development of the dimorphic chloroplasts of sugar cane. *Amer J Bot* 56: 77–87
- Lal A and Edwards GE (1996) Analysis of inhibition of photosynthesis under water stress in the C₄ species *Amaranthus cruentus* and *Zea mays*: Electron transport, CO₂ fixation and carboxylation capacity. *Aust J Plant Physiol* 23: 403–412
- Langdale JA and Kidner CA (1994) *bundle sheath defective*, a mutation that disrupts cellular differentiation in maize leaves. *Development* 120: 673–681
- Langdale JA and Nelson T (1991) Spatial regulation of photosynthetic development in C₄ plants. *Trends Genet* 7: 191–196
- Langdale JA, Rothermel BA and Nelson T. (1988a) Cellular patterns of photosynthetic gene expression in developing maize leaves. *Genes Dev* 2: 106–115
- Langdale JA, Zelitch I, Miller E and Nelson T (1988b) Cell position and light influence C₄ versus C₃ patterns of photosynthetic gene expression in maize. *EMBO J* 7: 3643–351
- Larkin JC, Young N, Prigge M and Marks MD (1996) The control of trichome spacing and number in *Arabidopsis*. *Development* 122: 997–1005
- Lepiniec L, Keryer E, Philippe H, Gadal P and Cretin C (1993) Sorghum phosphoenolpyruvate carboxylase gene family: Structure, function and molecular evolution. *Plant Molec Biol* 21: 487–502
- Liu YQ and Dengler NG (1994) Bundle sheath and mesophyll cell differentiation in the C₄ dicotyledon *Atriplex rosea*: Quantitative ultrastructure. *Can J Bot* 72: 644–657
- Lloyd CW, Vennerloo CJ, Goodbody KC and Shaw PJ (1992) Confocal laser microscopy and three-dimensional reconstruction of nucleus-associated microtubules in the division

- plane of vacuolated plant cells. *J Microsc* 166: 99–109
- Long JJ and Berry JO (1996) Tissue-specific and light-mediated expression of the C₄ photosynthetic NAD-dependent malic enzyme of amaranth mitochondria. *Plant Physiol* 112: 473–482
- Lucas WJ (1995) Plasmodesmata: Intercellular channels for macromolecular transport in plants. *Curr Opin Cell Biol* 7: 673–680
- Lucas WJ, Bouche-Pillon S, Jackson DO, Nguyen L, Baker L, Ding B and Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* 270: 1980–1983
- Lukowitz W, Mayer U and Jurgens G (1996) Cytokinesis in the *Arabidopsis* embryo involves the syntaxin-related *KNOLLE* gene product. *Cell* 84: 61–71
- Marshall JS, Stubbs JD and Taylor WC (1996) Two genes encode highly similar chloroplastic NADP-malic enzymes in *Flaveria*: Implications for the evolution of C₄ photosynthesis. *Plant Physiol* 111: 1251–1261
- Marshall JS, Stubbs JD, Chitty JA, Surin B and Taylor WC (1997) Expression of the C₄ *Mel* gene from *Flaveria bidentis* requires an interaction between 5' and 3' sequences. *Plant Cell* 9: 1515–1525
- Matsuoka M (1995) The gene for pyruvate, orthophosphate dikinase in C₄ plants: Structure, regulation and evolution. *Plant Cell Physiol* 36: 1–7
- Matsuoka M and Numazawa T (1991) Cis-acting elements in the pyruvate, orthophosphate dikinase gene in maize. *Molec Gen Genet* 228: 143–152
- Matsuoka M, Tada Y, Fujimura T and Kano-Murakami Y (1993) Tissue-specific light-regulated expression directed by the promoter of a C₄ gene, maize pyruvate, orthophosphate dikinase, in a C₃ plant, rice. *Proc Natl Acad Sci USA* 90: 9586–9590
- Matsuoka M, Kyozuka J, Shimamoto K, Kano-Murakami Y (1994) The promoters of two carboxylases in a C₄ plant (maize) direct cell-specific, light-regulated expression in a C₃ plant (rice). *Plant J* 6: 311–319
- McCormac D, Boinski JJ, Ramsperger VC and Berry JO (1997) C₄ gene expression in photosynthetic and non-photosynthetic leaf regions of *Amaranthus tricolor*. *Plant Physiol* 114: 801–815
- Meierhoff K and Westhoff P (1993) Differential biogenesis of Photosystem II in mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme-type C₄ plants: The non-stoichiometric abundance of the subunits of Photosystem II in the bundle sheath chloroplasts and the translational activity of the plastome-encoded genes. *Planta* 191: 23–33
- Miyake H and Nakamura M (1993) Some factors concerning the centripetal disposition of bundle sheath chloroplasts during the leaf development of *Eleusine coracana*. *Ann Bot* 72: 205–211
- Miyake H and Yamamoto Y (1987) Centripetal disposition of bundle sheath chloroplasts during the leaf development of *Eleusine coracana*. *Ann Bot* 60: 641–647
- Monson RK (1996) The use of phylogenetic perspective in comparative plant physiology and developmental biology. *Ann Missouri Bot Gard* 83: 3–16
- Moore PD (1982) Evolution of photosynthetic pathways in flowering plants. *Nature* 295: 647–648
- Moose SP and Sisco PH (1996) *Glossy15*, an APETALA2-like gene from maize that regulates leaf epidermal cell identity. *Genes Devel* 10: 3018–3027
- Nelson T and Dengler NG (1992) Photosynthetic tissue differentiation in C₄ plants. *Intl J Plant Sci* 153: 93–105
- Nelson T and Langdale JA (1992) Developmental genetics of C₄ plants. *Ann Rev Plant Physiol Mol Biol* 43: 25–47
- Nishioka D, Miyake H and Tamiguchi T (1996) Suppression of granal development and accumulation of rubisco in different bundle sheath chloroplasts of the C₄ succulent plant *Portulaca grandiflora*. *Ann Bot* 77: 629–637
- O'Brien TP and Carr DJ (1970) A suberized layer in the cell walls of the bundle sheath of grasses. *Aust J Biol Sci* 23: 275–287
- Oelmüller R (1989) Photooxidative destruction of chloroplasts and its effects on nuclear gene expression and extraplastidic enzyme levels. *Photochem Photobiol* 49: 229–239
- Ohnishi J and Kanai R (1983) Differentiation of photorespiratory activity between mesophyll and bundle sheath cells of C₄ plants. I. Glycine oxidation by mitochondria. *Plant Cell Physiol* 24: 1411–1420
- Oswald A, Streubel M, Ljungberg U, Hermans J, Eskins K and Westhoff P (1990) Differential biogenesis of photosystem-II in mesophyll and bundle-sheath cells of 'malic' enzyme NADP= type C₄ plants. *Eur J Biochem* 190: 185–194
- Przemack GH, Mattsson J, Hardtke CS, Sung ZR and Berleth T (1996) Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta* 200: 229–237
- Pyke KA and Leech RM (1992) Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana*. *Plant Physiol* 99: 1005–1008
- Pyke KA and Leech RM (1994) A genetic analysis of chloroplast division and expansion in *Arabidopsis thaliana*. *Plant Physiol* 104: 201–207
- Pyke KA, Rutherford SM, Robertson EJ and Leech RM (1994) *arc6*, a fertile *Arabidopsis* mutant with only two mesophyll cell chloroplasts. *Plant Physiol* 106: 1169–1177
- Raghavendra AS and Das VSR (1978) The occurrence of C₄-photosynthesis: A supplementary list of C₄ plants reported during late 1974–mid 1977. *Photosynthetica* 12: 200–208
- Ramsperger VC, Summers RG and Berry JO (1996) Photosynthetic gene expression in meristems and during initial leaf development in a C₄ dicotyledonous plant. *Plant Physiol* 111: 999–1010
- Reiter RS, Coomber SA, Bourett TM, Bartley GE and Scolnik PA (1994) Control of leaf and chloroplast development by the *Arabidopsis* gene *pale cress*. *Plant Cell* 6: 1253–1264
- Robinson-Beers K and Evert RF (1991a) Ultrastructure of and plasmodesmatal frequency in mature leaves of sugarcane. *Planta* 184: 291–306
- Robinson-Beers and Evert RF (1991b) Fine structure of plasmodesmata in mature leaves of sugarcane. *Planta* 184: 307–318
- Rosche E and Westhoff P (1995) Genomic structure and expression of the pyruvate, orthophosphate dikinase gene of the dicotyledonous C₄ plant *Flaveria trinervia* (Asteraceae). *Plant Molec Biol* 29: 663–678
- Rosche E, Chitty J, Westhoff P and Taylor WC (1998) Analysis of promoter activity for the gene encoding pyruvate, orthophosphate dikinase in stably transformed C₄ *Flaveria*. *Plant Physiol* 117: 821–829
- Roth R, Hall LN, Brutnell TP and Langdale JA (1996) *bundle*

- sheath defective2*, a mutation that disrupts the coordinated development of bundle sheath and mesophyll cells in the maize leaf. *Plant Cell* 8: 915–927
- Rydzik E, Boinski JJ, Long JJ and Berry JO (1996) Copy number of C₄ photosynthetic genes in *Amaranthus hypochondriacus*. *Legacy, Newsletter of the American Amaranth Institute* 9: 12–16
- Sachs T (1981) The control of patterned differentiation of vascular tissues. *Adv Bot Res* 9: 151–262
- Sachs T (1989) The development of vascular networks during leaf development. *Curr Top Plant Biochem Physiol* 8: 168–183
- Sachs T (1991) Cell polarity and tissue patterning in plants. *Development (Supplement)* 1: 83–93
- Schäffner AR and Sheen J (1991) Maize *rbcS* promoter activity depends on sequence elements not found in dicot *rbcS* promoters. *Plant Cell* 3: 997–1012
- Schäffner A and Sheen J (1992) Maize C₄ photosynthesis involves differential regulation of phosphoenolpyruvate carboxylase genes. *Plant J* 2: 221–232
- Schuster G, Ohad I, Martineau B and Taylor WC (1985) Differentiation and development of bundle sheath and mesophyll thylakoids in maize. *J Biol Chem* 260: 11866–11873
- Sheen J (1990) Metabolic repression of transcription in higher plants. *Plant Cell* 2: 1027–1038
- Sheen J (1991) Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. *Plant Cell* 3: 225–245
- Sheen J-Y and Bogorad L (1986) Differential expression of six light-harvesting chlorophyll *a/b* binding protein genes in maize leaf cell types. *Proc Natl Acad Sci USA* 83: 7811–7815
- Sheen J-Y and Bogorad L (1987) Expression of the ribulose-1,5-bisphosphate carboxylase large subunit gene and three small subunit genes in two cell types of maize leaves. *EMBO J* 5: 3417–3422
- Sinha NR and Kellogg EA (1996) Parallelism and diversity in multiple origins of C₄ photosynthesis in the grass family. *Amer J Bot* 83: 1458–1470
- Smith LG, Greene B, Veit B and Hake S (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development* 116: 21–30
- Sprey B and Laetsch WM (1978) Structural studies of peripheral reticulum in C₄ plant chloroplasts of *Portulaca oleracea*. *Z Pflanzenphysiol* 87: 37–53
- Stockhaus J, Poetsch W, Steinmüller K and Westhoff P (1994) Evolution of the C₄ phosphoenolpyruvate carboxylase promoter of the C₄ dicot *Flaveria trinervia*: An expression analysis in the C₃ plant tobacco. *Molec Gen Genet* 245: 286–293
- Stockhaus J, Schlu U, Koczor M, Chitty JA, Taylor WC and Westhoff P (1997) The promoter of the gene encoding the C₄ form of phosphoenolpyruvate carboxylase directs mesophyll specific expression in transgenic C4 *Flaveria*. *Plant Cell* 9: 479–489
- Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. *Ann Rev Plant Physiol Plant Molec Biol* 40: 211–233
- Telfer A, Bollman KM and Poethig RS (1997) Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* 124: 645–654
- Thompson WF and White MJ (1991) Physiological and molecular studies of light-regulated nuclear genes in higher plants. *Annu Rev Plant Physiol Plant Molec Biol* 42: 423–466
- Tobin EM and Silverthorne J (1985) Light regulation of gene expression in higher plants. *Annu Rev Plant Physiol* 36: 569–593
- Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF and Komeda Y (1996) The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* 8: 735–746
- Torres-Ruiz R and Jurgens G (1994) Mutations in the FASS gene uncouple pattern formation and morphogenesis in *Arabidopsis* development. *Development* 120: 2967–2978
- Traas JA, Bellini C, Macry P, Kronenberger P, Bouchez D and Caboche M (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. *Nature* 375: 676–677
- Ueno O (1996) Structural characterization of photosynthetic cells in an amphibious sedge, *Eleocharis vivipara*, in relation to C₃ and C₄ metabolism. *Planta* 199: 382–393
- Ueno O, Takeda T and Maeda E (1988) Leaf ultrastructure of C₄ species possessing different Kranz anatomical types in the Cyperaceae. *Bot Mag* 101: 141–152
- Viret J-F, Mabrouk Y and Bogorad L (1994) Transcriptional photo-regulation of cell-type-preferred expression of maize *rbcS-m3: 3'* and *5'* sequences are involved. *Proc Natl Acad Sci USA* 91: 8577–8581
- von Arnim A and Deng X-W (1996) Light control of seedling development. *Annu Rev Plant Physiol Plant Molec Biol* 47: 215–243
- Walker JC (1994) Structure and function of the receptor-like protein kinases of higher plants. *Plant Mol Biol* 26: 1599–1609
- Wang J-L, Long JJ, Hotchkiss T and Berry JO (1993a) Regulation of C₄ gene expression in light- and dark-grown amaranth cotyledons. *Plant Physiol* 102: 1085–1093
- Wang J-L, Turgeon R, Carr JP and Berry JO (1993b) Carbon sink-to-source transition is coordinated with establishment of cell-specific gene expression in a C₄ plant. *Plant Cell* 5: 289–296
- Williamson RE (1986) Organelle movements along actin filaments and microtubules. *Plant Physiol* 82: 631–634
- Woo KC, Anderson JM, Boardman NK, Downton WJS, Osmond CR and Thorne SW (1970) Deficient Photosystem II in agranal bundle sheath chloroplasts of C4 plants. *Proc Natl Acad Sci USA* 67: 18–25
- Wrzischa M (1989) Ultrastructural localization of photosynthetic activity in thylakoids during chloroplast development in maize. *Planta* 177: 18–23
- Yanagisawa S and Sheen J (1998) Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. *Plant Cell* 10: 75–89
- Yang M and Sack FD (1995) The *too many mouths and four lips* mutations affect stomatal production in *Arabidopsis*. *Plant Cell* 7: 2227–2239

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Chapter 21

The Physiological Ecology of C₄ Photosynthesis

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Summary

C₄ photosynthesis is an evolutionary syndrome that concentrates CO₂ around Rubisco and in so doing reduces photorespiratory inhibition of photosynthesis to negligible levels. It is not a single pathway, but a syndrome of functionally similar modifications that utilize phosphoenolpyruvate carboxylation in mesophyll cells, and transport of four-carbon acids to an enlarged bundle sheath tissue where Rubisco is localized. At least 14 distinct types of C₄ photosynthesis have been recognized, reflecting the use of one of three decarboxylating enzymes and one to two cell layers around the periphery of the vascular bundle. Despite substantial variation in how C₄ plants accomplish CO₂ concentration, the net effect on photosynthesis is similar in all forms. Relative to C₃ plants, C₄ plants have enhanced photosynthesis at CO₂ levels below the current atmospheric level of 360 μmol mol⁻¹. Increases in temperature above 25 °C favor C₄ relative to C₃ photosynthesis because photorespiration increases in C₃ species as temperatures rise while in C₄ species it remains minimal. Thus, in the current atmosphere, C₄ species have higher temperature optima relative to C₃ species of similar life form and higher CO₂ assimilation capacity at the temperature optimum. Because rising CO₂ inhibits photorespiration, the photosynthetic advantage of C₄ plants at warmer temperature is reduced or eliminated in high CO₂ conditions. C₄ plants have higher water and nitrogen use efficiencies than C₃ plants. This occurs because the capacity of C₄ systems to saturate Rubisco with CO₂ at low atmospheric CO₂ levels enables C₄ species to operate at lower stomatal conductances and Rubisco contents than C₃ species of equivalent CO₂ assimilation capacity. However, light use efficiency (quantum yield) differences between C₃ and C₄ depend on temperature. At current atmospheric CO₂ levels, C₃ species have higher quantum yields than C₄ plants below about 25 °C but lower above 30 °C. In C₄ plants, quantum yields do not change with temperature and CO₂ variation as they do in C₃ species, but do show differences between the biochemical subtypes. Species using NADP-malic enzyme generally have higher quantum yields than NAD-malic enzyme types for reasons that remain unclear. Differences in CO₂ leak rates had been suggested as a possible cause but recent permeability estimates do not show consistent variation between subtypes.

Ecologically, the C₄ pathway promotes fitness in warm environments receiving greater than approximately 30% of full sunlight intensities. C₄ species are generally absent in environments where average growing season temperatures are less than 15 to 18 °C, yet potentially dominate environments where the growing seasons are on average warmer than 22 °C. In warm climates, the dominance of C₄ species is largely dependent upon the availability of summer precipitation and conditions that inhibit establishment and dominance of woody vegetation. Where soil conditions (arid or infertile) and ecological disturbances such as fire restrict woody vegetation, C₄ species are abundant if not dominant. In general, however, moisture, salinity or low soil fertility have a subordinate role over C₄ abundance in that the dominant factors of temperature and light must be favorable or else C₄ species will not be competitive. Where intermediate temperatures favor neither photosynthetic pathway, however, drought, high salinity and nitrogen deficiency are important secondary controls, and appear to promote C₄ success in environments that otherwise would support C₃ dominance.

In the future, the distribution and abundance of C₄ species may become restricted because C₃ species generally respond more to rising atmospheric CO₂ than C₄ plants. Paleoecology studies indicate that the direct consequences of rising CO₂ will be most important in the tropics, with woodland ecosystems potentially spreading into C₄ grasslands. In temperate zones, paleoecological studies indicate that rising CO₂ and temperature could offset each other. If this occurs, other key ecological controls could become paramount; in particular, changes in the seasonality of precipitation could be important. Everywhere, human land use practices will have to be considered, given that people can radically alter vegetation characteristics depending upon their needs and desires.

I. Introduction

The discovery of the C₄ photosynthetic pathway in the mid-1960s is one of the more important developments in plant biology. In addition to improving understanding of photosynthetic physiology, the C₄ discovery provided mechanistic insights into many ecological and evolutionary phenomena. This understanding also has major economic significance because photosynthetic pathway is a determinant of where and when crops species can be successfully cultivated. C₄ crops and pasture grasses perform well in hot, tropical environments, yet are unproductive in cool temperate climates unless they have been bred to complete their lifecycle during short growing seasons, as is the case for maize (*Zea mays*, C₄) grown in Europe. C₄ species are important converters of solar energy into biologically-useful forms required by humanity to meet its food and animal protein needs. Presently, about one-third of world grain production is C₄, and half of the world's meat production is based on C₄ grains and forages (estimated from statistics in FAO, 1990). The contribution of C₄ biomass to the world's food supply will increase in coming decades because human population growth and the consequent development of new agricultural land is concentrated in low latitudes where C₄ species perform well.

While the ecological and economic significance of the C₄ pathway has long been recognized, understanding of the evolutionary significance of C₄ photosynthesis is a relatively recent development. C₄ photosynthesis independently evolved at least 30 times in widely diverse lines of monocots and dicots, and is now present in about 8000 species from 18 families of flowering plants (about 4500 grass species, 1500 sedges, and 2000 dicots; Sage et al., 1999a). The evidence points to a recent origin in geologic time, between 15 and 30 million years ago (Cerling, 1999; Kellogg, 1999), with a rise to dominance on open landscapes of the tropics and subtropics between 5 and 8 million years ago (Cerling et al., 1997). Currently, C₄ photosynthesis is common, if not dominant, in biomes that cover about 40% of the surface of the Earth. In addition to their current

significance, C₄ plants have played an important role in human affairs throughout our history on this planet, affecting the development of both ancient and modern civilizations (van der Merwe and Tschauner, 1999). Our existence as a species may in part depend upon the success of the C₄ syndrome. The spread of the C₄-dominated grasslands and savannas in the last 5 to 8 million years contributed to the opening of wooded landscapes in East-Africa where early humans first evolved (Cerling et al., 1997). The environmental challenges of this open landscape were substantially different than in wooded ecosystems, and may have selected for many distinguishing characteristics of our species, such as upright stature, sophisticated weaponry, and complex social organization (Stanley, 1995).

With the growing awareness of the importance of C₄ plants to natural and human ecosystems has come an increased need for an up-to-date summary of the physiological ecology of the C₄ syndrome. No longer is an understanding of C₄ physiology and ecology solely the domain of plant scientists; it is also important for land managers, conservationists, anthropologists, geologists, and atmospheric scientists, to name a few. In this chapter, we review the physiological, ecological and geographic significance of the C₄ syndrome in a format that scales from biochemistry to landscape ecology. We also discuss the relationship between global change and the dynamics of C₃/C₄ interactions, providing some educated speculation about how C₄ plants may fare in the human-dominated world of the future.

II. Physiological Considerations

The physiological rational for the existence of C₄ plants arises from a fundamental flaw in the reaction mechanism of the primary carboxylating enzyme in all plants, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

A. Oxygen Inhibition of Rubisco Activity

Rubisco is a dual-function enzyme, catalyzing either the carboxylation or oxygenation of RuBP (Andrews and Lorimer, 1987). A product of RuBP oxygenation is phosphoglycollate (PG), a two-carbon compound that has no apparent value to plants and whose accumulation is toxic. Plants metabolize PG to PGA using ATP and reducing power, but in the process,

Abbreviations: Ca – ambient partial pressure of CO₂; Ci – intercellular partial pressure of CO₂; N – nitrogen; NAD(P)-ME – malic enzyme; NUE – nitrogen use efficiency; PCK – PEP carboxykinase; PEP – phosphoenolpyruvate; PEPCase – PEP carboxylase; PG – phosphoglycollate; PPDK – pyruvate-phosphate dikinase; WUE – water use efficiency

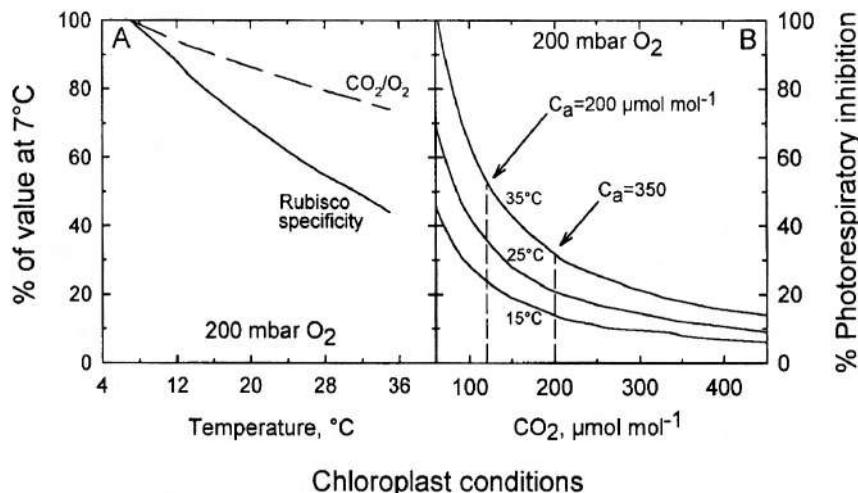


Fig. 1. A) The effect of temperature on the key variables controlling the rate of photorespiration in C₃ species, expressed relative to values at 7 °C. Rubisco specificity refers to the specificity of Rubisco for CO₂ relative to O₂, while CO₂/O₂ is the ratio of the aqueous concentrations of these gases. Developed from Jordan and Ogren (1984). B) The modeled response of photorespiratory inhibition of photosynthesis (expressed as the ratio of photorespiration to photosynthesis × 100%) as a function of stromal CO₂ concentration. Arrows indicate approximate atmospheric CO₂ concentrations (C_a) corresponding to conditions of the late-Pleistocene 20,000 years ago (180 $\mu\text{mol mol}^{-1}$), and of the current era (from Sage, 1995).

previously fixed carbon is lost as CO₂. Photorespiration (RuBP oxygenation and the metabolism of PG to PGA) inhibits photosynthesis by 1) competing for Rubisco active sites, 2) consuming RuBP, ATP and reducing power, and 3) releasing previously fixed CO₂ (Sharkey, 1985, 1988). The degree to which photorespiration inhibits photosynthesis depends upon temperature and the stromal concentration of CO₂ and O₂ (Andrews and Lorimer, 1987). Rising temperature substantially enhances the rate of photorespiration by reducing the specificity of Rubisco for CO₂ relative to O₂, and by reducing the solubility of CO₂ to a greater degree than it reduces the solubility for O₂ (Fig. 1A). At the current atmospheric CO₂ level, photorespiration is relatively modest below 15 °C; above 35 °C, photorespiration is highly inhibitory in all C₃ plants, reducing photosynthesis by over 30% (Fig. 1B).

Because of its potential to inhibit photosynthesis, there has been considerable discussion of the adaptive significance of photorespiration. No direct adaptive role has been identified, although indirectly photorespiration helps dissipate excess light energy (Takeba and Kozaki, 1998). A widely accepted view is that RuBP oxygenation is an unavoidable side reaction of the carboxylation mechanism and thus likely has no adaptive value. Its persistence through evolutionary time is a function of the critical

importance of Rubisco to photosynthetic organisms and the presence of high levels of atmospheric CO₂ through most of earth's history. Rubisco first evolved some 3 to 3.5 billion years ago in a setting where photorespiration was not possible because the atmosphere was highly enriched in CO₂ and lacking free O₂ (Hayes, 1994; Fig. 2A). Atmospheric conditions remained highly unfavorable for photorespiration for over three billion years, during which time an elaborate photosynthetic biochemistry centered on RuBP carboxylation evolved and became so successful that all photosynthetic life now employs the Rubisco-based pathway for the ultimate fixation of inorganic carbon into organic forms. With the possible exception of a low CO₂ event during the Carboniferous period 300 million years ago, estimated atmospheric CO₂ levels remained high enough to have restricted Rubisco oxygenase activity to minor levels. Only in the past 10 to 20 million years has low atmospheric CO₂ combined with abundant atmospheric oxygen to produce rates of photorespiration that would have been able to consistently inhibit photosynthesis in non-stressed plants by 35% or more above 30 °C. This would be particularly so during the Pleistocene era between 0.02 and 2 million years ago when atmospheric CO₂ fell below 200 μbar for extended periods (Figs. 2E, 2F).

By the time high rates of photorespiration became

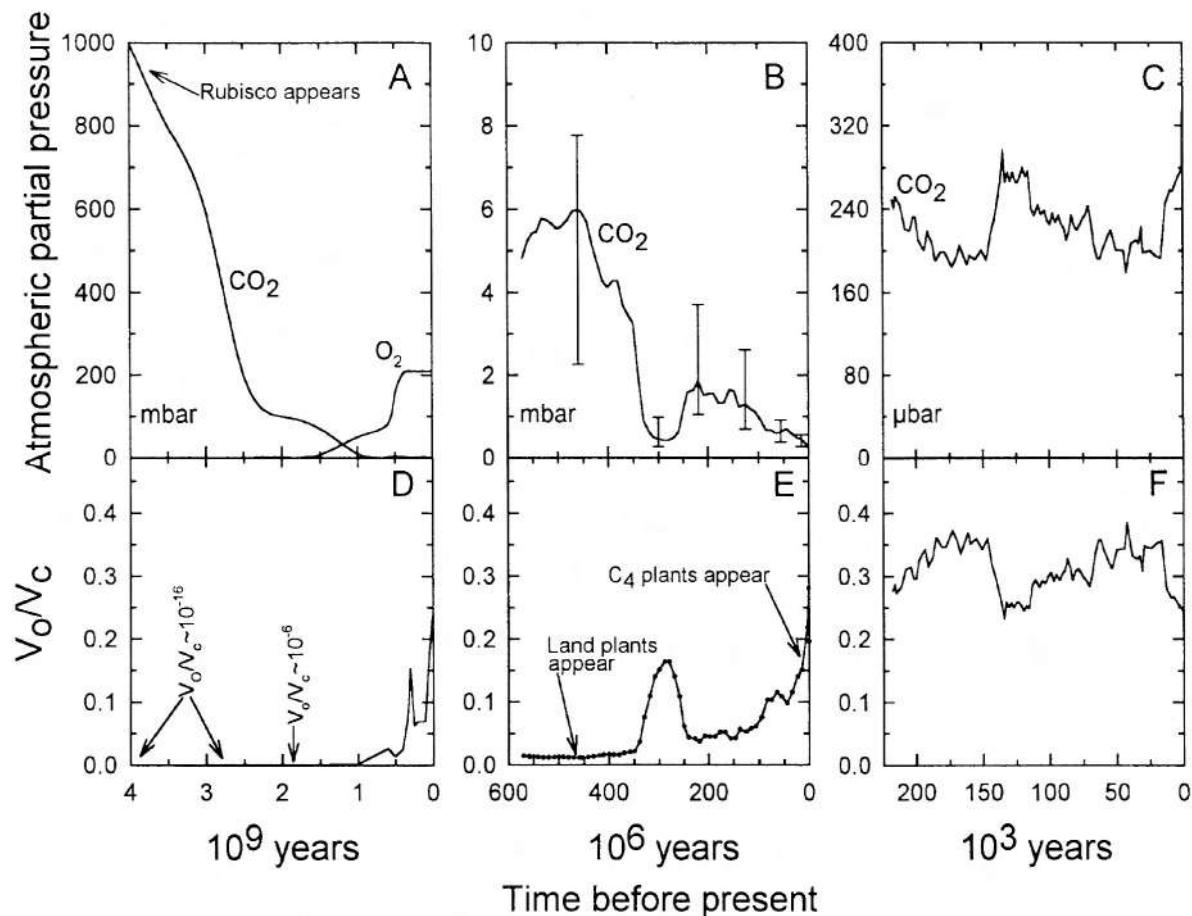


Fig. 2. Atmospheric CO₂ and O₂ content over the history of the earth, modeled for A) the last 4 billion years (Kasting, 1987, and Berner and Canfield, 1989), B) the past 600 million years (according to Berner, 1994), and C) the past 240,000 years (after Barnola et al., 1989, and Jouzel et al., 1993). Partial pressures are expressed as mbar in panels A and B, and μbar in panel C. Panels D, E, and F represent the modeled ratios of oxygenation (v_o) to carboxylation (v_c) at 30 °C for the corresponding CO₂ and O₂ levels in panels A, B and C, respectively, and assuming a spinach type Rubisco according to Jordan and Ogren, 1984.

a problem, the photosynthetic pathway centered on Rubisco was thoroughly established and intricately coordinated with metabolism throughout the plant. Thus, substantial constraints limited options for evolutionarily resolving the oxygenation problem. Oxygenation is likely an inherent feature of the reaction mechanism required for the formation of PGA from CO₂ and RuBP (Andrews and Lorimer, 1987), so that utilization of an entirely different carboxylation mechanism would likely be required to avoid photorespiration. While there are alternative carboxylases such as PEP carboxylase (PEPCase), their direct utilization would require a different pathway for carbon reduction and acceptor regeneration. Given this, a complete change of carboxylating enzyme and C-metabolism pathway may have been

too much to accomplish in the presence of competition from already successful, albeit photosynthetically inefficient vegetation.

B. C₄ Solutions to Photorespiratory Inhibition

Instead of evolving a new carboxylase that lacked oxygenase activity, plants solved the oxygenase problem by 1) localizing Rubisco in an internal compartment that is separated from the rest of the cell or tissue by a barrier that restricts CO₂ efflux, and then 2) biochemically pumping CO₂ into that compartment using ATP. In algae, the solution to photorespiration is to localize Rubisco within an internal cellular compartment into which CO₂ or bicarbonate is concentrated (Badger and Spalding,

chapter 16). In terrestrial plants, photorespiration is minimized by C₄ photosynthesis, a polyphyletic system where CO₂ is first fixed by PEPCase into four-carbon (C₄) acids within photosynthetic mesophyll cells. The resulting organic acids are then transported to a bundle sheath layer where Rubisco is localized. Here, they are decarboxylated, with the result being an approximate tenfold enhancement of bundle sheath CO₂ concentration. Substantial variation is present in how various C₄ species achieve CO₂ concentration, highlighting the diverse evolutionary pathways that land plants used to arrive at a common solution to photorespiratory inhibition.

C. Variations on the C₄ Theme

All C₄ species operate on the same basic theme of pumping CO₂ via C₄ acids from the mesophyll tissue where PEPCase activity is enhanced to a bundle sheath layer where Rubisco is localized and C₄ acids are decarboxylated. Other than this, the only common feature shared by C₄ plants is a reduction in the ratio of mesophyll to bundle sheath cells when compared to C₃ plants. Because diffusion of organic acids between the mesophyll and bundle sheath must be relatively rapid, mesophyll cells in C₄ plants are rarely more than one cell distance from bundle sheath cells. As a result, mesophyll to bundle sheath cell ratios are between 1 and 2 in C₄ plants, while they are over 4 in most laminate C₃ leaves (Dengler and Nelson, 1999).

Beyond these common features, C₄ plants exhibit substantial variation in how they accomplish CO₂ concentration. In all, at least 14 distinct types of C₄ species have been identified (eight grass, three sedge, and three dicot types) and numerous more will likely be described as less common groups are studied. These variations result from three distinct decarboxylation modes and multiple patterns of anatomical modification. Biochemically, the major distinguishing feature of C₄ subtypes is the enzyme used for the decarboxylation step in the bundle sheath (Hatch, 1987). The three decarboxylation modes are NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and PEP carboxykinase (PCK). Table 1 lists economically significant C₄ plants of each subtype. Notably, economically significant C₄ plants tend to be NADP-ME species (Brown, 1999). While most C₄ species primarily use one decarboxylating enzyme, a second decarboxylating enzyme may be employed in a back-up role in some species. For

Table 1. Economically important C₄ plant species with biochemical subtype. Modified from Brown (1999).

Crops – grasses	
<i>Digitaria exilis</i> (fonio)	NADP-ME
<i>Pennisetum glaucum</i> (pearl millet)	NADP-ME
<i>Saccharum officinalis</i> (sugar cane)	NADP-ME
<i>Sorghum bicolor</i> (sorghum)	NADP-ME
<i>Setaria italica</i> (foxtail millet)	NADP-ME
<i>Zea mays</i> (maize, corn)	NADP-ME
<i>Eleusine coracana</i> (finger millet)	NAD-ME
<i>Eragrostis tef</i> (tef)	NAD-ME
<i>Panicum miliaceum</i> (Proso millet)	NAD-ME
Crops – dicots	
<i>Amaranthus edulis</i> (grain amaranth)	NAD-ME
<i>Amaranthus tricolor</i> (vegetable amaranth)	NAD-ME
Forages (all grasses)	
<i>Cenchrus ciliaris</i>	NADP-ME
<i>Digitaria decumbens</i>	NADP-ME
<i>Paspalum</i> spp	NADP-ME
<i>Pennisetum</i> spp	NADP-ME
<i>Setaria anceps</i>	NADP-ME
<i>Sorghum alnum</i>	NADP-ME
<i>Cynodon dactylon</i>	NAD-ME
<i>Panicum coloratum</i>	NAD-ME
<i>Brachiaria mutica</i>	PCK
<i>Chloris guyana</i>	PCK
<i>Melinis minutiflora</i>	PCK
<i>Panicum maximum</i>	PCK
Weeds – grasses	
<i>Echinochloa colonum</i>	NADP-ME
<i>Echinochloa crusgalli</i>	NADP-ME
<i>Imperata cylindrica</i>	NADP-ME
<i>Sorghum halopense</i>	NADP-ME
<i>Digitaria sanguinalis</i>	NADP-ME
<i>Paspalum conjugatum</i>	NADP-ME
<i>Cynodon dactylon</i>	NAD-ME
<i>Eleusine indica</i>	NAD-ME
Weeds – sedges	
<i>Cyperus esculentus</i>	NADP-ME
<i>Cyperus rotundus</i>	NADP-ME
Weeds – dicots	
<i>Amaranthus retroflexus</i>	NAD-ME
<i>Amarantus spinosus</i>	NAD-ME
<i>Portulaca oleracea</i>	NAD-ME
<i>Tribulus terrestris</i>	NADP-ME

example, PCK species usually have some NAD-ME activity as a means of shuttling NADH into the bundle sheath for ATP generation (Leegood and

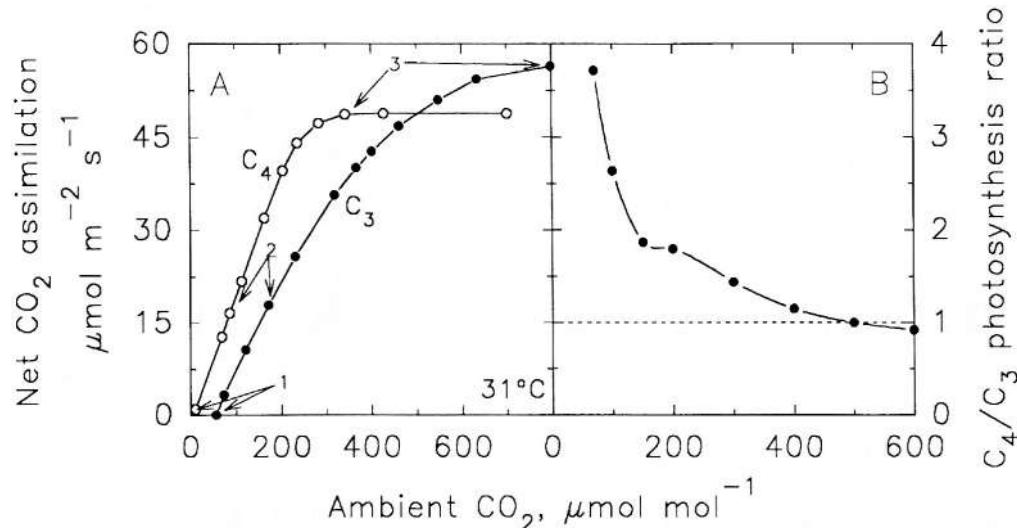


Fig. 3. A) The response of net CO₂ assimilation rate to ambient CO₂ level in *Amaranthus retroflexus* (C₄) and *Chenopodium album* (C₃) measured at 31 °C using a null balance gas exchange system. Arrows indicate notable points, namely 1) differences in the CO₂ compensation point, 2) differences in the initial slope of photosynthesis, and 3) differences in the CO₂ saturation point. Panel B shows the ratio of photosynthesis of *A. retroflexus* to *C. Album* presented in panel A (modified from Sage, 1995).

(Walker, 1999). Anatomically, two general distinctions are made depending upon the origin of the cell layer where Rubisco is localized. Many C₄ species localize Rubisco in a layer of parenchymatous bundle sheath cells that are derived from ground meristem. A significant number of grasses and sedges also utilize mesome sheath cells instead of or in addition to the parenchymatous bundle sheath (Dengler and Nelson, 1999). The mesome sheath is a cellular layer just inside the parenchymatous bundle sheath and is derived from procambium during development. Additional variation occurs in the metabolic steps associated with C₄ acid transport and PEP regeneration, the location and ultrastructure of chloroplasts, and properties of the wall separating Rubisco-containing cells from the mesophyll tissue (Hattersley and Watson, 1992). Many of these variations are associated with the biochemical decarboxylation step, and in some cases the association is causal. For example, in NADP-ME species, the movement of malate imports reducing power into the cell, leading to NADPH formation as a product of the decarboxylating step (Chapter 19, Leegood). As a consequence, bundle sheath chloroplasts in the NADP-ME species require less Photosystem II activity and evolve less O₂ in the bundle sheath. The reduced O₂ production within the bundle sheath allows for a bundle sheath wall that can be more resistive to CO₂ efflux, and correspondingly, NADP-ME grasses commonly have

a suberized wall that hypothetically is more effective at restricting gas diffusion.

D. Physiological Consequences of C₄ Biochemistry at the Leaf Level

The physiological significance of the C₄ system of CO₂ concentration is best demonstrated by comparing the response of photosynthesis to the intercellular partial pressure of CO₂ and to light in C₃ and C₄ species of similar life form and ecological habitat.

1. CO₂ Response

Differences between C₃ and C₄ responses to CO₂ are generally so striking that they are useful indicators of C₄ photosynthesis (Downton and Tregunna, 1968; Downton, 1975). As demonstrated in Fig. 3, the CO₂ response of net CO₂ assimilation rate (A) in C₄ plants differs from the C₃ response in four main points (Edwards and Walker, 1983). 1) The CO₂ compensation point (the CO₂ level where gross photosynthesis equals respiration and thus net photosynthesis is zero) is near zero in C₄ plants at all temperatures, but is near 50 μmol mol⁻¹ in C₃ species at moderate temperatures, rising to over 70 μmol mol⁻¹ above 35 °C (Sage et al., 1990). 2) The initial slope of the CO₂ response of A is steeper in C₄ than C₃ plants; 3) The CO₂ saturation point occurs below 200 to 300

Theoretical Controls Over Higher Plant Photosynthesis

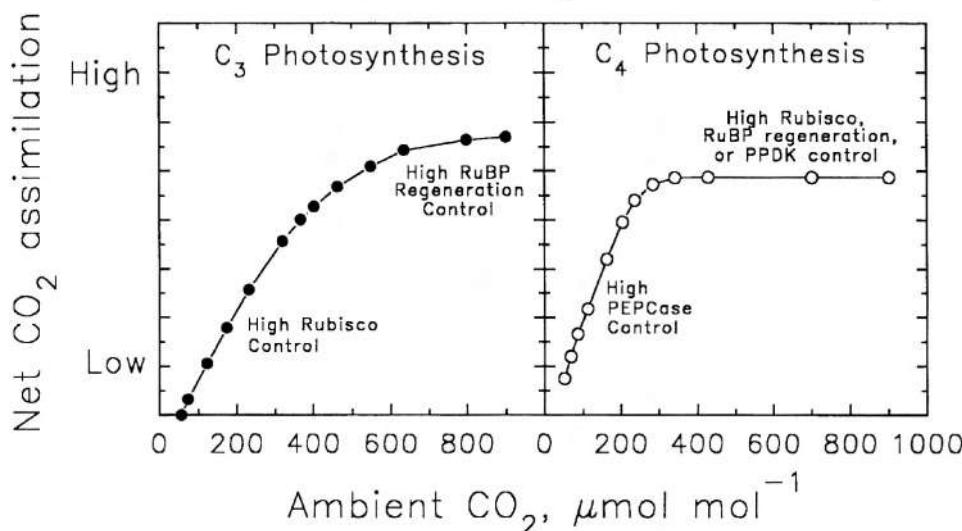


Fig. 4. Schematic CO₂ response curves of photosynthesis showing the theoretical limitations on net CO₂ assimilation rate for typical C₃ and C₄ plants.

$\mu\text{mol mol}^{-1}$ in C₄ species and is distinctly delineated. In contrast, the CO₂ saturation point in C₃ species commonly occurs at much higher CO₂ than ambient and is not sharply delineated, except at low temperature (<~15 °C; Sage and Sharkey, 1987). Finally, the CO₂ saturated rate of photosynthesis is typically lower in C₄ than in C₃ species of similar life-form. These responses lead to the commonly recognized view that C₄ species have superior photosynthetic performance at CO₂ levels below ambient while C₃ species have superior values at elevated CO₂ (Fig. 3B). These trends are, however, influenced by temperature (Berry and Raison, 1982).

Differences in the CO₂ responses of C₃ and C₄ species are explained by the limitation profiles of the respective photosynthetic types across a range of CO₂ (Fig. 4). In C₃ species, photosynthetic rates in the initial slope region of the CO₂ response curves at light saturation are generally dependent on availability of Rubisco active sites (Rubisco-limited A). In contrast, when light is limiting, the regeneration capacity of RuBP limits CO₂ assimilation (Fig. 4; von Caemmerer and Farquhar, 1981; Sage and Reid, 1994). In both cases, photosynthesis is sensitive to photorespiratory inhibition, which increases the CO₂ compensation point and reduces the initial slope of the CO₂ response curve (Edwards and Walker, 1983; Sage et al., 1990).

In C₄ plants, the initial slope of the CO₂ response

is dependent upon PEP carboxylation rather than Rubisco carboxylation (Fig. 4B; von Caemmerer and Furbank, 1999). PEPCase utilizes bicarbonate rather than CO₂ as a substrate. Although the K_m of PEPCase for HCO₃⁻ is similar to the K_m for CO₂ of Rubisco (PEPCase K_m ~20 μM at 25 °C; Rubisco K_m ~10–20 μM), at cellular pH, the bicarbonate/CO₂ equilibrium favors a HCO₃⁻ concentration that is approximately eight times greater than the concentration of CO₂ (Edwards and Walker, 1983). Consequently, at CO₂ levels that are below the K_m for Rubisco and therefore highly limiting for RuBP carboxylation, enough HCO₃⁻ is available for high PEPCase activity. PEPCase also experiences no inhibitory side reactions such as oxygenase activity of Rubisco and its specific activity is about eight times greater than that of Rubisco (Sage et al., 1987). Thus, in the cellular environment, a mole of PEPCase can turn over about 20 to 30 times faster than a mol of Rubisco from C₃ plants. In practical terms, these differences mean that a large quantity of CO₂ can be pumped into the bundle sheath of C₄ plants so that Rubisco is substrate-saturated even at mesophyll CO₂ levels below the current atmospheric value. In C₃ plants at current atmospheric levels of CO₂, Rubisco functions at only 20% to 30% of its maximum activity at 30 °C (Sage et al., 1987).

Maximum photosynthetic rates in C₄ plants (that is, the CO₂-saturated rate) are potentially determined

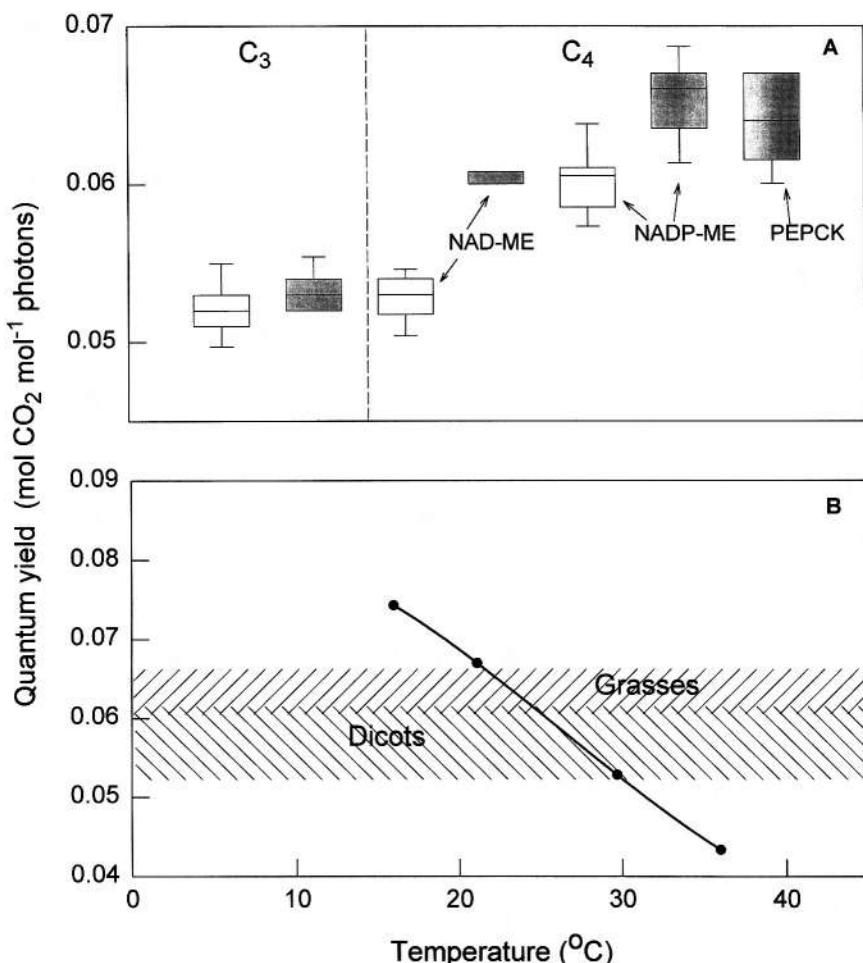


Fig. 5. A. Variation in quantum yield of C₃ (left side) and C₄ plants (right side) among photosynthetic subtypes and between monocots and dicots. The top and bottom of the boxes give the 75th and 25th percentile, respectively, and the line through the center gives the median value for each. Error bars express the 10th and 90th percentile. The open and shaded boxes are for dicots and monocots, respectively. B. The dependence of quantum yield on temperature. The line gives the typical temperature dependence of quantum yields in C₃ species. Quantum yields are independent of temperature in C₄ species; the diagonally hatched areas give the range of values in quantum yield shown in panel A. The intersection of the line for C₃ species and the ranges for the C₄ species shows the range of crossover temperatures possible. (Redrawn from data in Ehleringer and Pearcy, 1983).

by either Rubisco activity, the rate of RuBP regeneration, the rate of PEP regeneration, PEPCase activity, or any combination thereof (von Caemmerer and Furbank, 1999). Examples where each is limiting have recently been described using transgenic *Flaveria* and *Amaranthus* species in which antisense constructs were used to modify enzyme levels (Dever, 1997; Furbank et al., 1997).

2. Quantum Yield

The quantum yield of photosynthesis is the ratio of photosynthetic product ($\mu\text{mol CO}_2$ taken up or O₂

released) expressed relative to absorbed light intensity. At very low light (for example, less than 100 μmol photons $\text{m}^{-2} \text{s}^{-1}$ (< 5% of full sunlight)), a maximum proportion of absorbed photons is used for photosynthetic carbon fixation and the metabolism of photorespiratory products. At these photon fluxes, the initial slope of the light response of photosynthesis is linear and reflects the maximum quantum yield of CO₂ fixation. At higher light intensities, light absorption exceeds light utilization by the cellular photochemistry. When this occurs, the excess light energy is dissipated as heat causing the instantaneous quantum yield to decline because absorbed light

energy is not used in the biochemical reactions of photosynthesis (Björkman and Demmig-Adams, 1994; Genty and Harbinson, 1996).

In studies of C_4 photosynthesis, the maximum quantum yield has become an important index of photosynthetic performance because it directly reflects the energy costs of the two photosynthetic pathways. The major energetic cost of the C_4 pathway is the two extra ATP per CO_2 fixed that are required to regenerate PEP. In the absence of photorespiration, these energy costs reduce the maximum quantum yield of C_4 plants by 30% to 40% relative to C_3 plants (Fig. 5A). Under current atmospheric conditions of $360 \mu\text{mol mol}^{-1} CO_2$, $210 \text{ mmol mol}^{-1}$ (21%) O_2 , and 20 to 30 °C, energy losses due to photorespiration in C_3 leaves are approximately equivalent to the additional energy costs of the C_4 concentrating mechanism. Thus quantum yields are similar (Fig. 5A). Because of the temperature, CO_2 and O_2 dependence of photorespiration, the quantum yields of C_3 plants also exhibit a high dependence on these factors. At current atmospheric CO_2 contents, maximum quantum yields tend to be greater in C_4 than C_3 species above 30 °C, but lower below 25 °C (Fig. 5B). Reducing atmospheric CO_2 shifts the equivalency point to lower temperature, such that at CO_2 levels encountered during the Pleistocene (~200 $\mu\text{mol mol}^{-1}$), the temperature where quantum yields of C_3 and C_4 species are equal would be less than 20 °C (Ehleringer et al., 1997). In contrast to C_3 plants, for a given C_4 species, maximum quantum yields are constant, showing no temperature, CO_2 or O_2 dependency (Fig. 5B). There is, however, significant variation in quantum yields between the various classes of C_4 plants.

3. Variation of Quantum Yield of C_4 Species

When expressed on the basis of absorbed quanta, the quantum yields of C_3 species under non-photorespiratory conditions have been found to be remarkably invariant (Björkman and Demmig, 1987). By contrast, in C_4 species, considerable difference exists between species of varying decarboxylation type and evolutionary origin. NADP-ME plants have higher quantum yields than NAD-ME plants of similar taxonomic group, and within the NADP-ME and NAD-ME decarboxylation types, grasses consistently have somewhat higher quantum yields than dicots (Fig 5A; Ehleringer and Pearcy, 1983).

The reasons for differences in quantum yield between decarboxylation types have proved elusive

to identify. The costs for CO_2 concentration in NADP-ME and NAD-ME type plants consist of 2 ATP per CO_2 fixed for regeneration of PEP, plus the amount of ATP required to pump in extra CO_2 (overcycle CO_2) to compensate for the CO_2 that leaks out of the bundle sheath. In PCK type plants, one extra ATP and 0.5 extra NADPH are required in addition to the amount required for overcycling (Hatch, 1987).

In the absence of overcycling, there is little theoretical difference among C_4 decarboxylation types in the energy required for the CO_2 concentration cycle. However, leakage is unavoidable because the numerous plasmodesmata required for C_4 acid transport provide a low resistance path for diffusive efflux relative to the surrounding wall region of the bundle sheath (Hatch, 1987). The degree of leakage may also vary between photosynthetic subtypes because of the need to allow for efflux of any O_2 produced by Photosystem II (PS II) activity in the bundle sheath (BS) cells. NADP-ME species have little PS II activity in the BS chloroplasts, and therefore have little need to allow for O_2 efflux. In contrast, NAD-ME and PCK species have substantial PS II activity in the BS tissues and thus it was initially hypothesized that plants of these subtypes have bundle sheath walls that were more permeable to gaseous efflux than NADP-ME species (Farquhar, 1983). However, estimates of leak rates show little correlation between C_4 subtype and quantum yield. The fraction of CO_2 produced in the bundle sheath that actually escapes is difficult to quantify and estimates have ranged from 0.08 to 0.5 (Farquhar, 1983; Henderson et al., 1992; Hatch et al., 1995). The upper value, based on measurement of carbon isotope discrimination in leaf tissue, would be energetically costly because it would require two turnovers of the C_4 cycle, and hence two extra ATP, for each CO_2 assimilated. It may, however, be an overestimate because of other, non-photosynthetic sources of isotopic discrimination in leaf tissues (Henderson et al., 1992). Short-term fractionation studies, which should reflect photosynthetic processes, yielded leakage estimates close to 0.21 for a wide range of species of different C_4 types (Henderson et al., 1992). Recently, application of a new pulse-chase technique for direct measurement of the leak rate gave values for 11 species ranging from 0.08 to 0.14 (Hatch et al., 1995). At the present time, the consensus is that the leak rate is generally between 10% to 20% of the net CO_2 assimilation rate but it is not dependent upon bundle-sheath wall properties such as the degree of suberization, nor on

C₄ subtype. Thus, permeability differences do not explain variation in quantum yields between C₄ subtypes.

An alternative possibility is that variation in quantum yields is related to differences in the efficiency of light absorption and partitioning of quanta between bundle sheath and mesophyll cells. Differences in quantum yields between monocots and dicots have been attributed to a less optimum spacing between vascular bundles and therefore to a lower efficiency of light absorption (Ehleringer et al., 1997). This is an intriguing possibility that requires experimental verification.

III. Primary Environmental Controls—Temperature and Light

The geographic distributions of C₄ plants is highly dependent upon temperature during the growing season, and light regimes, so much so that these parameters can be considered primary controls over the distribution of C₄ plants. They effectively override influences of other environmental parameters such as water or soil nutrient supply. C₄ plants require warm to hot growing seasons and with only a few exceptions, do not occur in deeply shaded environments. While the mechanistic causes of these distribution patterns arise from the differential responses of C₃ and C₄ photosynthesis to light and temperature, numerous ecological factors, notably disturbances such as fire, interact with physiological differences to control where, and when, C₄ plants are successful. In this section, we discuss light and temperature controls over C₄ plant distribution, describing first the pattern of C₄ distribution along these environmental gradients, followed by a discussion on how photosynthetic properties may explain the trends.

A. Temperature

1. Geographic Distribution

a. Grasses

The first distinctive environmental characteristic noted for C₄ photosynthesis was its high representation among grasses of tropical origin and summer crops, weeds, and forages (Downton and Tregunna, 1968; Black et al., 1969). Subsequent work has shown that C₄ grass abundance is highly

correlated with temperature along latitude, altitude and seasonal gradients. Below approximately 25° latitude, both grass floras and grassland primary productivity are dominated (>75% representation) by C₄ species. Only where woody species are established will C₄ productivity be minimal or absent. With the exception of species adapted to salinized, sandy, or arid soils, tree and shrub species are almost exclusively C₃.

In the low elevation tropics, C₃ grasses are uncommon in open grasslands and deserts but increase in importance in tropical marshes and swamps. They dominate grass floras under forest canopies and at high elevation (Skerman and Riveros, 1990; Sage et al., 1999b). The shift from C₄ dominated to C₃ dominated grasslands along altitude gradients always occurs above 1000 m, but the precise altitude of crossover varies with precipitation. In wet tropical areas such as Hawaii and New Guinea, C₄ grasses dominate the grass cover up to 1500 to 2000 m (Rundel, 1980; Bird et al., 1994). On Mt. Kenya in East Africa, by contrast, C₄ grasses can predominate up to 2700 m, and can be locally dominant at even higher elevations on the drier microsites (Tieszen et al., 1979; Young and Young, 1983). However, above 3000 to 3500 m, C₃ cover and floristic representation always predominates over that of C₄ plants (Sage et al., 1999b).

Altitude limits of C₄ grasses in natural vegetation are modified by competitive interactions with both C₃ grasses and woody species. In agricultural systems, human management offsets much of the competitive effects from C₃ vegetation and the physiological potential for production becomes a major control over crop distribution. In montane regions at low latitudes, C₄ crops predominate at low elevation, but give way to C₃ crops and forages at high elevation (Skerman and Riveros, 1990). In the Ethiopian highlands, for example, C₄ crops predominate below 1500m, but are rarely cultivated above about 2500 m (Fig. 6). Instead, many temperate-zone crops and forages are grown in higher elevations in the tropics. In East Africa, C₃ ryegrass (*Lolium perenne*) and *Festuca* spp become important forage above 2200 m. Below this elevation, all the major forages are C₄, even on irrigated soils (Boonman, 1993).

At latitudes above 55°N and 46°S, C₄ grasses are rare at all elevations. At 50°N, C₄ grasses make up between 5% to 15% of regional grass floras, but are rarely dominant except in localized situations where microsites exhibit a combination of factors favorable to C₄ vegetation (Sage et al., 1999b). Microsites with

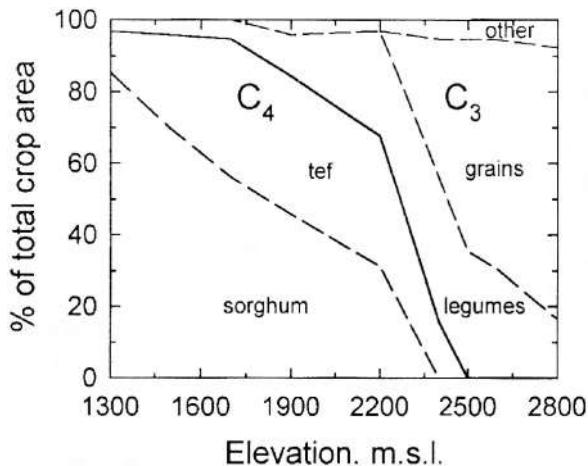


Fig. 6. The percentages of crop area devoted to major crops of non-vertisol soils expressed as a function of elevation in the central highlands of Ethiopia, Africa. C₄ crop area falls below the solid line, C₃ crop area is above it. C₄ crops contributing to the figure are *Sorghum bicolor* and *Eragrostis tef* (tef). C₃ crops include legumes in *Pisum* (chickpeas, fieldpeas) and *Vicia* (horsebeans), and the cereal grains wheat, barley and oats. Other C₃ crops are noug (*Gultozia abyssinica*) and linseed (*Linum* spp). Modified from Pearson (1992).

high solar insolation, saline and/or dry soils, and slope aspects facing the equator favor C₄ plants in generally cool climates (Guy et al., 1986; Schwarz and Redman, 1988; Pyankov and Makronosov, 1993). In many regions, C₄ species of high latitude are largely weedy species dependent upon human action for their continued success. When weeds are factored out, the C₄ contribution to a regional grass flora declines by 50% to 100%. In the British Isles, for example, 90% of the C₄ grass flora is introduced (Stace, 1997).

Between 50°N and 30°N, and 45°S and 35°S, the contribution of C₄ grasses to grassland cover and productivity increases with decreasing latitude until C₄ grasses predominate at the warmer ends of the temperate zones (Pyankov and Mokronosov, 1993; Bird and Pousai, 1997; Tieszen et al., 1997). In this zone, the latitude at which C₄ grasses become dominant is variable, and reflect interactions with other environmental factors (Epstein et al., 1997).

b. Sedges and Dicots

C₄ sedge taxa generally do not show the same degree of ecosystem dominance as do C₄ grasses. This is probably because sedges (plants of the Cyperaceae) are more likely to occur on flooded soils that also

support a rich C₃ sedge flora, even at low latitudes. While the proportional representation of C₄ sedges shows similar latitudinal responses as C₄ grasses, they do not extend to as high of latitude or elevation and they have reduced representation within a sedge flora than do C₄ grasses within the grass flora from the same region (Sage et al., 1999b). For example, in localities where C₄ grasses may represent over 80% of the grass flora, C₄ sedges may represent only 40% to 60% of the sedge flora (Teeri and Stowe, 1976; Teeri et al., 1980).

C₄ dicot distribution is more difficult to characterize in terms of environmental correlates than grasses and sedges because C₄ dicots rarely account for more than 5% of the dicot flora. Thus variation in C₄ dicot estimates is often due more to variation in total dicot species, rather than C₄ dicot numbers. Instead, presenting the occurrence of C₄ dicots in their respective families or functional groups is generally more meaningful. When this is done, similar latitudinal patterns as exhibited by grasses become apparent. In the Chenopodiaceae, less than 20% of the species in Europe north of the Mediterranean basin are C₄. In the Mediterranean basin, approximately 50% of the Chenopods are C₄, while over 95% are C₄ in central Africa (Akhani et al., 1997). Similarly, along the Nile river in Egypt, the proportion of species in the Euphorbiaceae that are C₄ rises from less than 20% along the Mediterranean sea in Northern Egypt, to 50% in southern Egypt near the Sudanese border (Batanouey et al., 1991).

2. Seasonal Trends

In temperate climates where C₄ species are abundant, their activity is nearly always centered on the summer months. Typically, they break dormancy some three to six weeks after C₃ species of similar functional groups. Seeds of annual C₃ weeds germinate in early to mid-spring, while C₄ weeds germinate mid-to-late spring (Baskin and Baskin, 1977). Similarly, in the Sonoran desert, C₃ species germinate under the temperatures and photoperiods characteristic of autumn and winter while C₄ annuals germinate under late-spring conditions (Mulroy and Rundel, 1977; Kemp, 1983). Among grasses from the Great Plains of North America, C₃ grasses break dormancy in March to April, while C₄ grasses appear late April to May (Dickinson and Dodd, 1976; Ode et al., 1980; Monson et al., 1983; Sage et al., 1999b). The result of these differences is the long-recognized segregation

of grasses and weeds into warm season (largely C₄) and cool season (largely C₃) species, and management practices have adjusted to account for these physiological differences (Skerman and Riveros, 1990; Boonman, 1993)

3. Temperature Thresholds

It is now well recognized that growth season temperature is the principle controlling factor over the abundance of C₄ plants along latitude and elevation gradients, particularly with grasses and sedges. In North America and Australia, C₄ grass and sedge distribution is well correlated ($r^2 > 0.8$) with numerous indices of growth season temperature including mean temperature of the warmest month, mean minimum temperature of the warmest month, mean maximum of the warmest month, number of days above 32 °C, summer pan evaporation, and potential evapotranspiration (Teeri and Stowe, 1976; Hattersley, 1983; Epstein et al., 1997). Close correlation between C₄ abundance and growth season temperature has also been observed along altitude gradients (Long, 1983; Cavagnaro, 1988). In the tropics, annual temperature is a good index of C₄ abundance along altitude gradients while in temperate zones annual temperature is less meaningful because winter temperature is poorly correlated with summer temperature. Winter cold does not appear to be any more harmful to dormant C₄ species than their dormant C₃ associates (Long, 1983; Schwarz and Reaney, 1989).

From analysis of geographical gradients, the following temperature thresholds are critical for C₄ plants. Below mean minimum temperatures of the warmest month of 6 to 10 °C, C₄ plants are largely absent (although a few hardy species can survive nightly lows averaging 2 °C) (Hattersley, 1983; Long, 1983; Pyankov and Mokronosov, 1983; Sage et al., 1999b). Transition from C₃ to C₄ dominance in grass floras occurs at minimum temperatures of the warmest month of 15° to 18 °C; and mean daily temperatures of about 22 °C (Teeri and Stowe, 1976; Hattersley, 1983; Bird and Pousai, 1997). Above mean daily temperatures of 24 °C, C₄ plants largely dominate a regional grass flora of non-shaded habitats.

4. Physiological Explanations for the Temperature Dependency

Rarely in ecology do single physiological mechanisms

explain distinct biogeographical patterns. In the case of the correlation between C₄ dominance and temperature, a strong argument can be made that the underlying mechanism explaining the trend is a result of the distinct temperature response of photosynthesis in C₄ plants relative to C₃ species of similar life form.

a. The Temperature Response of C₄ Relative to C₃ Photosynthesis

The temperature response of net CO₂ assimilation in C₃ and C₄ species show distinct differences: C₄ species have a pronounced rise in assimilation between 10 °C and the thermal optimum for photosynthesis, which usually occurs between 30 °C and 40 °C (Fig. 7; Björkman et al., 1975, 1980). Atmospheric CO₂ variation has little effect on this response above the current ambient of 365 μmol mol⁻¹, but reductions in CO₂ below 365 μmol mol⁻¹ reduce the rate of C₄ photosynthesis at the thermal optimum. In C₃ species, the temperature response of photosynthesis is highly dependent upon CO₂ partial pressure at all temperatures. At low CO₂ partial pressures, the temperature response of photosynthesis is relatively flat, which reflects a high control over photosynthetic rate by Rubisco. Rubisco has low thermal responsiveness at CO₂ partial pressures below its K_m, because both the K_m and V_{max} have similar thermal dependencies (Berry and Raison, 1981). At elevated CO₂, by contrast, C₃ photosynthesis shows a high thermal dependency at temperatures below the thermal optimum, similar to C₄ species. This is because the RuBP regeneration capacity, which has a high thermal dependency (Q₁₀ near 2) dominates the control of CO₂ assimilation in C₃ species at elevated CO₂ (von Caemmerer and Farquhar, 1981; Berry and Raison, 1981).

Comparison of the thermal responses of C₄ and C₃ species show that at low temperature (<18 °C), C₃ species typically have equivalent or higher CO₂ assimilation rates than ecologically similar C₄ species, even at the low CO₂ levels of the recent geological past. At elevated temperature, the C₄ species outperform C₃ species, especially at the low CO₂ level of the past 20,000 years (200 to 270 μmol mol⁻¹). The superiority of C₄ photosynthesis at elevated temperatures is commonly accepted as an important mechanism promoting C₄ success. Growth and competition studies commonly show that where temperatures favor C₄ photosynthesis, the overall performance of C₄ species is superior to C₃ species of similar life form (Table 2; Pearcy et al., 1981; Christie

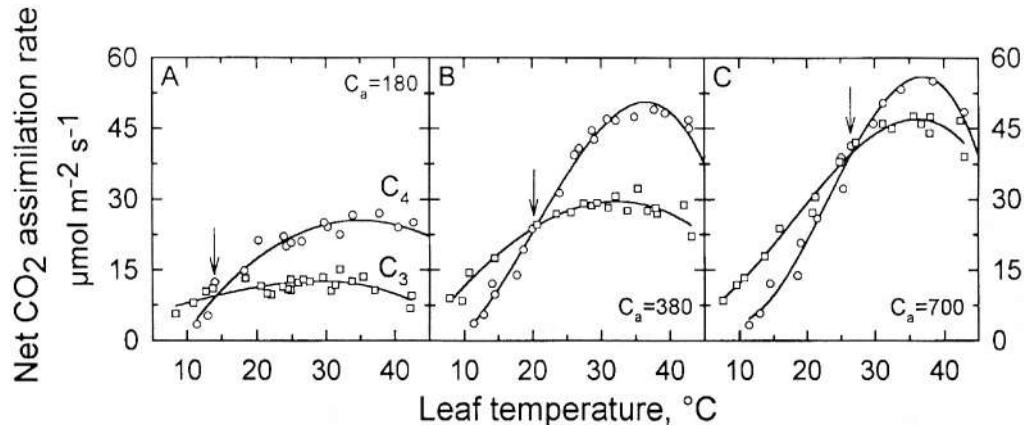


Fig. 7. Net CO_2 assimilation rate as a function of temperature in *Amaranthus retroflexus* (C_4 , circles) and *Chenopodium album* (C_3 , squares) measured at the indicated ambient CO_2 levels (in $\mu\text{mol mol}^{-1}$). Plants were grown at the current ambient CO_2 level of $360 \mu\text{mol mol}^{-1}$ and measured using a null balance gas exchange system. From M.-R. Li and R. F. Sage (unpublished). The arrows indicate the temperature where photosynthesis is equivalent between the two species. This equivalency temperature rises with CO_2 .

and Detling, 1982; Grise, 1996). In turn, superior C_3 photosynthesis at cooler temperature is well correlated with superior C_3 performance at the whole plant level (Table 2).

b. Why Do C_4 Plants Fail in Cool Climates?

The reasons for failure of all but a few C_4 species to grow in climates where growing season minimums average below $\sim 8^{\circ}\text{C}$ has not been resolved and continues to be discussed at length (for example, Long, 1999). Most C_4 species are chilling sensitive, leading to speculation that inherent properties in the C_4 syndrome may predispose C_4 plants to chilling injury (Long, 1983). For example, PPDK and PEPCase can dissociate upon exposure to low temperature, indicating the C_4 pump may be sensitive to chilling (Leegood and Edwards, 1996). Alternatively, chilling intolerance may simply reflect the tropical origin of most C_4 species, especially the economically important ones such as maize that have been heavily studied. If true, C_4 plants from high altitude and latitude may not exhibit chilling sensitivity. Research on C_4 plants from the low temperature extremes of the C_4 distribution support the latter possibility, as little evidence exists that they are damaged by low temperature any more than their C_3 neighbors. Enzymes of the C_4 pump from high latitude and altitude plants are relatively cold stable in low temperature, and the degree of photoinhibition is less in chilling conditions when compared with low latitude relatives (Simon and Hatch, 1994;

Leegood and Edwards, 1996; Matsuba et al., 1997; Pittermann, 1998; Long, 1999).

A common viewpoint is that C_4 plants perform poorly in cool climates; however, this may not reflect physiological possibilities, because if given adequate resources, high altitude C_4 species perform very well, and can even exhibit productivity in excess of local C_3 crops. Beale and Long (1997) and Kao (1997) for example, report that *Miscanthus* species grown in cool temperatures in Europe and Asia can exhibit CO_2 assimilation rates and yields exceeding those of many C_3 species of similar functional type. Similarly, one of us (RFS) observed in the alpine zone of White Mountains, California, that robust swards of the montane C_4 grass *Muhlenbergia richardsonis* occurred within 200 m of its known altitude limit in North America of 3950 m.

Given these observations, the failure of C_4 species to grow in cold environments with growth minima below about 8°C appears to reflect competitive suppression caused by C_3 species (as seen by Pearcy et al., 1981, and Christie and Detling, 1982). The dominance of the C_3 species is probably due in large part to their higher photosynthetic capacity observed in low temperature (Fig 7), and their superior quantum yields. This likely gives the C_3 plants more of an edge in the competition for other scarce resources, and thus prevent C_4 species from acquiring the resources they need to exhibit the strong performance that can be observed in cool climates when they are grown in isolation under resource rich conditions.

Table 2. Net photosynthesis per unit leaf area at light saturation, relative growth rate and shoot productivity in mixed stands in C₃ and C₄ annuals grown and measured at the indicated growth temperature regimes. From Pearcy et al. (1981).

Species	Day/night temperature °C	Net CO ₂ uptake μmol m ⁻² s ⁻¹	RGR g g ⁻¹ d ⁻¹	Yield in mixed stands ^a g dry matter
<i>Chenopodium album</i> (C ₃)	17/14	31.2	0.23	600
	25/18	37.5	0.28	250
	34/28	32.5	0.26	60
<i>Amaranthus retroflexus</i> (C ₄)	17/14	23.1	0.19	40
	25/18	38.6	0.29	470
	34/28	48.7	0.35	725

^aYield in mixed stands refers to estimated shoot mass in 50:50 mixtures of *C. album* and *A. retroflexus* plants that were 8 weeks old.

B. Light

In warm environments, the major C₃ functional groups that are successful at the expense of C₄ plants are the shrubs and trees of forests and scrubland. While much of the competition between grasses and woody species is affected by disturbance factors such as fire and browsing, modification of the light environment by the woody canopy is a primary determinant of the their ability to suppress C₄ vegetation. In C₄ plants, access to moderate to high light is, along with warm temperature, a prerequisite for the dominance of C₄ species.

1. Distribution Along Light Gradients

'Moderate to high light' is vague, but necessary, terminology because it is difficult to precisely state the minimum light requirement for C₄ success. Full sunlight to modest shade (that is, midday photon flux densities greater than about 1000 μmol photons m⁻² s⁻¹) fulfill the light requirement for C₄ success wherever C₄ plants are found. C₄ vegetation shows little reduction in species coverage or biomass contribution until shade intensities below 50% of full sunlight (Sage et al., 1999b). In the shade of trees and shrubs in tropical savannas, C₄ grasses remain abundant down to at least 30% of full sunlight values, and often show an increase in abundance in partial shade between 40% and 60% of full sun (Belsky et al., 1989; Weltzin and Coughenour, 1990). In a northern mixed grass prairie/savanna of Minnesota and Wisconsin, C₄ grasses begin to have less coverage relative to open sites only below 50% of canopy openness and they become rare below about 25% of canopy openness. (Bray, 1958; Ko and Reich, 1993;

Means, 1997). C₄ plants are almost always absent in full shade of a forest canopy, where daily light intensities are typically 10% or less than the light availability in open areas (Medina and Klinge, 1983; Pearcy, 1990).

Shade adaptation has been reported for a number of C₄ grasses. For example, the genera *Microstegium*, *Muhlenbergia*, and *Paspalum* each have numerous species that are found in deep shade (Brown, 1977; Smith and Wu, 1994; Horton and Neufeld, 1998). However, examination of the microsite descriptions of these shade-adapted species indicates that they often grow on sandy soils, or drought prone sites (Brown, 1977). Such areas often have slightly more open overstories where light intensity may be higher than 10% of full sky exposure. Several species of *Chaemasyce* (= *Euphorbia*) are native to the understory of moist Hawaiian forests. Some of these species (for example, *Chaemasyce forbesii*) have shade adaptation syndromes equivalent to co-occurring C₃ understory species (Pearcy and Calkin, 1983). The occurrence of shade adaptation in C₄ *Chaemasyce* species may reflect early invasion of the Hawaiian Islands by high-light-adapted ancestors of the current shade-adapted C₄ species. Because of the remoteness of the Hawaiian archipelago, shade-adapted C₃ species may have been rare early in the islands formation, thereby leaving open understory niches for colonization by C₄ *Chaemasyce* species. In the absence of competition from C₃ shade specialists, the C₄ invaders are hypothesized to have eventually evolved shade adaptations characteristic of most understory specialists (Robichaux and Pearcy, 1980; Pearcy and Calkin, 1983).

Little has been reported on the light requirement for ecological success of C₄ sedges and dicots. C₄

sedges probably show similar responses as grasses, given that they are often found among dense vegetation in tropical wetlands. C_4 dicots appear to have higher light requirements than grasses and sedges based on quantum yield comparisons (Ehleringer and Pearcy, 1983). With the exception of *Chaemasyce* spp, C_4 dicots are rare in understory environments.

The occurrence of shade-adapted C_4 species demonstrates that the C_4 syndrome does not preclude the growth and survival of C_4 plants in low light environments; however, the rarity of C_4 plants in forest shade indicates they are poor competitors against C_3 plants in low light environments. This poor competition is not simply a matter of grasses against woody functional types, because C_4 dicots are often woody, and some develop into robust shrubs and small trees. Moreover, hundreds of C_3 grass species are shade-adapted understory species, demonstrating that the grass functional type is able to adapt well to forest shade (Renvoize and Clayton, 1992).

What then, might explain the general absence of C_4 species in forest shade?

2. Physiological Controls

a. Photosynthetic Capacity

Early comparisons of C_3 and C_4 species commonly showed C_4 plants had substantially higher light saturated rates of photosynthesis and higher light saturation points (see Pearcy and Ehleringer, 1984 for review). However, much of the early work was based on comparisons between species of different growth form or habitat requirements; for example, temperate versus tropical crop species. When ecologically similar C_4 and C_3 plants are compared at moderate temperatures (25 to 30 °C) and current ambient CO_2 pressures, there is often little difference in light-saturated photosynthetic rates and light saturation points (Pearcy and Ehleringer, 1984). Desert annuals, weeds and annual crop species are known to have the highest photosynthetic capacities, and within each of these groups C_4 and C_3 species exhibit substantial overlap, with photosynthesis of either group often exhibiting light responsiveness at full sunlight (Pearcy and Ehleringer, 1984; Sage and Pearcy, 1987). In turn, many C_4 plants perform well in shaded environments, where light intensities may not commonly exceed 30% of full sun. Consequently, at moderate temperature, there is little obvious

difference in photosynthetic light requirements at the leaf level. At higher temperature (30 to 40 °C), however, increasing rates of photorespiration limit photosynthetic rates in C_3 species, while photosynthesis in C_4 species is stimulated. Under these conditions, C_4 species will typically exhibit higher light saturation points than C_3 species and superior photosynthetic performance at subsaturating light intensities. In turn, at cooler temperatures (<20 °C), C_3 species often have higher light requirements for photosynthetic saturation.

These differences in light response largely reflect the ability to exploit an abundance of light energy by C_4 plants. In forests and shrub understories, by contrast, carbon balance is most dependent upon low light performance, which largely reflects quantum yield, respiration, and the ability to exploit brief, high light events (sunflecks).

b. Quantum Yield and C_4 Performance in Shade

The rarity of C_4 photosynthesis in the shade has been attributed to a disadvantage in C_4 quantum yield relative to those of C_3 species (Ehleringer, 1978). In deep shade, quantum yields are important determinants of photosynthetic rate, and species with higher quantum yields should have higher photosynthetic carbon gain, all things being equal. On this parameter alone, the failure of C_4 species to occupy forest understories can be partially understood. Forest understories rarely experience thermal regimes that are highly favorable to C_4 species because understory temperatures do not commonly rise above 30 °C, even in the tropics (Pearcy and Calkin, 1983). Forest understories also experience elevated atmospheric CO_2 levels as a result of decomposition of forest detritus, which can increase the average daytime CO_2 level by 10% and with it the advantage of the C_4 quantum yield (Bazzaz and Williams, 1991). Understories are also more humid, favoring stomatal opening and relatively high intercellular CO_2 concentrations. Together, these factors contribute to potentially superior C_3 quantum yields and photosynthetic performance in the typical deep shade of the understory environment.

c. Respiratory Costs

Net carbon acquisition in low light is also influenced by respiratory costs for building and maintaining photosynthetic tissues. Species with high leaf respiration rates do poorly in shade, because it

subtracts from the carbon available for the remainder of the plant. It has been hypothesized that because C₄ photosynthesis has lower protein requirements, it may have reduced respiratory costs (Byrd et al., 1992). Byrd et al. evaluated this in a number of high light adapted C₃ and C₄ species, and found no evidence of respiration differences based on photosynthetic capacity. Respiratory costs have not been compared in shade adapted C₃ and C₄ species, although available evidence from light response curves indicate differences in dark CO₂ exchange and light compensation points are not striking (Pearcy and Calkin, 1983; Horton and Neufeld, 1998).

d. Exploitation of Sunflecks

Variation in irradiance because of cloud movement and sunflecks in leaf canopies are important controls over daily carbon gain in most field environments. In shade-adapted C₃ species, 30% to 70% of daily carbon gain is typically acquired from sunflecks (Pearcy, 1990). The ability to exploit sunflecks is an important factor determining performance in low light environments, with sun-adapted species having a reduced ability to utilize sunflecks relative to shade-adapted species (Sharkey et al., 1986). Could this explain the general absence of C₄ species from deep shade?

The basic responses to transient light differ between C₄ and C₃ species because of the absence of a photorespiratory CO₂ burst in C₄ species and, as demonstrated for maize, a burst of CO₂ evolution at the beginning of short (1–10 s) sunflecks (Krall and Pearcy, 1993). The photorespiratory burst has no net effect on sunfleck utilization in C₃ species but the CO₂ burst at the beginning of short sunflecks in maize substantially reduces the efficiency with which sunflecks are utilized by this species. The burst may be due to imbalances between metabolite pools and reducing power created during short sunflecks in the bundle sheath of NADP-ME species, such that some of CO₂ initially fixed into C₄ acids in the mesophyll cannot be refixed within the bundle sheath. For longer sunflecks, no initial burst is present, presumably because metabolite pools and energy supplies have had sufficient time to come into balance. Although there is a substantial restriction in the ability to utilize short sunflecks in maize, the overall consequence may not be that great. Short sunflecks, although present in significant numbers, contribute much less of the total PFD than longer sunflecks, and

therefore inefficiencies in their use would not be a significant limitation to carbon gain in understory microenvironments. Indeed, its significance may be somewhat greater in crop canopies where a larger fraction of the available PFD is contributed by short sunflecks. Utilization of longer sunflecks is qualitatively similar in C₄ and C₃ species (Pearcy et al., 1985); with most of the quantitative differences being related to the faster stomatal responses in C₄ grasses as compared to C₃ dicots (Fay and Knapp, 1995), or to differences in photosynthetic capacity.

Comparisons of shade-adapted understory species, such as *Chamaesyce forbesii* (C₄) and an ecologically similar C₃ tree, *Claoxylon sandwicense* led to the conclusion that C₄ photosynthesis was adaptively neutral relative to C₃ photosynthesis in the Hawaiian forest understory where they occur (Pearcy and Calkin, 1983). No advantage in utilization of sunflecks for photosynthesis or growth could be associated with the C₄ pathway in *Chamaesyce forbesii*. More recently Horton and Neufeld (1998) examined shade performance of *Microstegium vimineum*, a shade-tolerant C₄ grass that has become a noxious weed in warm-temperate zones of eastern North America. This species does well in moist, low-light habitats generally favorable to C₃ plants, where its aggressive expansion is somewhat of a surprise. *Microstegium vimineum* exhibits typical shade-adaptations often found in C₃ plants adapted to low light conditions but some important differences indicate it may be more of a gap species than true deep shade species. First, the induction state of photosynthesis declined far more rapidly in *M. vimineum* than is apparent in C₃ species from understory environments. This could lead to substantially less photosynthetic carbon gain in environments where sunflecks are separated by 2 to 5 min, as is often the case. Second, stomatal conductance tended to track light variation as is observed in gap-adapted species. While this saves water, it could lead to reduced exploitation of light flecks in rapidly changing light environments. In contrast to *M. vimineum*, understory C₃ species maintain open stomates between sunflecks, thereby avoiding transient stomatal limitations (Horton and Neufeld, 1998).

3. Synopsis

As with temperature, differences in quantum yields, respiration and sunfleck utilization do not indicate an obvious reason why C₄ species are ecologically

unfit in low light habitats. While C_4 plants are able to exploit low light, the temperature and CO_2 conditions generally do not favor greater low light performance of C_4 plants, but will often favor slightly higher C_3 quantum yields. Second, C_4 species can exploit dynamic light environments effectively, but may have a slightly reduced ability to exploit short sunflecks, and because of more rapid loss of induction state, may not be as effective exploiting intermediate sunflecks. Taken together, these slight reductions in C_4 performance relative to C_3 may be enough to shift the competitive balance in favor of C_3 species except where other factors such as poor soil fertility or drought may offset any C_3 advantage.

The exclusion of C_4 species from forests has great ecological significance, because where woody C_3 species can become established, they will, with sufficient time, become dominant. To some degree, the ability of woody species to establish and persist in grasslands uncouples ecological success from photosynthetic pathway. Establishment is not solely a consequence of photosynthetic potential, but is often more a function of stress tolerance and life history characteristics. In turn, persistence depends on the disturbance regime of the landscape. Ecological disturbances that select against woody plants, namely fire, severe drought, large animal herbivory, wind, floods, and human activity are critical modifiers of woodland distribution and are often a prerequisite for C_4 success (Sage et al., 1999b). This understanding has often led ecologists to ignore photosynthetic pathway as an important control over grass/woodland dynamics. It is important, however, to realize that ecological factors that affect woodland success often act by modifying light competition between interacting C_3 and C_4 vegetation.

IV. Secondary Environmental Controls— Water Supply, Salinity, Nitrogen

In addition to growth season warmth and moderate to high light requirements, the success of C_4 species is influenced by water availability, salinity and soil nitrogen (N) supply. Here, we discuss the physiological basis for the different responses of C_3 and C_4 species to water supply, salinity, and drought, and then examine how these differences modify the geographical patterns established by temperature and light.

A. Water Availability

1. Physiological Considerations

Differences in performance along gradients of water availability can arise if species have inherently different tolerances of extremes of water supply, or have different efficiencies of water use. C_3 and C_4 plants have no inherent differences in their tolerance of drought or flooding (Pearcy and Ehleringer, 1984). In response to drought, they show similar levels of physiological impairment at similar water potentials or relative water contents as C_3 species of similar life form. There are, however, consistent differences between C_3 and C_4 species in the efficiency with which water is used that result from the ability of the C_4 cycle to concentrate CO_2 . These differences promote C_4 over C_3 vegetation in specific situations.

Instantaneous water use efficiency is expressed as the ratio of photosynthesis to transpiration. Assuming all things are equal, the higher water use efficiency in C_4 plants occurs because the relative stomatal aperture (indexed by stomatal conductance) is regulated at a lower value in C_4 than C_3 species having the same CO_2 assimilation capacity. This is possible because the CO_2 concentration system allows C_4 plants to pump enough CO_2 into the bundle sheath to saturate Rubisco, even at intercellular CO_2 levels in the mesophyll tissue as low as $100 \mu\text{mol mol}^{-1}$. On average, C_4 plants will have stomatal conductance values that are 50% to 70% less than C_3 species with identical CO_2 assimilation rates (Schulze and Hall, 1982; Sage and Pearcy, 1987). Assuming equivalent leaf temperatures, this will translate into transpiration rates that are 50% to 70% less in the C_4 than the C_3 , and WUE values that are two to three times higher. However, because of the lower level of evaporative cooling, C_4 leaf temperatures are on average higher, increasing transpiration and reducing the WUE differences between C_4 and C_3 plants in field situations.

High water savings can also be achieved through stomatal closure in C_3 plants but this comes at substantial reductions in photosynthetic capacity. Except at low temperature ($<15^\circ\text{C}$), C_3 photosynthesis decreases proportionally with reduction in stomatal conductance, reflecting the reduction in CO_2 supply to the chloroplast (Björkman, 1976; Farquhar and Sharkey, 1982). C_3 species that attempt to save water through stomatal closure lose much of their potential for carbon gain and experience high

levels of photorespiration, which reduces nutrient and light use efficiency. Thus, in environments where water is limiting, C₃ plants that are conservative in their water use may compete with C₄ plants that have similar water demands, but twice the CO₂ acquisition potential and therefore a much greater growth capacity. This greater growth potential may enable the C₄ to produce roots and leaves faster, thereby capturing the remaining water, nutrient, and light resources in the environment.

Differences in WUE between C₃ and C₄ species are most significant in warmer climates because the transpiration demand rises exponentially with increasing temperature. In warm-climate C₃ plants, stomatal closure for water conservation combines with thermal enhancement of photorespiration to give a synergistic reduction in CO₂ assimilation capacity. In C₄ plants, rising temperatures stimulate PEPCase and the other enzymes of the C₄ pump so that the ability to supply Rubisco with high levels of CO₂ is increased, even at the lower intercellular CO₂ levels that may result if stomatal aperture was reduced. As a consequence, rising temperature can stimulate photosynthesis in C₄ plants at low intercellular CO₂, while in C₃ species, increasing temperature above 30 °C reduces the rate of net CO₂ assimilation at low intercellular CO₂ levels (Fig. 8; Björkman, 1976; Björkman et al., 1980). Correspondingly, WUE in C₄ species can rise with increasing temperature above 30 °C, while in C₃ plants it will almost always decline.

2. Geographical Distribution

Within a few years of the discovery of the C₄ syndrome, the large water use efficiency differences between C₃ and C₄ plants were noted, as was a pattern where C₄ species often predominated in hot, dry environments (Black, 1973). The associations between an apparent drought adaptation (higher WUE) and occurrence in arid sites was instrumental in the development of the textbook paradigm that C₄ photosynthesis is an adaptation for aridity (for example, see Raven et al., 1992). This view has some merit, in that greater water use efficiency can improve C₄ performance relative to C₃ in arid environments, possibly enabling C₄ species to occur in climates where temperature and light might otherwise favor C₃ species. For example, at the cold end of their distribution range, C₄ grasses are commonly noted to occur on dry sites, or sandy soils with reduced water-holding capacity. In central Asia and in Canada, for

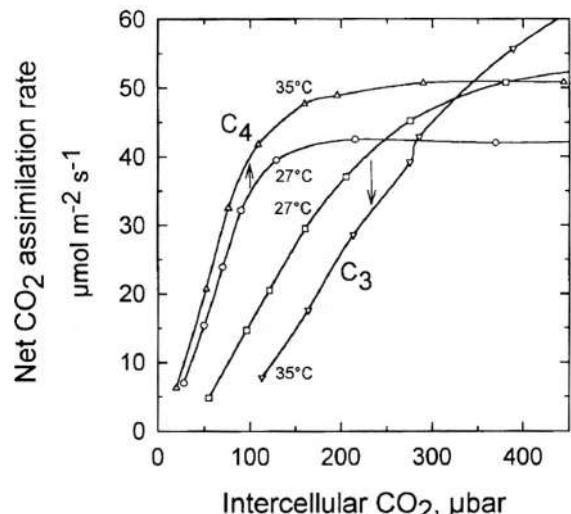


Fig. 8. The response of net CO₂ assimilation in *Amaranthus retroflexus* (C₄) and *Chenopodium album* (C₃) to intercellular CO₂ partial pressure at 27 °C and 35 °C. Arrows show that at typical CO₂ levels corresponding to ambient CO₂ levels of 360 μbar, a rise in temperature from 27 to 35° increases photosynthesis in the C₄ plant, but reduces photosynthesis in the C₃ plant.

example, the native C₄ species occurring north of 50° largely occur in arid sites (or saline soils, which will be discussed below) (Schwarz and Redman, 1988; Pyankov and Mokronosov, 1993). C₄ species occurring above 3500 m in Asia, North America and the Peruvian Andes are also reported to occur on xeric soils (Sage et al., 1999b). On Mount Kenya, Young and Young (1983) show that in the zone of approximate equivalent frequency, C₄ grasses occurred on significantly drier sites than C₃.

In each of these instances, however, the direct role of aridity versus other microclimate factors has to be resolved. For example, increasing aridity brings about a gradual loss of canopy cover, thus opening the canopy and enabling more light penetration to lower leaves, and greater solar heating of the canopy.

The adaptive advantage of higher water use efficiency is probably greatest in hot arid zones where the combination of high temperature and low rainfall can preclude sufficient net primary productivity by most C₃ species (Schulze et al., 1996). Consistently, in the hot deserts of the world (in Somalia, Namibia, the Punjab of India, Northern Australia, and southwestern North America) grass and sedge floras are over 90% C₄, and large numbers of C₄ dicots are found (Sage et al., 1999b). Similarly, C₄ floristic representation is very high on arid tropical

islands. For example, on the Galapagos Islands, the grass flora is over 90% C_4 and C_4 dicots are common as well (Wiggins and Porter, 1971). In addition, in warm temperate deserts, summer active herbaceous species are primarily C_4 , in contrast to winter active species that are almost all C_3 (Shreve and Wiggins, 1964; Smith and Nobel, 1986). The high proportion of C_4 herbs in these deserts may result in part from a failure of C_3 herbs to establish under the harsh conditions that often include soil temperatures above 50 °C and relative humidity below 10%. Whereas the high WUE of C_4 species may allow for substantial carbon gain under the very high evaporative demands of these regions, the low WUE of C_3 species may prevent positive carbon gain except at dangerously high rates of transpiration (Schulze et al., 1996). These differences in WUE may be most significant during the seedling stage, when plants live in the hot boundary layer of the soil yet do not have the root volume required to supply the necessary amounts of water for sustained C_3 photosynthesis.

While enhanced water use efficiency has apparent value in specific instances, it will not guarantee C_4 success in dry environments. Similarly, arid conditions are not required for C_4 success. Cold, dry environments such as polar or alpine deserts lack a C_4 flora, and cold deserts of western North America and Northern Mongolia have few C_4 grasses (Sage et al., 1999b). In Mediterranean type climates of Southern Europe, North Africa, South Africa, Chile, California and Southwest Australia, C_4 plants are uncommon except as summer active weeds where irrigation provides soil moisture, or in marshes. Mediterranean zones are characterized as having wet winters and dry summers. Although summer days are commonly hot, the lack of summer precipitation prevents the establishment of a native C_4 flora in most Mediterranean-type habitats. Mediterranean zones now have a rich exotic C_4 flora, however, due largely to agricultural irrigation which provides the necessary summer moisture.

In contrast to Mediterranean zones, monsoon regions often have relatively dry winters and wet summers. In these regions, C_4 plants predominate so long as there is not an established canopy of woody C_3 species. Thus, monsoon grasslands and grass areas of savannas are heavily dominated by C_4 graminoids (Solbrig, 1996; Sage et al., 1999b).

The role of summer precipitation can be seen in a comparison of climate diagrams from southern South Africa (Fig. 9). The climate around Capetown is Mediterranean, and the grass flora is >90% C_3 ,

Durban, South Africa occurs at a similar latitude but in a monsoon zone where 70% of the annual precipitation occurs during the warm season and >75% of the regional grass flora is C_4 (Fig. 9; Vogel et al., 1978; Ellis et al., 1980). In South Africa, the best predictor for C_4 occurrence is not total precipitation but the proportion of precipitation that falls during the warm season. Where the warm season precipitation is proportionally high, C_4 grasses dominate the grass flora (Vogel et al., 1978). Similarly, in the Great Plains of North America, regional dominance by C_4 grasses rises with increasing summer precipitation (Epstein et al., 1997).

Stowe and Teeri (1978) reported high correlation between measures of aridity and floristic occurrence of C_4 dicots. This work has been cited frequently in support of the hypothesis that C_4 photosynthesis is an adaptation to aridity. The high correlation Stowe and Teeri report, however, may be an anomaly arising from changes in life form in different communities. For example, many of the C_4 species in Arizona are summer-active annuals that germinate in response to summer rains, and are not necessarily drought-adapted because they complete their life cycles before experiencing severe moisture deficiency.

Numerous studies of local distributions of arid zone species show little relationship between photosynthetic pathway and occurrence along gradients of moisture availability. In Arizona, the C_3 grass *Stipa neomexicana* occupied dry ridge-top sites whereas the lower topographic positions with more soil moisture were occupied by C_4 species (Gurevitch, 1986). Along local topographic gradients in the Nebraska Sand Hills, C_3 species are most abundant on swales between sandy ridges (Barnes and Harrison, 1982). Soils here are finer than on the ridges, and hold significantly more moisture in the early spring to the benefit of the C_3 grasses. By early-to-mid summer, this moisture becomes exhausted, to the detriment of the C_4 species. C_4 species are more abundant on the sandy ridge-tops, where moisture remains available during the summer. While soil properties rather than photosynthetic performance may be the more immediate cause of the ecological success on these soils, these patterns ultimately arise from physiological controls that reflect interactions between the timing of water availability, differences in phenology and photosynthetic performance of C_3 and C_4 species.

A clear example that aridity is not a prerequisite for C_4 success is the dominance of flooded savannas, riverbanks and lake margins in tropical regions by C_4

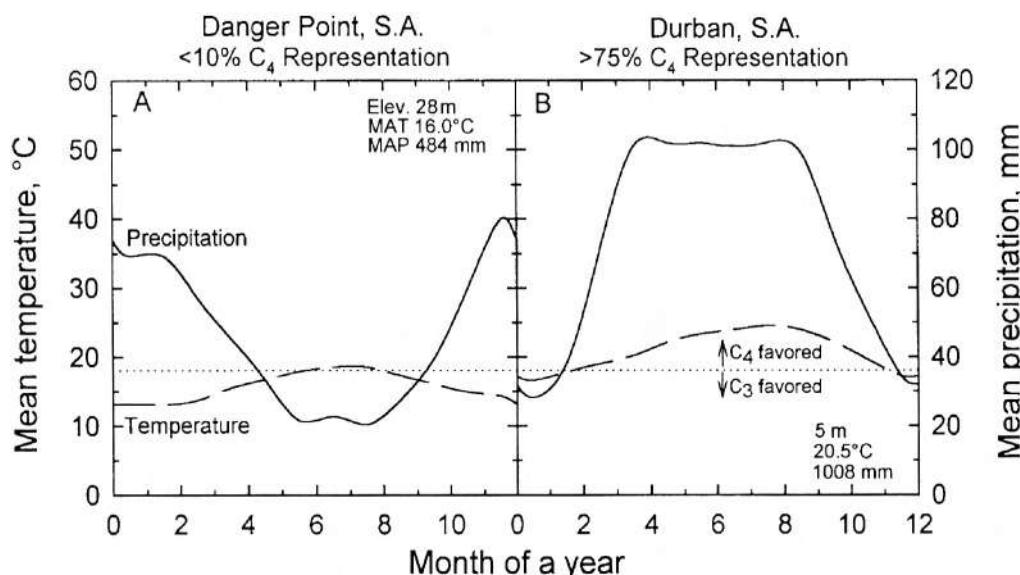


Fig. 9. Walter-type climate diagrams for A) the Capetown-Danger point region of South Africa, which has a Mediterranean climate and low C₄ representation; and B) the Durban, South Africa region which is monsoonal with high C₄ representation. In Walter-type diagrams where the curve for mean monthly temperature is higher than the curve of mean monthly precipitation, a water deficit is indicated. The dotted lines indicate the approximate temperature where conditions favor equal C₃ and C₄ biomass. MAT is mean annual temperature. MAP is mean annual precipitation (after Walter et al., 1975 and Vogel et al., 1978).

grasses and sedges. Along floodplains of the Amazon, the C₄ grass *Echinochloa polystachya* forms dominant stands that establish on exposed riverbanks when the river is low, and form monocultures that grow as fast as the river rises, eventually forming dense mats of river vegetation (Junk, 1983; Piedade et al., 1994). Similarly, in Africa, the C₄ sedge *Cyperus papyrus* (papyrus), forms dense mats along river and lake shallows, where drought is rarely a factor (Jones, 1986). There is obviously no need to conserve water in these flooded settings. Instead, the ability of these C₄ species to attain high photosynthetic and productivity rates under warm to hot conditions is more critical. An important feature of plants in flooded locations is the ability to become established during low water, and then outgrow the rising waters following the start of the wet season. Only through C₄ photosynthesis does the ability to exhibit the necessarily high growth rates appear to be realized (Long, 1999).

3. The Importance of Ecological Disturbance in Arid Climates

The association of C₄ species with aridity also arises because of a number of indirect factors related to disturbance events such as fire and grazing. Aridity promotes fire that suppresses C₃ woody competitors,

therefore favoring C₄ grasses. Also, the slower growth of woody vegetation in arid zones prolongs the period where it is vulnerable to disturbance events (Goldammer, 1993; Bond and van Wilgen, 1996). If disturbance is prevented, for example, by reducing populations of large herbivores such as elephants and giraffes, or suppressing fire, then many, if not most, C₄-dominated ecosystems will shift to C₃ dominated shrublands or forest. Exceptions to this pattern occur in saline areas, seasonally flooded soils, hot deserts, and possibly soils with very low fertility, where fire frequencies are already low.

B. Salinity

Plant communities of saline habitats are known to have a relatively high proportion of C₄ species. Large areas of temperate coastal salt marshes are often dominated by C₄ grasses in *Spartina*, *Distichlis*, *Sporobolus* and *Zoysia* (Archibold, 1995) while saline and alkaline soils in deserts are rich with C₄ members of the family Chenopodiaceae (Walter and Box, 1983a,b). In salt marshes, C₄ species are able to maintain dominance into much cooler climatic settings than is the case on adjoining non-saline soils (Long, 1983; Guy et al., 1986). In the cold deserts of North America, lowland saline soils dominated by the C₄ species *Atriplex confertifolia* and *Distichlis*

spicata give way to well-drained alluvial soils dominated by C₃ species such as *Artemesia tridentata* (West, 1983). C₄ halophytes are common as high as 50°N in Atlantic coastal marshes of eastern Canada and the British Isles, yet immediately inland at latitudes above 45°N, C₄ species are rare (Sage et al., 1999b).

Despite this common occurrence of C₄ plants in saline conditions, the functional reasons for this association, if any, have yet to be confirmed. C₄ plants have an obligate requirement for trace amounts of Na⁺ (Brownell and Crossland, 1972) but the concentration needed to meet this requirement is present in nearly all terrestrial habitats. Halophytes survive in saline habitats mostly because of a) selective exclusion of salts at the roots, b) partitioning of salt that does enter the transpiration stream into storage vacuoles, salt glands or salt bladders, and c) because they are able to osmotically adjust to the low water potentials (Flowers et al., 1977; Larcher, 1995). C₄ photosynthesis plays little direct role in these processes. Consistently, the most salt tolerant plants along salinity gradients are succulent, C₃ halophytes such as *Sarcobatus* and *Salicornia* spp. In saltmarshes where C₄ grasses often form pure stands, C₄ photosynthesis is most important in zones of intermediate salinity (MacDonald, 1977; Pomeroy and Wiegert, 1983). Here, the success of C₄ species has been hypothesized to result from the inherently greater WUE, which would reduce the water flux through the plant per unit of growth and therefore reduce the amount of salt that must be screened, extruded, stored, or secreted (Osmond et al., 1982; Adam, 1990). For C₄ species, this possibility has not yet been experimentally confirmed. Evidence from comparative studies of C₃ and C₄ salt-marsh halophytes show slightly greater performance of photosynthesis in C₄ *Spartina* species at intermediate salinities than C₃ *Scirpus* spp which often occurs nearby (Pearcy and Ustin, 1984); others show no difference (DeJong et al., 1982).

Ironically, the best evidence that high WUE is important to success in saline areas comes from mangroves, which are all woody and all C₃. In tropical regions, C₄ grasses, sedges and dicots are well represented in saline areas, and tend to dominate early succession stages in coastal salt marshes (MacDonald, 1977; Costa and Davy, 1992). Eventually mangroves invade and convert the salt marsh into a mangrove swamp. As with inland forests, arborescence confers ecological advantage to the C₃

species. Salt tolerance in mangroves has been extensively studied (Ball, 1988), and provides a model to explain why C₄ plants are successful in saline areas. In mangroves, water use efficiency is very high for C₃ species because of conservative stomatal behavior and leaf orientations that minimize heat loading. Stomata show pronounced midday closure, and will close under high evaporative demand and during high transient salinity (Ball and Farquhar, 1984; Ball et al., 1988). Only during cooler periods of greater relative humidity, when evaporative demand is lowest, will stomata reach maximum apertures. By minimizing transpiration in this manner, salt uptake via the transpiration stream is minimized. If the plants then grow fast enough so that the salt that is accumulated is sequestered into new storage tissues and vacuoles, accumulation of salt to lethal levels can be prevented. Similarly, in C₄ plants, the inherently high water use efficiency should allow for relatively low salt loading at the roots, while the high photosynthetic capacity at low CO₂ should allow for high productivity (Adam, 1990).

For unknown reasons, mangroves fail to colonize temperate zones (MacDonald, 1977). Perhaps, low temperature impairs their salt exclusion mechanisms. In any case, where mangroves cannot grow, C₄ plants dominate salt marshes up to latitudes where the growing season is too cold, at which point C₃ graminoids such as *Puccinellia* dominate the marshes (Archibald, 1995). This gives rise to the phenomenon where C₃ mangroves dominate coastal estuaries in tropical and subtropical zones, C₄ graminoids dominate estuaries in temperature zones where mid-winter cold excludes the mangroves, and C₃ graminoids dominate the estuaries where mid-summer cold excludes the C₄ species. Identifying the mechanisms governing this biogeographical pattern will be one of the important contributions to ecophysiology in the next century.

C. Mineral Nutrition

1. Physiology

An important advantage of the C₄ relative to the C₃ pathway is that it is less expensive to terms of nitrogen and other mineral resources invested in the photosynthetic machinery (Brown, 1977). The more efficient use of N in C₄ than C₃ photosynthesis has three primary causes. First, the maintenance of high CO₂ in the bundle sheath means that C₄ Rubisco

operates near its V_{max}, in contrast to C₃ plants where the enzyme operates below the K_m. Thus, for a given photosynthetic rate above 20 °C, C₄ species require only 20% to 40% of the Rubisco that is required by C₃ species. Second, the suppression of photorespiration in C₄ plants leads to a further increase in the efficiency of Rubisco use. Third, the form of Rubisco expressed by C₄ species has on average, a 20% to 30% higher turnover capacity, so that less of the enzyme is required to support a given photosynthetic rate (Seemann et al., 1984; Sage and Seemann, 1992). In agreement with the theoretical predictions, C₃ species have three to four times as much Rubisco on average as C₄ species with equivalent assimilation rates (Table 3; Sage et al., 1987; Sage and Seemann, 1993). Because Rubisco is large (550 kilodaltons), and relatively inefficient (with a maximum specific activity that is 10% of PEPCase), the enzyme is an expensive sink in terms of nitrogen. C₄ species maintain 5% to 10% of their total leaf nitrogen in Rubisco, while C₃ species maintain 15% (shade plants) to over 30% (crops) of leaf nitrogen in Rubisco (Sage et al., 1987; Evans, 1989). C₄ pump enzymes represent a N cost to C₄ photosynthesis not shared by C₃ species but this is relatively low and is offset somewhat by the N cost associated with the photorespiratory pathway in C₃ species. For example, in *Amaranthus retroflexus* (C₄), PEPCase contains about 5% of total leaf N, which combined with

Rubisco accounts for a total of 14% of the leaf N in this species (Table 3). By contrast, in *Chenopodium album* (C₃), about 1.5 times N is invested in carboxylating enzymes at equivalent leaf N levels. At equivalent CO₂ assimilation rates, the N cost of carboxylating enzymes rises to be over 2.5 times as much N in the C₃ species than the C₄.

Nitrogen use efficiency (NUE) can be expressed in a variety of ways such as the biomass produced per unit of nitrogen, harvest yield per unit nitrogen input, instantaneous photosynthetic rate per unit leaf nitrogen, and the slope of the relationship between photosynthetic capacity and leaf nitrogen content. The slope of net CO₂ assimilation versus leaf nitrogen content is one of the more robust expressions of C₃ and C₄ differences in NUE because it most closely reflects the consequence of the differences between C₃ and C₄ photosynthetic physiology. At moderate temperature (20 to 27°), the A/N slope is 50% greater in C₄ than C₃ species, but it is over 100% greater at warmer temperature (34 °C) (Table 4). Differences in NUE are most pronounced at high N levels, but at high N, C₃ species often allocate more nitrogen to leaves, thereby compensating for the greater NUE of the C₄ species. For example, *Chenopodium album* (C₃) plants grown at high N had 30% more N per unit leaf area than *Amaranthus retroflexus* (C₄) plants grown at the same N levels (Sage and Pearcy, 1987a). Similarly, Li (1993) observed that the maximum leaf

Table 3. The content of carboxylating enzymes in the C₃ plant *Chenopodium album* and *Amaranthus retroflexus* (C₄), expressed on an absolute basis, as a ratio between the species, and as a percent of total leaf nitrogen (in parentheses). Leaf nitrogen (N) contents were selected to represent ratios at equivalent leaf N, and at equivalent CO₂ assimilation capacities. Data were estimated from regression equations of net CO₂ assimilation versus leaf N, Rubisco content versus leaf N, and total carboxylase content versus leaf N. From Sage et al. (1987).

	Leaf Nitrogen Content ^a , mmol m ⁻²		
	160	215	215/160
C ₃ rate of CO ₂ uptake ^b	29	45	—
C ₄ rate of CO ₂ uptake	47	—	—
C ₃ Rubisco content	3.3 (24%)	4.9 (26%)	—
C ₄ Rubisco content	1.3 (9%)	—	—
C ₃ /C ₄ Rubisco Ratio	2.4	—	3.7
C ₃ Carboxylase content ^c	3.3 (24%)	4.9 (26%)	—
C ₄ Carboxylase content	1.9 (14%)	—	—
C ₃ /C ₄ Carboxylase ratio	1.5	—	2.4

^a *A. retroflexus* did not exhibit leaf N levels above 170 mmol m⁻².

^b Units: Net CO₂ uptake rate in $\mu\text{mol m}^{-2} \text{s}^{-1}$; Rubisco and total carboxylase content in g m⁻².

^c Carboxylase content refers to Rubisco + PEPCase. In *C. album*, PEPCase content was insignificant compared to Rubisco content.

Table 4. Photosynthetic Nitrogen Use Efficiencies (PNUE) of Herbaceous Plant Species. PNUE is expressed as the slope of net CO₂ assimilation versus leaf nitrogen where both parameters are expressed on an area basis.

Species	PNUE μmol CO ₂ mol ⁻¹ N	Reference
C₄ Plants		
<i>Cyperus papyrus</i>	280	Li, 1993
<i>Cyperus longus</i>	391	Li, 1993
<i>Cyperus japonicus</i>	380	Li, 1993
<i>Echinochloa crusgalli</i>	580 @ 34 °C	R. F. Sage unpublished
<i>Miscanthus sinensis</i>	247	Li, 1993
<i>Spartina cynosuroides</i>	377	M. B. Jones (in Li, 1993)
<i>Amaranthus retroflexus</i>	350 @ 20 °C 420 @ 27 °C 520 @ 34 °C	Sage and Pearcy, 1987b
C₃ Plants		
<i>Cyperus alternifolium</i>	252	Li, 1993
<i>Cyperus involucratus</i>	256	Li, 1993
<i>Cyperus vegetus</i>	180	Li, 1993
<i>Typha domingensis</i>	53	M. B. Jones (in Li, 1993)
<i>Oryza sativa</i>	200 to 250 @ 25 °C	Makino et al., 1994; Makino et al., 1997
<i>Solidago altissima</i>	140	Hirose and Werger, 1987
<i>Chenopodium album</i>	230 @ 20 °C 280 @ 27 °C 240 @ 34 °C	Sage and Pearcy, 1987b

N of two C₄ *Cyperus* species was 60 to 80 mmol m⁻², while that of two C₃ *Cyperus* spp was about 30 mmol m⁻² higher.

2. Ecological Aspects

The high photosynthetic nitrogen use efficiency of C₄ photosynthesis could be expected to provide a significant advantage under nitrogen limitation. To effectively evaluate this hypothesis, plants adapted to some level of nutrient deficiency should be compared. In plants requiring high levels of N, such as weeds and crops, photosynthetic NUE is poorly correlated with performance (Sage and Pearcy, 1997a; Long, 1999). This is not surprising, given that on N enriched soils, high capacities for leaf production and growth are more closely related to performance, while on nutrient poor soils, storage, nutrient retention, and conservative growth patterns appear to be more fit (Chapin, 1980). A higher NUE may have significance on high N soils if it enables C₄ species to allocate more N to leaf area and root production, thereby giving C₄ plants better access to other resources essential to growth and competitive

ability. Some circumstantial evidence supports this possibility (Sage and Pearcy, 1987a; Long, 1999).

In contrast to crops and their weeds, soils of the mixed-grass prairies of central North America are commonly N deficient. The C₃ grasses (*Poapratensis*, *Agrostis scabra*, *Agropyron repens*) and C₄ grasses (*Schizachyrium scoparium*, *Andropogon gerardii*) of these soils show responses to soil N that directly reflect differences in NUE (Wedin, 1995; Wedin and Tilman, 1993, 1996). In this ecosystem, C₄ species are often dominant, particularly in late successional stages and on unfertilized soils (Wedin and Tilman, 1990). In a series of experiments designed to evaluate effects of N addition, Wedin and Tilman found that nitrogen use efficiency (plant biomass per N use) of the C₄ grasses was approximately double that of the C₃, and on plots receiving no N fertilizer, C₄ biomass was 20% to 60% of total biomass at midsummer (Wedin and Tilman, 1996). At N fertilization rates above 10 gN m⁻² year⁻¹, the C₃ grasses completely crowded out the C₄ species (Wedin and Tilman, 1992, 1996). The mechanisms underlying this competitive shift are complex, and show some of the intricacies of how physiological differences give rise

to ecological mechanisms (Wedin, 1995; Wedin and Tilman, 1992, 1996). In one of the C₄ species, *Schizachyrium scoparium*, high photosynthetic production potential enabled it to maintain a higher root fraction than C₃ competitors. In the absence of fertilization, this enabled the C₄ grasses to reduce available soil N to levels too low for growth of the C₃ plants. Second, the higher photosynthetic NUE enabled the C₄ species to maintain high C:N ratios in leaves, which reduced the forage quality of the C₄ grasses and thus, the probability of herbivory. In addition, high C:N in leaves gave rise to litter with high C:N, and this decomposed more slowly such that N cycling also slowed (Wedin, 1995). This further contributed to low soil N availability. Fertilization disrupted these controls, and by supporting rapid growth of the C₃ species, produced a dense C₃ canopy in early spring that was able to shade, and therefore suppress, the C₄ biomass during the summer (Wedin, 1995; Sage et al., 1999b).

V.The Functional Role of Photosynthetic Subtype

An important advance in the 1980's was the development of anatomical screen that segregated most (>80%) of the world's grass genera to either the malate-forming NADP-ME subtype, or the aspartate forming NAD-ME and PCK subtypes (Hattersley, 1983; Hattersley and Watson, 1992). Further refinements in recent years have enabled segregation of many NAD-ME and PCK species into their respective sub-types (Watson and Dallwitz, 1998). As a result of these advances, most grasses have now been classified as C₃ or into one of the C₄ sub-types (Sage et al., 1999a).

Significant correlation is evident between C₄ biochemical subtype and aridity despite there being no correlation between total C₄ representation and aridity. At mesic ends of aridity gradients, NADP-ME species dominate the C₄ grass flora; at xeric ends, NAD-ME species predominate (Fig. 10). These patterns have been well described for grasses from Australia (Prendergast, 1989; Henderson et al., 1994), Namibia (Schulze et al., 1996), South Africa (Ellis et al., 1980), central Asia (Pyankov and Mokronosov, 1993), and South America (Knapp and Medina, 1999). Our examination of regional North American floras indicates the correlation between photosynthetic subtype and precipitation is robust here as well.

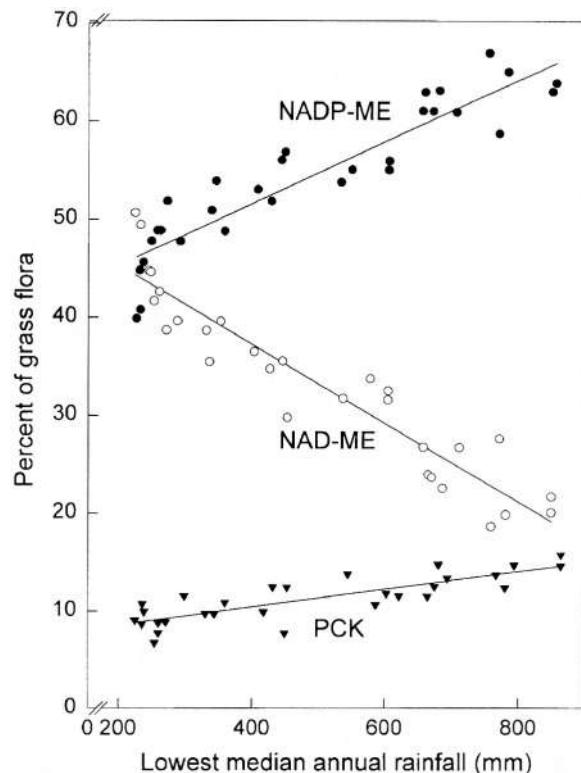


Fig. 10. Relationships between the percent representation of C₄ subtypes in regional C₄ grass floras in Australia, and the lowest median annual rainfall in the regions. (Adapted from Hattersley, 1992.)

Interestingly, Pyankov and Mokronosov (1993) report a similar observation between salinity and subtype, with NAD-ME species being common on high salinity soils, while NADP-ME species are floristically much less common.

No correlation between subtype and aridity or salinity has been observed among sedges and dicots. In sedges, the NAD-ME subtype is present only in *Eleocharis*, which contains about 200 of the estimated 1500 C₄ sedge species; all other sedges are NADP-ME (Sage et al.; 1999a). In dicots, the taxonomic distribution of photosynthetic subtypes is too poorly known to allow meaningful assessments; however, most C₄ dicots occur on arid or saline habitats, so it is likely that no pattern will arise.

These correlations indicate there is some advantage of the NAD-ME as compared to the NADP-ME decarboxylation mechanism under low rainfall conditions, but currently there is little known about the underlying mechanism (Hattersley, 1992). The advantage of the NADP-ME subtype under wetter

conditions might be related to quantum yield advantage. Higher quantum yields could be important in the dense canopies found where water supplies are greater. However, it must also be kept in mind that the patterns may not at all be mechanistically related to photosynthetic subtype per se. In Australia, the percent Andropogonae in the grass flora of different regions is also correlated with precipitation and these species are solidly of the NADP-ME subtype (Hattersley, 1992). This raises the possibility that other characteristics of the Andropogonae are involved. Likewise, characteristics of other taxonomic groups cannot be ruled out.

VI. C_4 Photosynthesis in the Future

A. Cursory Observations

Currently, human activity is restoring atmospheric CO_2 contents to levels not seen for millions of years. Theoretically, this will remove the selective pressure that may have supported C_4 evolution and radiation, leading to the possibility that C_3 vegetation will expand at the expense of C_4 vegetation, in some cases causing extinction of C_4 species. In recent years, extensive discussion and increasingly sophisticated experiments have focused on this issue. Rather than summarize the details of this work (readers are referred to Patterson and Flint, 1990; Henderson et al., 1994; Polley, 1997; Sage et al., 1999b for more detailed discussions), we highlight some key features that have arisen from discussions of C_4 and C_3 responses to future global change:

1. High CO_2 stimulates C_3 photosynthesis more than C_4 in the short term, but the ability to exploit CO_2 enrichment depends upon long-term enhancement of photosynthesis (Kirschbaum et al., 1995; Bowes, 1996). In C_3 plants, maintenance of photosynthetic stimulation by high CO_2 depends upon sustained availability of nutrients. Experiments conducted on soils with high nutrient availability show increased C_3 biomass at the expense of C_4 biomass in high CO_2 conditions (Patterson and Flint, 1990; Drake et al., 1996). With soil nutrient deficiency, a series of feedbacks at the leaf, whole plant and soil level become apparent that reduce long-term photosynthetic and growth responses of the C_3 species (Bazzaz, 1990; Stitt, 1991; Diaz et al., 1993). High CO_2 may also

aggravate soil nutrient deficiency, because it promotes higher levels of soil carbon, and creates high C:N litter that decomposes more slowly (Diaz et al., 1993; Ball, 1997). Both effects lead to sequestration of nutrients into less mobile pools and a slowing of nutrient cycling. Because many C_4 grasses are favored by N deficiency (Wedin and Tilman, 1996), such effects of high CO_2 on soil fertility could promote C_4 species at the expense of C_3 .

Alternatively, nitrogen eutrophication of landscapes by sewage, agricultural fertilizers, and industrial pollutants promote C_3 dominance by reducing the consequences of NUE advantages of C_4 species, and by increasing the ability of C_3 species to exploit CO_2 enrichment. Moreover, high CO_2 coupled with nitrogen (N) deposition will likely favor more rapid establishment of woody species, thereby accelerating succession from grasslands to forests (Sage et al., 1999b). Human production of N fertilizers has increased greatly in recent decades to the point where anthropogenic N fixation is approximately equal to natural N fixation (Vitousek, 1994). Much of this anthropogenic N is deposited onto terrestrial ecosystems. Because human demands for reduced N are increasing, it is certain that predictions of future C_3/C_4 vegetation dynamics will need to account for N enrichment effects.

2. High CO_2 reduces stomatal conductance in both C_4 and C_3 species, apparently by similar magnitudes (Sage, 1994; Polley, 1997). This enhances water use efficiency and leads to reductions in water consumption, delaying the onset of water stress and reducing its severity (Owensby et al., 1996, 1997). Although both C_4 and C_3 species potentially benefit from such responses, in natural ecosystems the C_4 species are more likely to benefit. The growing season of C_4 plants occurs when evaporative demand is greatest, and thus potential reductions in transpiration caused by stomatal closure are potentially more significant. Consistent with this hypothesis, exposure of North American grassland communities to double the current atmospheric CO_2 favored the C_4 grasses, largely as a result of a longer growing season in dry years and reduced inhibition from water stress (Owensby et al., 1996, 1997).

3. C₃ species often show substantial acclimation to high CO₂ that appears to be driven by carbohydrate status of the plants (Sage, 1994; Bowes, 1996). This can reduce the N cost of photosynthesis and may lead to enhanced NUE and performance in high CO₂ conditions (Sage et al., 1997). In C₄ species, acclimation to elevated CO₂ could also lead to a lower N cost of photosynthesis and increases in NUE. In high CO₂, C₄ photosynthesis operates well above the CO₂ saturation point, indicating PEPCase is nonlimiting (Sage, 1994). Theoretically, acclimation could reduce PEPCase without affecting A, and this would be observed as a reduction in the initial slope of the photosynthetic CO₂ response and a rise in the CO₂ saturation point. In reality, this does not appear to be the case, because C₄ plants grown and measured at high CO₂ show no qualitative shifts in their photosynthetic CO₂ responses (Wong, 1979; Sage, 1994). Some quantitative changes have been noted, in that modest reductions in A can occur following long term exposure to high CO₂ (Wong, 1979; Morgan et al., 1994).

4. Global warming may be expected to favor C₄ plants, given their greater response to high temperature. More important than the degree of warming, however, is likely going to be the seasonal timing of the warming. Models of climate change predict that wintertime and high latitudes will experience the greatest level of warming (Kattenberg et al., 1996). These periods are typically the time when C₃ species are active (Monson et al., 1993), so that where winters and early-spring may become milder, the 'cool' growing season favorable to C₃ plants may improve. Some evidence for this is present in recent data from the plains grasslands of south-central Canada where the C₃ grasses have expanded in recent decades, apparently because of milder winter temperatures and longer spring growing seasons (Peat, 1997; Skinner et al., 1998).

Similarly, the response of species to changing temperature and rising CO₂ will also depend on when precipitation falls, with the most dramatic shifts likely found at temperate latitudes where patterns of seasonal drought may be altered. If summer heating leads to more frequent and severe drought, C₄ species may become inhibited. Alternatively, where monsoon precipitation patterns become established, the C₄ species will

likely be favored. One region that may be affected by monsoon intensification is the southern Great Basin desert of Nevada and Utah, where summer precipitation is currently sparse. Monsoon rains that support a rich C₄ flora in the Mojave and Sonoran deserts to the south of the Great Basin are predicted to shift northward (Lin et al., 1996), and with it, many of the southern C₄ species may become common on landscapes further north.

5. Interactions between high CO₂ and climate warming are difficult to predict because warming alone favors C₄ species while high CO₂ alone favors C₃ species, particularly at warmer temperatures. Studies of interactive effects of temperature and CO₂ show that high CO₂ enables the C₃ species to narrow the gap in photosynthesis and growth between the C₃ and C₄ species in warmer temperatures but does not completely overcome the differences (Hunt et al., 1996; Grise, 1996). Part of the reason why C₄ species remain superior at warmer temperatures is because they have evolved many features that promote fitness in warm habitats. For example, allocation strategies, phenology of growth and reproduction, heat tolerance, and thermal optima of cellular processes are some of the non-photosynthetic parameters that can be critical to survival in hot environments. Because many potential C₃ competitors are adapted to habitats and seasons of lower temperature, they may remain unfit despite improved photosynthetic performance in high CO₂ (Bazzaz, 1990).

C. Paleoecological Perspectives

One of the best approaches to assessing the future of C₄ photosynthesis is to use the tools of paleoecology to evaluate how past episodes of CO₂ and climate change affected the distribution of C₄ relative to C₃ species. This has already provided some valuable insights.

1. Theoretical Issues

At the height of the last ice age 21,000 years ago, atmospheric CO₂ content was 180 $\mu\text{mol mol}^{-1}$ and mean global temperature about 7 °C cooler relative to today (Barnola et al., 1987; Kutzbach et al., 1998). Between 15,000 and 10,000 years ago, CO₂ levels rose from 180 $\mu\text{mol mol}^{-1}$ to the Holocene average of

270 $\mu\text{mol mol}^{-1}$ (Fig. 1C). Temperature also rose during this period, eventually peaking about 8,000 years ago during the Holocene Hypsithermal episode.

Theoretically, these changes should have profound effects on C_3 and C_4 vegetation dynamics. Reducing CO_2 levels to 180 $\mu\text{mol mol}^{-1}$ substantially reduces photosynthetic carbon gain of C_3 species but causes only marginal photosynthetic reductions in C_4 species (Sage, 1995). At the CO_2 levels of the last ice age and at temperatures of 25 °C to 30 °C, photosynthesis is approximately double in C_4 relative to functionally similar C_3 plants (Fig. 3; 7; Tissue et al., 1995). With respect to growth, *Amaranthus retroflexus* (C_4) produces 23 times more biomass than the ecologically similar weed *Abutilon theophrasti* (C_3) at 150 $\mu\text{mol mol}^{-1}$ and 28 °C, but only 2.6 times more biomass at 270 $\mu\text{mol mol}^{-1}$ (Dippery et al., 1995). At 350 $\mu\text{mol mol}^{-1}$, the two species are equivalent in biomass production. In soil plots extracted from Texas prairies, increasing growth CO_2 concentration from 200 to 270 $\mu\text{mol mol}^{-1}$ reduced frequency of C_4 vegetation by 37% and the C_4/C_3 biomass ratio by 78% (estimated from Johnson et al., 1993). These responses indicate C_4 plants could have expanded across landscapes during low CO_2 intervals of the Pleistocene. Not only would warm habitats have been affected, because C_4 species would theoretically become more competitive in cool temperate settings they do not currently dominate (Jolly and Haxeltine, 1997). However, the cooler global temperatures of the last glacial maximum would have partially offset the effect of low CO_2 , which combined with other ecological factors could have attenuated shifts in C_4 distributions (Collatz et al., 1998).

2. Empirical Observations

Consistent with the theoretical predictions, paleo-ecology studies indicate increased C_4 grass abundance during the low CO_2 episodes of the last glacial maximum; however, this expansion is not global, and may be related to increased aridity in addition to, or instead of, low CO_2 . Relative to the current era, central Africa experienced widespread expansion of C_4 grasslands during the last glacial maximum 15 to 25 kYA. Both upland and lowland sites from Ghana in West Africa to Kenya in East Africa show increases in grass and sedge pollen (most likely C_4 species in these latitudes) (Maley, 1991; Sowunmi, 1991; Aucour et al., 1994; Giresse et al., 1994; Street-Perot, 1994). Correspondingly, carbon isotope ratios

from central-African lakes are significantly more positive in late Pleistocene than early Holocene sediments that are 12,000 to 8,000 years old (Hillaire-Marcel et al., 1989; Ehleringer et al., 1997; Street-Perot et al., 1997). At these sites, the observed shifts in carbon isotope ratio from C_4 to C_3 like-values correspond to the 30% increase in CO_2 at the end of the Pleistocene. This correlation is interpreted as evidence for a CO_2 -control over C_4 distribution, with the rise in CO_2 causing, in part, the contraction of C_4 -dominated grasslands (Ehleringer et al., 1997; Street-Perot et al., 1997).

While consistent with theoretical predictions, these studies must be interpreted with caution for a number of reasons. First, drier conditions are apparent over much of central Africa at the last glacial maximum (Street-Perot, 1994; Jolly et al., 1998) and this would have strong influences over the distribution of grasslands and forest regardless of CO_2 level. Effects of aridity versus CO_2 change remain to be disentangled. Second, in some of the studies where C_4 grass retreat is evident 10,000 to 15,000 years ago, the cores extend to deposits older than 30,000 years. Between 25,000 and 35,000 years old, CO_2 levels remained below 200 $\mu\text{mol mol}^{-1}$, yet some of the cores of this age show strong shifts in carbon isotope ratios from values indicative of C_3 vegetation (Talbot and Johannesson, 1992; Aucour et al., 1994; Street-Perot, 1994). Third, the African trends are not apparent for many areas of the planet. In Kashmir, India, and western Java, for example, isotopic change indicates shifts from C_4 to C_3 vegetation during the low CO_2 levels of the late Pleistocene (Krishnamurthy et al., 1982; Kaars and Ram, 1997). Pollen evidence also indicates much of the tropical South America was woodland at the end of the Pleistocene (Bush et al., 1990; Colinvaux, 1993). However, consistent with the results from Africa, savanna-type vegetation appears to have expanded into rainforest over parts of Amazonia and gallery forest in southeastern Brazil during the period corresponding to the end-Pleistocene CO_2 low (van der Hamer and Absy, 1994; Behling and Lichte, 1997). As in Africa, this expansion is also associated with greater aridity in this region at this time (van der Hamer and Absy, 1994; Behling and Lichte, 1997).

In the tropics, life form differences often dominate influences of photosynthetic pathway to favor woody C_3 species over herbaceous C_4 species. It is possible that the failure to see an expansion of C_4 vegetation in many areas reflects the persistent dominance of

the woody biota at low CO₂. To more precisely evaluate how C₃ and C₄ vegetation respond to past change in climate and CO₂, it may be better to examine responses in landscapes where C₃ and C₄ vegetation of similar life form occur, namely the steppes and grasslands of North America, Asia, and temperate Africa.

In the temperate zones, pollen evidence shows no consistent correlation between possible C₄ vegetation occurrence and the CO₂ rise 15,000 to 10,000 years ago (Villagran and Armento, 1993; Fredlund, 1995; Sun et al., 1997). It is difficult, however, to detect the presence of C₄ species from pollen evidence alone, so these results cannot be considered definitive and carbon isotopic evidence is necessary to compliment pollen and plant macrofossil evidence. In contrast to predictions, however, carbon isotope data don't show obvious signs of enhanced C₄ dominance during the last glacial maximum. In temperate South Africa and east-central China, no strong shift between C₃ and C₄ dominance are apparent in carbon isotope records from deposits 10,000 to 27,000 years old (Lee-Thorp and Beumont, 1995; Wang et al., 1997). A number of detailed studies from the southwestern U.S. also show no consistent trend. In eastern New Mexico, carbon isotope ratios indicate enhanced C₄ dominance at the last glacial maximum, with C₃ shrubs encroaching into C₄ grasslands about 8,000 years ago (Cole and Monger, 1994). Cole and Monger interpret this shift as a response to the CO₂ rise of a few thousand years earlier, although this interpretation has been questioned (Boutton et al., 1994). In a study of carbon isotopes from tooth enamels of ice-age grazers (*Mammuthus*, *Bison*, *Equus* and *Camelops* spp) of New Mexico and Arizona, Connin et al. (1998) inferred that regional patterns of C₄ abundance of the late-Pleistocene changed little in the Holocene, despite the changes in CO₂ and temperature. As today, C₄ grasses were abundant in southeast Arizona and southern New Mexico, but uncommon further north and west. While this lack of change in C₄ distribution could result from antagonistic effects of rising CO₂ and temperature, Connin et al. (1998) note that summer rainfall during the Pleistocene may have been the critical control over C₄ success during the ice age.

In north-central Texas, Humphrey and Ferring (1994) observed reductions in carbon isotope ratios in soil deposits that are 14,000 to 10,000 years old, indicating contraction of C₄ biomass. Further west, in central Texas, soil organic matter show increases

in carbon isotope ratio between 15,000 and 8,000 years ago, indicating C₄ expansion in the Holocene (Nordt et al., 1994). In the southern high plains of west Texas, a similar C₃ to C₄ transition is observed, based on changes in carbon isotope ratio of *Bison* collagen (Stafford et al., 1994, cited in Connin, 1998). A survey of carbon in grass phytoliths from the northern Great Plains indicates increased C₄ grass expansion into C₃ grasslands of North America at the termination of the last ice age (Kelly et al., 1991), which is consistent with increasing temperature favoring the expansion of C₄ vegetation. One additional shift from C₄ to a C₃-enriched flora has been reported in the desert southwest of North America. Here, a desert of C₃ thornscrub and CAM succulents replaced a C₄ grassland as conditions warmed and became more arid between the last glacial maximum and the Holocene (Liu et al., 1996).

In summary, the evidence to date is consistent with the hypothesis that low CO₂ levels of the late Pleistocene favored more extensive C₄ grasslands in tropical areas; however, C₃ vegetation remained common and was often dominant in the tropics, particularly outside of central African locations. In temperate zones, there is no consistent evidence for a C₃ expansion as a result of higher CO₂, although there are a number of studies that indicate the warming of the early to mid Holocene period between 5,000 and 10,000 years ago promoted greater C₄ dominance. Taken together, these results indicate that higher CO₂ can promote C₃ species over C₄, but mainly in tropical areas, and mainly by promoting woody vegetation over graminoids. As is the case today, the timing of precipitation may be one of the most important determinants of the abundance of C₄ species in coming centuries.

VII. Conclusion: C₄ Plants and the Human Factor

To a certain extent, discussions of the response of natural vegetation to climate change are academic, because the rising human population is becoming so pervasive across the face of the earth and so demanding of resources that only marginal land will not be subjected to extensive human management. With regards to the future of C₄ vegetation, recent history serves as a pretty good guide to the probable trends that will occur as a result of increasing human dominance of ecosystem processes. The strength of

human controls is so great that trends already observed will likely overwhelm effects of any C_4 response to climate and atmospheric change.

In recent decades, humans have greatly altered the distribution of C_3 and C_4 species on the planet through heavy logging, landscape conversion to agriculture, burning, fire suppression, establishment of timber plantations, overhunting of large herbivores, and overgrazing. In the tropics, natural forest landscapes have been greatly reduced in scope by conversion to pasture, croplands or exotic grasslands (Myers, 1991; D'Antonio and Vitousek, 1992). Dry tropical forests have been severely affected, with over 80% of the natural forest area lost (Maas, 1995). Wet tropical forests are going rapidly and only remnants are predicted to exist in a few decades, with C_4 grasses occupying much of the former forested landscape (Myers, 1991). In West Africa, for example, human exploitation of landscapes has resulted in a 500 km extension of C_4 grasslands and savanna into forested regions by 1983 (Hopkins, 1983). These changes represent the largest expansion of C_4 biomass since the mid-Holocene, and have occurred during substantial increases in atmospheric CO_2 and nitrogen deposition.

Human action does not simply favor C_4 grasses, however. Savanna grasslands of tropical regions are increasingly being converted to timber plantations (Soares, 1990). In temperate regions, widespread loss of native C_4 grasslands has resulted because overgrazing has led to shrubland encroachment, while fire suppression and timber plantations have generated woodlands in large areas of what were once C_4 grasslands and savannas (Sage et al., 1999b). In some cases, C_4 dominated biomes have been largely eliminated, as is demonstrated by the conversion of the eastern portion of the tall-grass prairie of North America into row crop agriculture. C_4 plants are common on this former grassland, but they are mainly maize or one of its weeds.

From these observations, what appears clear is that the future of C_4 plants will largely depend upon human decisions concerning how the landscape will be managed. Where C_4 plants fit into the management scheme, or, as weeds, are able to exploit the management scheme, they will do extremely well. However, if they are of little use to humans, then it is possible they could, like much of the rest of the biota, be marginalized in the future, human-dominated world.

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References

- Adam P (1990) Saltmarsh Ecology. Cambridge University Press, Cambridge
- Akhani H, Trimborn P and Ziegler H (1997) Photosynthetic pathways in Chenopodiaceae from Africa, Asia and Europe with their ecological, phytogeographical and taxonomical importance. Plant Syst Evol 206: 187–221
- Andrews TJ and Lorimer GH (1987) Rubisco: Structure, mechanisms, and prospects for improvement. In: Hatch MD and Boardman NK (eds) The Biochemistry of Plants, Vol 10, pp 131–128. Academic Press, San Diego
- Archibald OW (1995) Ecology of World Vegetation. Chapman and Hall, London
- Aucour A, Hillaire-Maracel C and Bonnefille R (1994) Late Quaternary biomass changes from ^{13}C measurements in a highland peatbog from equatorial Africa (Burundi). Quaternary Res 41: 225–233
- Ball AS (1997) Microbial decomposition at elevated CO_2 levels: Effect of litter quality. Global Change Biol 3: 379–386
- Ball MC (1988) Ecophysiology of mangroves. Trends Ecol Evol 2:129–142
- Ball MC and Farquhar GD (1984) Photosynthetic and stomatal responses of two mangrove species, *Aegiceras corniculatum* and *Avicennia marina*, to long term salinity and humidity conditions. Plant Physiol 74: 1–6
- Ball MC, Cowan IR and Farquhar GD (1988) Maintenance of leaf temperature and the optimization of carbon gain in relation to water loss in a tropical mangrove forest. Aust J Plant Physiol 15: 263–276
- Barnes PW and Harrison AT (1982) Species distribution and community organization in a Nebraska sandhills mixed prairie as influenced by plant/soil-water relationships. Oecologia 52: 192–201
- Barnola JM, Raynaud D, Korotkevich YS and Lorius CD (1987) Vostok ice core provides 160,000-year record of atmospheric CO_2 . Nature 329: 408–414
- Baskin JM and Baskin CC (1977) Role of temperature in the germination ecology of three summer annual weeds. Oecologia 30: 377–382
- Batanouny KH, Stichler W and Ziegler H (1991) Photosynthetic pathways and ecological distribution of *Euphorbia* species in Egypt. Oecologia 87: 565–569
- Bazzaz FA (1990) The response of natural ecosystems to the rising global CO_2 levels. Annu Rev Ecol Syst 21: 167–196
- Bazzaz FA and Williams WE (1991) Atmospheric CO_2 within a mixed forest: Implications for seedling growth. Ecol 72:12–16
- Beale CV and Long SP (1997) Seasonal dynamics of nutrient accumulation and partitioning in the perennial C_4 grasses *Miscanthus × giganteus* and *Spartina cynosuroides*. Biomass Bioenergy 12: 419–428
- Behling H and Lichte M (1997) Evidence of dry and cold

- climatic conditions at glacial times in tropical southeastern Brazil. *Quaternary Res* 48:348–358
- Belsky AJ, Amundson RG, Duxbury JM, Riha SJ, Ali AR and Mwonga SM (1989) The effects of trees on their physical, chemical, and biological environments in a semi-arid savanna in Kenya. *J Appl Ecol* 26: 1005–1024
- Berner RA (1994) 3Geocarb II: a revised model of atmospheric CO₂ over Phanerozoic time. *Amer J Sci* 291: 339–376
- Berner RA and Canfield DE (1989) A new model for atmospheric oxygen over Phanerozoic time. *Amer J Sci* 289: 333–361
- Berry JA and Raison JK (1981) Responses of macrophytes to temperature. In: Lange OL, Nobel PS, Osmond CB and Ziegler H (eds) *Encyclopedia of Plant Physiology*. New Series Vol. 12A, pp 277–338. Springer-Verlag, Berlin
- Bird MI and Pousai P (1997) Variations of Delta ¹³C in the surface soil organic carbon pool. *Global Biogeochem Cycles* 11:313–322
- Bird MI, Haberle SG and Chivas AR (1994) Effect of altitude on the carbon-isotope composition of forest and grassland soils from Papua New Guinea. *Global Biogeochem Cycles* 8: 13–22
- Björkman O (1976) Adaptive and genetic aspects of C₄ photosynthesis. In: Burris RH and Black CC (eds) *CO₂ Metabolism and Plant Productivity*, pp 287–310. University Park Press, Baltimore
- Björkman O and Demmig B (1987) Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origin. *Planta* 170: 489–504
- Björkman O and Demmig-Adams B (1994) Regulation of photosynthetic light energy capture, conversion and dissipation in leaves. In: Schulze ED and Caldwell MM (eds) *Ecophysiology of Photosynthesis*, pp 17–48. Springer-Verlag, Berlin
- Björkman O, Badger MR and Armond P (1980) Response and adaptation of photosynthesis to high temperatures. In: Turner NC and Kramer PJ (eds) *Adaptation of Plants to Water and High Temperature Stress*, pp 233–250. John Wiley and Sons, New York
- Björkman O, Mooney H and Ehleringer J (1975) Photosynthetic characteristics of plants from habitats with contrasting thermal regimes: comparisons of photosynthetic responses of intact plants. *Carnegie Inst Wash Yearbook* 74: 743–748
- Black CC (1973) Photosynthetic carbon fixation in relation to net CO₂ uptake. *Annu Rev Plant Physiol* 24: 253–286
- Black CC, Chen TM and Brown RH (1969) Biochemical basis for plant competition. *Weed Sci.* 17: 338–344
- Bond WJ and van Wilgen BW (1996) *Fire and Plants*. Chapman and Hall, London
- Boonman JG (1993) East Africa's Grasses and Fodders: Their Ecology and Husbandry. Kluwer Academic Publishers, Dordrecht
- Boutton TW, Harrison AT and Smith BN (1994) Distribution of biomass of species differing in photosynthetic pathway along an altitudinal transect in a southeastern Wyoming grassland. *Oecologia* 45: 287–298
- Bowes G (1996) Photosynthetic responses to changing atmospheric carbon dioxide concentration. In: Baker NR (ed) *Photosynthesis and the Environment*, pp 387–407. Kluwer Academic Publishers, Dordrecht
- Bray JR (1958) The distribution of savanna species in relation to light intensity. *Can J Bot* 36: 671–681
- Brown RH (1999) Agronomic implications of C₄ photosynthesis. In: Sage RF and Monson RK (eds) *C₄ Plant Biology*, pp 473–507. Academic Press, San Diego
- Brown WV (1977) The Kranz syndrome and its subtypes in grass systematics. *Mem Torrey Bot Club* 23: 1–97
- Brownell PF and Crossland CJ (1972) The requirement for sodium as a micronutrient by species having the C₄ dicarboxylic photosynthetic pathway. *Plant Physiol* 49:794–797
- Byrd GT, Sage RF and Brown RH (1992) A comparison of dark respiration in C₃ and C₄ plants. *Plant Physiol* 100:191–198
- Cavagnaro JB (1988) Distribution of C₃ and C₄ grasses at different altitudes in a temperate arid region of Argentina. *Oecologia* 76: 273–277
- Cerling TE (1999) Paleorecords of C₄ plants and ecosystems. In: Sage RF and Monson RK (eds) *C₄ Plant Biology*, pp 445–469. Academic Press, San Diego
- Cerling TE, Harris JM, MacFadden BJ, Leacey MG, Quade J, Eisenmann V and Ehleringer JR (1997) Global vegetation change through the Miocene/Pliocene boundary. *Nature* 389: 153–158
- Chapin FS III (1980) The mineral nutrition of wild plants. *Annu Rev Ecol Syst* 11:233–260
- Christie EK and Detling JK (1982) Analysis of interference between C₃ and C₄ grasses in relation to temperature and soil nitrogen supply. *Ecology* 63: 1277–1284
- Cole DR and Monger HC (1994) Influence of atmospheric CO₂ on the decline of C₄ plants during the last deglaciation. *Nature* 368: 533–536
- Colinvaux P (1993) Pleistocene biogeography and diversity in tropical forests of South America. In: Goldblatt P (ed) *Biological Relationships Between Africa and South America*, pp 473–499. Yale University Press, New Haven
- Collatz GJ, Berry JA and Clark JS (1998) Effects of climate and atmospheric CO₂ partial pressure on the global distribution of C₄ grasslands: Present, past, and future. *Oecologia* 114: 441–454
- Connin SL, Betancourt J and Quade J (1998) Late Pleistocene C₄ plant dominance and summer rainfall in the southwestern United States from isotopic study of herbivore teeth. *Quaternary Res* 50:179–193
- Costa CSB and Davy AJ (1992) Coastal salt marsh communities of Latin America. In: Seeliger U. (ed) *Coastal Plant Communities of Latin America*, pp 179–199. Academic Press, San Diego
- D'Antonio CM and Vitousek PM (1992) Biological invasions by exotic grasses, the grass/fire cycle, and global change. *Annu Rev Ecol Syst* 23: 63–87
- DeJong TM, Drake BG and Pearcy RW (1982) Gas exchange responses of Chesapeake Bay tidal marsh species under field and laboratory conditions. *Oecologia* 52: 5–11
- Dengler NG and Nelson T (1999) Leaf structure and development in C₄ plants. In: Sage RF and Monson RK (eds) *C₄ Plant Biology*, pp 133–172. Academic Press, San Diego
- Dever LV (1997) Control of photosynthesis in *Amaranthus edulis* mutants with reduced PEP carboxylase. *Aust J Plant Physiol* 24: 469–476
- Diaz S, Grime JP, Harris J and McPherson E (1993) Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. *Nature* 364: 616–617
- Dickinson CE and Dodd JL (1976) Phenological pattern in the Shortgrass Prairie. *Amer Mid Nat* 96: 367–378
- Dipperly JK, Tissue DT, Thomas RB and Strain BR (1995) Effects of low and elevated CO₂ on C₃ and C₄ annuals I.

- Growth and biomass allocation. *Oecologia* 101: 13–20
- Downton, WJS (1975) The occurrence of C₄ photosynthesis among plants. *Photosynthetica* 9: 96–105
- Downton WJS and Tregunna EB (1968) Carbon Dioxide compensation—its relation to photosynthesis carboxylation reactions, systematics of the Gramineae, and leaf anatomy. *Can J Bot* 46: 207–215
- Drake BG, Peresta G, Beugeling E and Matamala R (1996) Long-term elevated CO₂ exposure in a Chesapeake Bay wetland: Ecosystem gas exchange, primary production, and tissue nitrogen. In: Koch GW and Mooney HA (eds) *Carbon Dioxide and Terrestrial Ecosystems*, pp 197–214. Academic Press, San Diego
- Edwards GE and Walker DA (1983) C₃, C₄: Mechanism, and Cellular and Environmental Regulation, of Photosynthesis. Blackwell Scientific Publications, Oxford
- Ehleringer JR (1978) Implications of quantum yield differences on the distributions of C₃ and C₄ grasses. *Oecologia* 31:255–267
- Ehleringer JR and Pearcy RW (1983) Variation in quantum yield for CO₂ uptake among C₃ and C₄ plants. *Plant Physiol* 73: 555–559
- Ehleringer JR, Ceding, TE and Helliker BR (1997) C₄ photosynthesis, atmospheric CO₂ and climate. *Oecologia* 112: 285–299
- Ellis RP, Vogel JC and Fuls A (1980) Photosynthetic pathways and the geographical distribution of grasses in southwest Africa/Namibia. *S Afr J Sci* 76: 307–314
- Epstein HE, Lauenroth WK, Burke IC and Coffin DP (1997) Productivity patterns of C₃ and C₄ functional types in the U.S. Great Plains. *Ecology* 78: 722–731
- Evans JR (1989) Photosynthesis and the nitrogen relationship in leaves of C₃ plants. *Oecologia* 78: 9–19
- FAO (1990) Production yearbook, 1989, Vol 43. Food and Agricultural Organization of the United Nations, Rome
- Farquhar GD (1983) On the nature of carbon isotope discrimination in C₄ species. *Aust J Plant Physiol* 10: 205–226
- Farquhar GD and Sharkey TD (1982) Stomatal conductance and photosynthesis. *Annu Rev Plant Physiol* 33: 317–345
- Fay PA and Knapp AK (1995) Stomatal and photosynthetic responses to variable light in sorghum, soybeans and eastern gammagrass. *Physiol Plant* 94:613–620
- Flowers TJ, Troke PF and Yeo AR (1977) The mechanism of salt tolerance in halophytes. *Annu Rev Plant Physiol* 28: 89–121
- Fredlund GG (1995) Late Quaternary Pollen Record from Cheyenne Bottoms, Kansas. *Quaternary Res* 43: 67–79
- Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S, and Ashton AR (1997) Genetic manipulation of key photosynthetic enzymes in the C₄ plant *Flaveria bidentis*. *Aust J Plant Physiol* 24: 477–485
- Genty B and Harbinson J (1996) Regulation of light utilization for photosynthetic electron transport. In: Baker NR (ed) *Photosynthesis and the Environment*, pp 67–99. Kluwer Academic Publishers, Dordrecht
- Giresse P, Maley J and Brenac P (1994) Late quaternary palaeoenvironments in the Lake Barombi Mbo (West Cameroon) deduced from pollen and carbon isotopes of organic matter. *Palaeogeography Palaeoclimatology Paleoecology* 107:65–78
- Goldammer J G (1993) Historical biogeography of fire: tropical and subtropical. In: Crutzen PJ and Goldammer JG (eds) *The Ecological, Atmospheric, and Climatic Importance of Vegetation Fire*, pp 297–314. John Wiley and Sons, New York
- Grise DJ (1996) Effects of Elevated CO₂ and High Temperature on the Relative Growth Rates and Competitive Interactions between a C₃ (*Chenopodium album*) and a C₄ (*Amaranthus hybridus*) Annual. PhD thesis, University of Georgia, Athens
- Gurevitch J (1986) Restriction of a C₃ grass to dry ridges in a semiarid grassland. *Can J Bot* 64: 1006–1011
- Guy R D, Reid DM and Krouse HR (1986) Factors affecting ¹³C/¹²C ratios of inland halophytes. II. Ecophysiological interpretations of patterns in the field. *Can J Bot* 64: 2700–2707
- Hatch MD (1987) C₄ photosynthesis: A unique blend of modified biochemistry, anatomy and ultrastructure. *Biochim Biophys Acta* 895: 81–106
- Hatch MD, Agostino A and Jenkins CLD (1995) Measurement of the leakage of CO₂ from bundle-sheath cells of leaves during C₄ photosynthesis. *Plant Physiol* 108: 173–181
- Hattersley PW (1983) The distribution of C₃ and C₄ grasses in Australia in relation to climate. *Oecologia* 57: 113–128
- Hattersley PW (1992) C₄ photosynthetic pathway variation in grasses (Poaceae): Its significance for arid and semi-arid lands. In: Chapman EP (ed) *Desertified Grasslands: Their Biology and Management*, pp 181–212. Academic Press, London
- Hattersley PW and Watson L (1992) Diversification of photosynthesis. In: Chapman EP (ed) *Grass Evolution and Domestication*, pp 38–116. Cambridge University Press, New York.
- Hayes JM (1994) Global methanotrophy at the Archean-Proterozoic transition. In: Bengston S (ed) *Early Life on Earth*, pp 220–236. Columbia University Press, New York
- Henderson S, Hattersley P, von Caemmerer S and Osmond CB (1994) Are C₄ pathway plants threatened by global climatic change? In: Schulze E-D and Caldwell MM (eds) *Ecophysiology of Photosynthesis*, pp 529–549. Springer-Verlag, New York.
- Henderson SA, von Caemmerer S and Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C₄ species. *Aust J Plant Physiol*, 19: 263–285
- Hillaire-Marcel C, Aucour A-M, Bonnefilie R, Rioulet G, Vincens A and Williamson D (1989) ¹³C palynological evidence of differential residence times of organic carbon prior to its sedimentation in east African rift lakes and peat bogs. *Quaternary Sci Rev* 8: 207–212
- Hirose T and Weger MJA (1987) Nitrogen use efficiency in instantaneous and daily photosynthesis of stand leaves in the canopy of *Solidago altissima*. *Physiol Plant* 70: 215–222
- Hopkins B (1983) Successional processes. In: Bourliere F (ed) *Ecosystems of the World* 13: Tropical Savannas, pp 605–616. Elsevier, Amsterdam
- Horton JL and Neufeld HS (1998) Photosynthetic responses of *Microstegium vimineum* (Trin.) A. Camus, a shade-tolerant, C₄ grass, to variable light environments. *Oecologia* 114: 11–19
- Humphrey JD and Ferring CR (1994) Stable isotopic evidence for latest Pleistocene and Holocene climatic change in North-Central Texas. *Quaternary Res* 41: 200–213
- Hunt HW, Elliott ET, Detling JK, Morgan JA and Chen D-X (1996) Responses of a C₃ and C₄ perennial grass to elevated CO₂ and temperature under different water regimes. *Global Change Biol* 2: 35–47

- Johnson HB, Polley HW and Mayeux HS (1993) Increasing CO₂ and plant-plant interactions: effects on natural vegetation. *Vegetatio* 104/105: 157–170
- Jolly D and Haxeltine A (1997) Effect of low glacial atmospheric CO₂ on tropical African montane vegetation. *Science* 276: 786–787
- Jolly D, Harrison SP, Dammati B, and Bonnefille R (1998) Simulated climate and biomes of Africa during the late Quaternary: comparison with pollen and lake status data. *Quaternary Sci Rev* 17:629–657
- Jones MB (1986) Wetlands. In: Baker NR and Long SP (eds) *Photosynthesis in Contrasting Environments*, pp 103–138. Elsevier, London
- Jordan DB and Ogren WL (1984) The CO₂/O₂ specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. *Planta* 161: 308–313
- Jouzel J, Barkov NI, Barnola JM, Bender M, Chappellaz J, Genthon C, Kotlyakov VM, Lipenkov V, Lorius C, Petit JR, Raynaud D, Raisbeck G, Ritz C, Sowers T, Stievenard M., Yiou F, Yiou P (1993) Extending the Vostok ice-core record of palaeoclimate to the penultimate glacial period. *Nature* 364: 407–412
- Junk WJ (1983) Ecology of swamps on the middle Amazon. In: Gore AJP (ed) *Ecosystems of the World* 4B: MIRES: Swamp, Bog, Fen and Moor, pp 269–294. Elsevier, Amsterdam
- Kaars S van der and Ram D (1997) Vegetation and climate change in west-Java, Indonesia during the last 135,000 years. *Quaternary Int* 37: 67–71
- Kao, WY (1997) Contribution of *Miscanthus transmorrisonensis* to soil organic carbon in a mountain grassland: Estimated from stable carbon isotope ratio. *Bot Bull Acad Sinica* 38:45–48
- Kasting JF (1987) Theoretical constraints on oxygen and carbon dioxide concentrations in the Precambrian atmosphere. *Precambrian Res* 34: 205–229
- Kattenberg A, Giorgi F, Grassl H, Meehl GA, Mitchell JFB, Stouffer RJ, Tokioka T, Weaver AJ and Wigley TML (1996) Climate models—projections of future climate. In: Houghton JT, Filho LGM, Callander BA, Harris N, Kattenberg A and Maskell K (eds) *Climate Change, 1995: The Science of Climate Change*, pp 285–357. Cambridge University Press, Cambridge
- Kellogg EA (1999) Phylogenetic aspects of the evolution of C₄ photosynthesis. In: Sage RF and Monson RK (eds) *C₄ Plant Biology*, pp 411–444. Academic Press, San Diego
- Kelly EF, Amundson RG, Marino B and Deniro MJ (1991) Stable isotope ratios of carbon in phytoliths as a quantitative method of monitoring vegetation and climate change. *Quaternary Res* 35: 222–233
- Kemp PR (1983) Phenological patterns of Chihuahuan Desert plants in relation to the timing of water availability. *J Ecol* 71:427–436
- Kirschbaum MUF, Bullock P, Evans JR, Goulding K, Jarvis PG, Nobel IR, Rounsevell M and Sharkey TD (1995) Ecophysiological, ecological, and soil processes in terrestrial ecosystems: A primer on general concepts and relationships. In: Watson RT, Zinyowera MC and Moss RH (eds) *Climate Change, 1995: Impacts, Adaptations and Mitigation of Climate Change: Scientific-Technical Analysis*, pp 57–74. Cambridge University Press, Cambridge
- Knapp AK and Medina E (1999) Success of C₄ photosynthesis in the field: Lessons from communities dominated by C₄ plants. In: Sage RF and Monson RK (eds) *C₄ Plant Biology*, pp 251–283. Academic Press, San Diego
- Ko LJ and Reich PB (1993) Oak tree effects on soil and herbaceous vegetation in savannas and pastures in Wisconsin. *Am Midl Nat* 130: 31–42
- Krall JP and Pearcy RW (1993) Concurrent measurements of oxygen and carbon dioxide exchange during lightflecks in maize (*Zea mays* L). *Plant Physiol* 103: 823–828
- Krishnamurthy RV, Deniro MJ and Pant RK (1982) Isotope evidence for Pleistocene climate changes in Kashmir, India. *Nature* 298: 640–641
- Kutzbach J, Gallimore R, Harrison S, Behling P, Selin R and Laarif F (1998) Climate and biome simulations for the past 21,000 years. *Quaternary Sci Rev* 17:473–506
- Larcher W (1995) *Physiological Plant Ecology*. 3rd Ed. Springer, New York
- Leegood RC and Edwards GE (1996) Carbon metabolism and photorespiration: Temperature dependence in relation to other environmental factors. In: Baker NR (ed) *Photosynthesis and the Environment*, pp, 191–221. Kluwer Academic Publishers, Dordrecht
- Leegood RC and Walker RP (1999) Regulation of the C₄ pathway. In: Sage RF and Monson RK (eds) *C₄ Plant Biology*, pp 89–131. Academic Press, San Diego
- Lee-Thorp JA and Beaumont PB (1995) Vegetation and Seasonality Shifts during the Late Quaternary Deduced from ¹³C/¹²C Ratios of Grazers at Equus Cave, South Africa. *Quaternary Res* 43: 426–432
- Li M-R (1993) Leaf photosynthetic nitrogen-use of C₃ and C₄ Species of *Cyperus*. *Photosynthetica* 29: 117–130
- Lin G, Philips SL and Ehleringer JR (1996) Monsoonal precipitation responses of shrubs in a cold desert community on the Colorado Plateau. *Oecologia* 106: 8–17
- Liu BL, Phillips FM, and Campbell AR (1996) Stable carbon and oxygen isotopes of pedogenic carbonates, Ajo Mountains, Southern Arizona—Implications for paleoenvironmental change. *Palaeogeogr Palaeoclimatol PalaeoEcol* 124: 233–246
- Long SP (1983) C₄ Photosynthesis at low temperatures. *Plant Cell Environ* 6: 345–363
- Long SP (1999) Environmental responses. In: Sage RF and Monson RK (eds) *The Biology of C₄ photosynthesis*, pp. 215–249. Academic Press, San Diego
- Maass JM (1995) Conversion of tropical dry forest to pasture and agriculture. In: Bullock SH, Mooney HA and Medina E (eds) *Seasonal Dry Tropical Forests*, pp 399–422. Cambridge University Press, Cambridge
- MacDonald KB (1977) Plant and animal communities of Pacific North American salt marshes. In: Chapman VJ (ed) *Ecosystems of the World 1: West Coastal Ecosystems*, pp 167–191. Elsevier, Amsterdam
- Makino A, Nakano H and Mae T (1994) Responses of Ribulose-1,5-bisphosphate carboxylase, cytochrome f, and sucrose synthesis enzymes in rice leaves to leaf nitrogen, and their relationships to photosynthesis. *Plant Physiol* 105: 173–179
- Makino A, Shimada T, Takumi S, Kaneko K, Matsuoka, M, Shimamoto K, Nakano H, Miyao-Tokotomi M, Mae T and Yamamoto N (1997) Does decrease in Ribulose-1,5-bisphosphate carboxylase by antisense RbcS lead to a high N-use efficiency of photosynthesis under conditions of saturating CO₂ and light in rice leaves? *Plant Physiol* 114: 483–491
- Maley J (1991) The African rain forest vegetation and

- Rowan F. Sage and Robert W. Pearcy
- palaeoenvironments during late Quaternary. *Climatic Change*, 19:79–98
- Matsuba K, Imaizuma N, Kaneko S, Samejima M and Ohsugi R (1997) Photosynthetic responses to temperature of phosphoenolpyruvate carboxykinase type C₄ species differing in cold sensitivity. *Plant, Cell and Environ* 20: 268–274
- Means DB (1997) Wiregrass restoration: Probable shading effects in a slash pine plantation. *Restoration Manage Notes* 15: 52–55
- Medina E and Klinge H (1983) Productivity of tropical forests and tropical woodlands. In: Lange OL, Nobel PS, Osmond CB and Ziegler H (eds) *Physiological Plant Ecology IV. Ecosystem Processes: Mineral Cycling, Productivity and Man's Influence*, pp 281–304. Springer-Verlag, Berlin
- Monson RK, Littlejohn RO Jr and Williams GJ III (1983) Photosynthetic adaptation to temperature in four species from the Colorado shortgrass steppe: A physiological model for coexistence. *Oecologia* 58: 43–51
- Morgan ME, Kingston JD and Marino BD (1994) Expansion and emergence of C₄ plants. *Nature* 371: 112–113
- Mulroy TW and Rundel PW (1977) Annual Plants: Adaptation to Desert Environments. *BioScience* 27: 110–114
- Myers N (1991) Tropical forests: Present states and future outlook. *Climatic Change*, 19: 3–32
- Nordt LC, Boutton TW, Hallmark CT, and Waters MR (1994) Late Quaternary Vegetation and Climate Changes in Central Texas Based on the Isotopic Composition of Organic Carbon. *Quaternary Res* 41: 109–120
- Ode DJ, Tieszen LL and Lerman JC (1980) The seasonal contribution of C₃ and C₄ plant species to primary production in a mixed prairie. *Ecology* 61: 1304–1311
- Osmond CB, Winter K and Ziegler H (1982) Functional Significance of different pathways of CO₂ fixation in photosynthesis. In: Lange OL, Nobel PS, Osmond CB and Ziegler H (eds) *Encyclopedia of Plant Physiology, New Series Vol. 12B. Physiological Plant Ecology II. Water Relations and Carbon Assimilation*, pp 479–547. Springer-Verlag, Berlin
- Owensby CE, Ham JM, Knapp A, Bremer D and Auen LM (1997) Water vapor fluxes and their impact under elevated CO₂ in a C₄-tallgrass prairie. *Global Change Biol* 3: 189–195
- Owensby CE, Ham JM, Knapp A, Rice CW, Coyne PI and Auen LM (1996) Ecosystem-level responses of tallgrass prairie to elevated CO₂. In: Koch GW and Mooney HA (eds) *Carbon Dioxide and Terrestrial Ecosystems*, pp 147–162. Academic Press, San Diego
- Patterson DT and Flint EP (1990) Implications of increasing carbon dioxide and climate change for plant communities and competition in natural and managed ecosystems. In: *Impact of Carbon Dioxide, Trace Gases, and Climate Change on Global Agriculture*, Special publication 53, pp 83–109. Amer Soc Agron, Madison
- Pearcy RW (1990) Sunflecks and photosynthesis in plant canopies. *Annu Rev Plant Physiol Plant Mol Biol* 41: 421–453
- Pearcy RW and Calkin HW (1983) Carbon dioxide exchange of C₃ and C₄ tree species in the understory of a Hawaiian forest. *Oecologia* 58: 26–32
- Pearcy RW and Ehleringer J (1984) Comparative ecophysiology of C₃ and C₄ plants. *Plant Cell Environ.* 7:1–13.
- Pearcy RW and Ustin SL (1984) Effects of salinity on growth and photosynthesis of three California tidal marsh species. *Oecologia* 62: 68–73
- Pearcy RW, Tumosa N and Williams K (1981) Relationships between growth, photosynthesis and competitive interactions for a C₃ and a C₄ plant. *Oecologia* 48: 371–376
- Pearcy RW, Osteryoung K and Calkin HW (1985) Photosynthetic responses to dynamic light environments by Hawaiian trees. *Plant Physiol* 79:896–902
- Pearson CJ (1992) Cereal-based systems of the highlands of North-East Africa. In: Pearson CJ (ed) *Ecosystems of the Worlds volume 18: Field Crop Ecosystems*, pp 277–289. Elsevier, Amsterdam
- Peat HCL (1997) Dynamics of C₃ and C₄ Productivity in Northern Mixed Grass Prairie. MSc thesis, University of Toronto
- Piedade MTF, Long SP and Junk WJ (1994) Leaf and canopy photosynthetic CO₂ uptake of a stand of *Echinochloa polystachya* on the Central Amazon floodplain: Are the high rates associated with the C₄ syndrome realized under the near-optimal conditions provided by this exceptional natural habitat? *Oecologia* 97: 193–201
- Pittermann J (1998) Control of C₄ Photosynthesis at Low Temperatures in High Elevation C₄ Grasses. MSc thesis, University of Toronto
- Polley HW (1997) Implications of rising atmospheric carbon dioxide concentration for rangelands. *J Range Manage* 50: 561–577
- Pomeroy LR and Wiegert, RG (eds) (1983) *The Ecology of a Salt Marsh*. Springer-Verlag, Berlin
- Prendergast HDV (1989) Geographical distribution of C₄ acid decarboxylation types and associated structural variants in native Australian C₄ grasses (Poaceae). *Aust J Bot* 37: 253–273
- Pyankov VI and Mokronosov AT (1993) General trends in changes of the earth's vegetation related to global warming. *Russ J Plant Physiol* 40: 443–458
- Raven PH, Evert RF and Eichhorn SE (1992) *Biology of Plants* (5th Ed). Worth Publishers, New York
- Renvoize SA and Clayton WD (1992) Classification and evolution of grasses. In: Chapman GP (ed) *Grass Evolution and Domestication*, pp 3–37. Cambridge University Press, Cambridge
- Robichaux RH and Pearcy RW (1980) Photosynthetic responses of C₃ and C₄ species from cool shaded habitats in Hawaii. *Oecologia* 47:106–109
- Rundel PW (1980) The ecological distribution of C₄ and C₃ grasses in the Hawaiian Islands. *Oecologia* 45: 354–359
- Sage RF (1994) Acclimation of photosynthesis to increasing atmospheric CO₂: The gas exchange perspectives. *Photosynth Res* 39: 351–368
- Sage RF (1995) Was low atmospheric CO₂ during the Pleistocene a limiting factor for the origin of agriculture? *Global Change Biology* 1: 93–106
- Sage RF and Pearcy RW (1987a) The nitrogen use efficiency of C₃ and C₄ plants. I. Leaf nitrogen, growth, and biomass partitioning in *Chenopodium album* L. and *Amaranthus retroflexus* L. *Plant Physiol* 84: 954–958
- Sage RF and Pearcy RW (1987b) The nitrogen use efficiency of C₃ and C₄ plants. II. Leafnitrogen effects on the gas exchange characteristics of *Chenopodium album* L. and *Amaranthus retroflexus* L. *Plant Physiol* 84: 959–963
- Sage RF and Reid CD (1994) Photosynthetic response mechanisms to environmental change in C₃ plants. In: Wilkinson RE (ed) *Plant-Environment Interactions*, pp 413–

499. Marcel Dekker, New York
- Sage RF and Seemann JR (1993) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in response to reduced light intensity in C₄ plants. *Plant Physiol* 102: 21–28
- Sage RF and Sharkey TD (1987) The effect of temperature on the occurrence of O₂ and CO₂ insensitive photosynthesis in field grown plants. *Plant Physiology* 84: 658–664
- Sage RF, Pearcy RW, and Seemann JR (1987) The nitrogen use efficiency of C₃ and C₄ plants. III. Leaf nitrogen effects on the activity of carboxylating enzymes in *Chenopodium album* L. and *Amaranthus retroflexus* L. *Plant Physiol* 85: 355–359
- Sage RF, Sharkey TD and Pearcy RW (1990) The effect of leaf nitrogen and temperature on the CO₂ response of photosynthesis in the C₃ dicot *Chenopodium album* L. *Aust J Plant Physiol* 17: 135–148
- Sage RF, Schäppi B and Körner C (1997) Effect of atmospheric CO₂ enrichment on Rubisco content in herbaceous species from high and low altitude. *Acta Oecologia* 18: 183–192
- Sage RF, Li M-R and Monson RK (1999a) The taxonomic distribution of C₄ photosynthesis. In: Sage RF and Monson RK (eds) C₄ Plant Biology, pp 551–584. Academic Press, San Diego
- Sage RF, Wedin DA and Li M-R (1999b) The Biogeography of C₄ photosynthesis: patterns and controlling factors. In: Sage RF and Monson RK (eds) C₄ Plant Biology, pp 313–373. Academic Press, San Diego
- Schulze E-D and Hall AE (1982) Stomatal responses, water loss, and CO₂ assimilation rates of plants in contrasting environments. In: Lange OL, Nobel PS, Osmond CB and Ziegler H (eds) Encyclopedia of Plant Physiology, New Series Vol. 12B. Physiological Plant Ecology II. Water Relations and Carbon Assimilation, pp 181–230. Springer-Verlag, Berlin
- Schulze E-D, Ellis R, Schulze W and Trimborn P (1996) Diversity, metabolic types and Delta ¹³C carbon isotope ratios in the grass flora of Namibia in relation to growth form, precipitation and habitat conditions. *Oecologia* 106: 352–369
- Schwarz AG and Reaney JT (1989) Perennating structures and freezing tolerance of northern and southern populations of C₄ grasses. *Bot Gaz* 150: 239–246
- Schwarz AG and Redmann RE (1988) C₄ grasses from the boreal forest region of northern Canada. *Can J Bot* 66: 2424–2430
- Seemann JR, Badger MR and Berry JA (1984) Variations in specific activity of ribulose-1,5-bisphosphate carboxylase between species utilizing differing photosynthetic pathways. *Plant Physiol* 74: 791–794
- Sharkey TD (1985) O₂-insensitive photosynthesis in C₃ plants. Its occurrence and a possible explanation. *Plant Physiol* 78: 71–75
- Sharkey TD (1988) Estimating the rate of photorespiration in leaves. *Physiologia Plantarum* 73: 147–152
- Sharkey TD, Seemann JR and Pearcy RW (1986) Contribution of metabolites of photosynthesis to post illumination CO₂ assimilation in response to lightflecks. *Plant Physiol* 82: 1063–1068
- Shreve F and Wiggins IL (1964) Vegetation and Flora of the Sonoran Desert. Stanford University Press, Stanford
- Simon J-P and Hatch MD (1994) Temperature effects on the activation and inactivation of pyruvate, Pi dikinase in two populations of the C₄ weed *Echinochloa crus-galli* (barnyard grass) from sites of contrasting climates. *Aust J Plant Physiol* 21: 463–473
- Skerman PJ and Riveros F (1990) Tropical Grasses—FAO Plant Production and Protection Series No. 23, Food and Agriculture Organization of the United Nations, Rome
- Skinner WR, Jefferies RL, Carleton TJ and Rockwell RF (1998) Prediction of reproductive success and failure in lesser snow geese based on early climatic variables. *Global Change Biol* 4: 3–16
- Smith M and Wu Y (1994) Photosynthetic characteristics of the shade-adapted C₄ grass *Muhlenbergia sobolifera* (Muhl.)Trin.: Control of development of photorespiration by growth temperature. *Plant Cell Environ* 17: 763–769
- Smith SD and Nobel PS (1986) Deserts. In: Baker NR and Long SP (eds) Photosynthesis in Contrasting Environments, pp 13–62. Elsevier, London
- Soares RV (1990) Fire in some tropical and subtropical South American vegetation types: An overview. In: Goldammer JG (ed) Fire in the Tropical Biota—Ecosystem Processes and Global Challenges, pp 63–81. Springer-Verlag, Berlin
- Solbrig OT (1996) The diversity of savanna ecosystems. In: Solbrig OT, Medina E and Silva JF (eds) Biodiversity of Savanna Ecosystem Processes—a Global Perspective, pp 1–27. Springer-Verlag, Berlin
- Sowunmi MA (1991) Late Quaternary environments in equatorial Africa: Palynological evidence. *Palaeoecol Afr* 22: 213–238
- Stace C (1997) New Flora of the British Isles, 2nd Ed. Cambridge University Press, Cambridge
- Stafford TJ, Fogel ML, Brendel K and Hare PE (1994) Late-Quaternary paleoecology of the southern high plains based on stable nitrogen and carbon isotope analysis of fossil bison collagen. In: Schafer H, Carlson D and Sobolik K (eds) The Archaic of the Southern North American Deserts. Texas A&M Press, College Station
- Stanley SM (1995) Climatic forcing and the origin of the human genus. In: Effects of Past Global Change of Life, pp 233–243. Board on Earth Sciences and Resources Commission on Geosciences, Environment, and Resources, National Research Council, National Academic Press, Washington, D. C.
- Stitt M (1991) Rising CO₂ levels and their potential significance for carbon flow in photosynthetic cells. *Plant, Cell and Environ* 14: 741–762
- Stowe LG and Teen JA (1978) The geographic distribution of C₄ species of the dicotyledonae in relation to climate. *Amer Nat* 112: 609–623
- Street-Perrott FA (1994) Paleo-perspectives: Changes in terrestrial ecosystems. *Ambio* 23: 37–44
- Street-Perrott FA, Huang Y, Perrott RA, Eglington G, Barker P, Khelifa LB, Harkness DD, and Olago DO (1997) Impact of lower atmospheric carbon dioxide on tropical mountain ecosystems. *Science* 278: 1422–1426
- Sun X, Song C, Wang F and Sun M (1997) Vegetation history of the Loess Plateau of China during the last 100,000 years based on pollen data. *Quaternary Int* 37: 25–36
- Takeba G and Kozaki A (1998) Photorespiration is an essential mechanism for the protection of C₃ plants from photooxidation. In: Satoh K and Murata N (eds) Stress Responses of Photosynthetic Organisms, pp 15–36. Elsevier, Amsterdam
- Talbot MR and Johannessen TA (1992) High resolution palaeoclimatic record for the last 27,500 years in tropical West Africa from the carbon and nitrogen isotopic composition of lacustrine organic matter. *Earth and Planetary Science Letters* 110: 23–37

- Teeri JA and Stowe LG (1976) Climatic patterns and the distribution of C_4 grasses in North America. *Oecologia* 23: 1–12
- Teeri JA, Stowe LG and Livingston DA (1980) The distribution of C_4 species of the Cyperaceae in North America in relation to climate. *Oecologia* 47: 307–310
- Tieszen LL, Hein D, Qvortrup SA, Troughton JH and Imbamba SK (1979) Use of $\delta^{13}C$ Values to Determine Vegetation Selectivity in East African Herbivores. *Oecologia* 37: 351–359
- Tieszen LL, Reed BC, Bliss NB, Wylie BK and DeJong DD (1997) NDVI, C_3 and C_4 production, and distributions in Great Plains grassland land cover classes. *Ecol Appl* 7: 59–78
- Tissue DT, Griffen KL, Thomas RB and Strain BR (1995) Effects of low and elevated CO_2 on C_3 and C_4 annuals. II. Photosynthesis and leaf biochemistry. *Oecologia* 101: 21–28
- Van der Merwe NJ and Tschauner H (1999) C_4 plants and the development of human societies. In: Sage RF and Monson RK (eds) C_4 Plant Biology, pp 509–549. Academic Press, San Diego
- Villagrán C and Armesto JJ (1993) Full and late glacial paleoenvironmental scenarios for the west coast of southern South America. In: Mooney HA, Fuentes ER and Kronberg BI (eds) Earth System Responses to Global Change—Contrasts between North and South America, pp. 195–207. Academic Press, San Diego
- Vitousek PM (1994) Beyond global warming: Ecology and global change. *Ecology* 75: 1861–1876
- Vogel JC, Fuls A and Ellis RP (1978) The geographical distribution of Kranz grasses in South Africa. *S Afr J Sci* 74: 209–215
- von Caemmerer S and Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153: 376–387
- von Caemmerer S and Furbank RT (1999) Modeling C_4 Photosynthesis. In: Sage RF and Monson RK (eds) C_4 Plant Biology, pp. 89–131. Academic Press, San Diego
- Walter H and Box EO (1983a) Middle Asian deserts. In: West NE (ed) Ecosystems of the World 5: Temperate Deserts and Semi-deserts, pp 79–104. Elsevier, Amsterdam
- Walter H and Box EO (1983b) The Karakum Desert, an example of a well-studied EUBIOME. In: West NE (ed) Ecosystems of the World 5: Temperate Deserts and Semi-deserts, pp 105–159. Elsevier, Amsterdam
- Walter H, Harnickell E and Mueller-Dombois D (1975) Climate—Diagram Maps of the Individual Continents and the Ecological Climatic Regions of the Earth—Supplement to the Vegetation Monographs. Springer-Verlag, Berlin
- Wang H, Ambrose SH, Liu CJ and Follmer LR (1997) Paleosol Stable Isotope Evidence for Early Hominid Occupation of East Asian Temperate Environments. *Quaternary Res* 48: 228–238
- Watson L and Dallwitz MJ (1998) Grass Genera of the World: Descriptions, Illustrations, Identification, and Information Retrieval; including Synonyms, Morphology, Anatomy, Physiology, Cytology, Classification, Pathogens, World and Local Distribution, and References, URL <<http://biodiversity.uno.edu/delta/>>
- Wedin DA (1995) Species, nitrogen and grassland dynamics: The constraints of stuff. In: Jones C and Lawton JH (eds) Linking Species and Ecosystems, pp 253–262. Chapman and Hall, London
- Wedin DA and Tilman D (1990) Species effects on nitrogen cycling: A test with perennial grasses. *Oecologia* 84: 433–411
- Wedin DA and Tilman D (1992) Nitrogen cycling, plant competition, and the stability of tallgrass prairie. In: Smith DD and Jacobs CA (eds) Proceedings of the Twelfth North American Prairie Conference: Recapturing a Vanishing Heritage, pp 5–8. University of Northern Iowa Press, Cedar Falls
- Wedin DA and Tilman D (1993) Competition among grasses along a nitrogen gradient: Initial conditions and mechanisms of competition. *Ecol Monogr* 63: 199–229
- Wedin, DA and Tilman D (1996) Influence of nitrogen loading and species composition on the carbon balance of grasslands. *Science* 274: 1720–1723
- Weltzin, JF and Coughenour MB (1990) Savanna tree influence on understory vegetation and soil nutrients in northwestern Kenya. *J Veg Sci* 1: 325–334
- West NE (1983) Intermountain salt-desert shrubland. In: West NE (ed) Ecosystems of the World 5: Temperate Deserts and Semi-deserts, pp 375–397. Elsevier, Amsterdam
- Wiggins IR and Porter DM (1971) Flora of the Galapagos Islands. Stanford University Press, Stanford
- Wong SC (1979) Elevated atmospheric partial pressure of CO_2 and plant growth. I. Interactions of nitrogen nutrition and photosynthetic capacity in C_3 and C_4 plants. *Oecologia* 44: 68–74.
- Young HJ and Young TP (1983) Local distribution of C_3 and C_4 grasses in sites of overlap on Mount Kenya. *Oecologia* 58: 373–377

Chapter 22

CO₂ Assimilation in C₃-C₄ Intermediate Plants

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Summary

The mode of CO₂ assimilation known as C₃-C₄ intermediate photosynthesis provides intriguing insight into a novel mechanism for reducing photorespiratory CO₂ loss and possible evolutionary pathways to C₄ photosynthesis. Plants with the C₃-C₄ pathway have been reported from twenty-five species in nine genera representing six families, and they are principally associated with warm or hot habitats. The ecological distribution of C₃-C₄ plants is consistent with an adaptive role for re-assimilation of photorespired CO₂ at warm temperatures. Photorespired CO₂ is re-assimilated through the differential partitioning of photorespiratory organelles between mesophyll cells and bundle-sheath cells in C₃-C₄ intermediates, including total isolation of glycine decarboxylase activity to the bundle-sheath. Thus, glycine diffuses to the bundle sheath tissue, where photorespired CO₂ is released and assimilated by surrounding chloroplasts before it can escape the leaf. Models of this ‘glycine shuttle’ reveal possible advantages in terms of CO₂ assimilation rate, water-use efficiency, and nitrogen-use efficiency, though there are strong constraints on the fraction of RuBP carboxylation capacity that can be allocated to the bundle sheath to assimilate the photorespired CO₂. The models further predict distinctive gas-exchange patterns with respect to the CO₂ compensation point and discrimination against ¹³CO₂. These predictions have been validated with gas-exchange measurements. One group of C₃-C₄ species (those belonging to the genus *Flaveria*) exhibit evidence of functional C₄ biochemistry and assimilation of at least some atmospheric CO₂ through the C₄ pathway. In the *Flaveria* intermediates, photorespiration rates are

reduced as in C_3 - C_4 intermediates from other genera, and in several *Flaveria* species O_2 inhibition of photosynthesis is reduced, suggesting the presence of a CO_2 -concentrating mechanism. It is not understood how photorespiration rates are reduced in these 'biochemical intermediates,' though there is evidence that at least part of it is due to the same glycine shuttle found in other C_3 - C_4 intermediates. The glycine shuttle may represent an initial step in the evolutionary path to C_4 photosynthesis.

I. Introduction

Plants that have been classified as C_3 - C_4 , or C_3 - C_4 intermediates, exhibit a unique mode of photorespiratory compartmentation between mesophyll and bundle-sheath cells. This unique mode of photorespiration is common to all C_3 - C_4 plants so far examined. It is important to realize from the outset, that the unique gas-exchange traits used to classify plants as C_3 - C_4 (i.e., low CO_2 compensation point and curvilinear response of the CO_2 compensation point to O_2) are due to unique aspects of photorespiratory compartmentation, not changes in photosynthetic metabolism (as occurs in C_4 plants). Most of the C_3 - C_4 intermediates have photosynthetic metabolism that is similar to C_3 plants. In a couple of genera, C_4 -type photosynthesis can be found, but its role in producing the unique gas-exchange traits that typify C_3 - C_4 plants is uncertain. In fact, the presence of C_4 photosynthesis in these species does not need to be invoked to explain the presence of C_3 - C_4 intermediate traits. Given that photorespiratory processes underlie C_3 - C_4 intermediate traits, it is somewhat misleading that this type of CO_2 assimilation is commonly labeled C_3 - C_4 photosynthesis or C_3 - C_4 intermediate photosynthesis.

C_3 - C_4 plants were first described, albeit with naiveté, in a report of anatomical variations among plants thought to possess C_4 photosynthesis (Laetsch, 1971). Among these observations was the report that the bundle sheath cells of *Mollugo verticillata*, a plant with C_3 -like carbon isotope ratios, contained numerous mitochondria and chloroplasts arranged in a tight centripetal pattern, with mitochondria lining the innermost tangential wall, surrounded by chloroplasts. A few years later, Kennedy and Laetsch (1974) reported C_4 -like CO_2 compensation points in this otherwise C_3 -like species. *Mollugo verticillata*

became the first reported C_3 - C_4 species. Since that time, a number of important discoveries have been made concerning the mechanism of C_3 - C_4 metabolism, the basis for C_3 - C_4 gas-exchange patterns, the nature of genetic control over C_3 - C_4 traits, and the significance of C_3 - C_4 intermediates to the evolution of C_4 photosynthesis (for reviews see, Monson et al., 1984; Edwards and Ku, 1987; Monson and Moore, 1989; Brown and Bouton, 1993; Rawsthorne and Bauwe, 1997). In this chapter we focus on the metabolic and physiological basis for C_3 - C_4 traits.

II. The Distribution of C_3 - C_4 Intermediates and the Advantages of CO_2 Assimilation in C_3 - C_4 Plants

C_3 - C_4 intermediates have been reported in twenty-five species from nine genera representing six families (Rawsthorne and Bauwe, 1997). Many of the genera occur in families that also have fully-expressed C_4 species, although this is not universal (e.g., *Moricandia* and *Parthenium* have C_3 - C_4 intermediates, but no C_4 species). Arguments have been posed to explain C_3 - C_4 intermediates as evolutionary precursors to C_4 photosynthesis (Monson et al., 1984; Brown and Hattersley, 1989; Monson, 1989a), although alternative explanations are possible (Monson and Moore, 1989). Recent studies using formal phylogenetic mapping on the basis of morphological traits (Monson, 1996) and molecular markers (Kopriva et al., 1996) have solidified the conclusion that C_3 - C_4 intermediate traits are the evolutionary antecedents to C_4 photosynthesis, at least in the genus *Flaveria*.

When grouped according to growth habit and ecological relationships, C_3 - C_4 intermediate traits tend to occur in fast-growing herbaceous species, that often exhibit the ruderal ecological habit, and undergo their greatest growth rates during the warmest part of the growing season (Monson, 1989a). The existence of these ecological patterns has been used to argue that the C_3 - C_4 mode of CO_2 assimilation provides adaptive advantages to carbon gain in warm

Abbreviations: A_{max} – light saturated photosynthesis; GDC – glycine decarboxylase; p_a – ambient CO_2 partial pressure; PEP – phosphoenolpyruvate; p_i – intercellular CO_2 partial pressure; PNUE – photosynthetic nitrogen use efficiency; PWUE – photosynthetic water-use efficiency; RUBP – ribulose 1,5-bisphosphate; SHMT – serine hydroxymethyltransferase

environments and in plants that exhibit high relative growth rates which must be accommodated by rapid rates of CO₂ assimilation (Monson, 1989a; Schuster and Monson, 1990). At warm temperatures photorespiration rate reaches a maximum due to decreases in the CO₂/O₂ solubility ratio and decreases in the affinity of Rubisco for CO₂ relative to O₂ (Ogren, 1984). Given that CO₂ assimilation through the C₃-C₄ intermediate path is actually a modification of photorespiratory patterns, the advantages of this metabolic mode should be greatest at warm leaf temperatures.

III. Photorespiratory Metabolism and Compartmentation in C₃ Versus C₃-C₄ Intermediate Species

A. Anatomy and Ultrastructure

The C₃-C₄ intermediate character is determined by anatomical and biochemical components which, when integrated, give rise to the gas exchange phenotypes that are described below. The leaves of C₃-C₄ intermediate species have a distinctive anatomy which differentiates them from either C₃ or C₄ species (Fig. 1) (Brown and Hattersley, 1989). The vascular bundles are surrounded by chlorenchymatous bundle-sheath cells which are reminiscent of the pronounced Kranz anatomy of leaves of C₄ plants. In C₃-C₄ intermediates, however, mesophyll cells are not arranged in a concentric ring around the bundle-sheath as in a C₄ leaf (Fig. 1). Instead, the mesophyll cells are arranged as in leaves of C₃ species with greater interveinal distances than in the leaves of C₄ plants (Brown and Hattersley, 1989). Bundle-sheath cells in the leaves of C₃ species in the genera which include C₃-C₄ intermediate species are chlorenchymatous but do not have specialized development that would differentiate them from the surrounding mesophyll cells (Brown and Hattersley, 1989; S. Rawsthorne, unpublished observations).

In all intermediate species studied to date, the bundle-sheath cells contain large numbers of organelles. Numerous mitochondria, peroxisomes, and many of the chloroplasts are located centripetally in the bundle-sheath cells (Edwards and Ku, 1987). Mitochondria are found along the cell wall adjacent to the vascular tissue and are overlain by chloroplasts (Fig. 2). Quantitative studies have shown that the mitochondria and peroxisomes are four times more

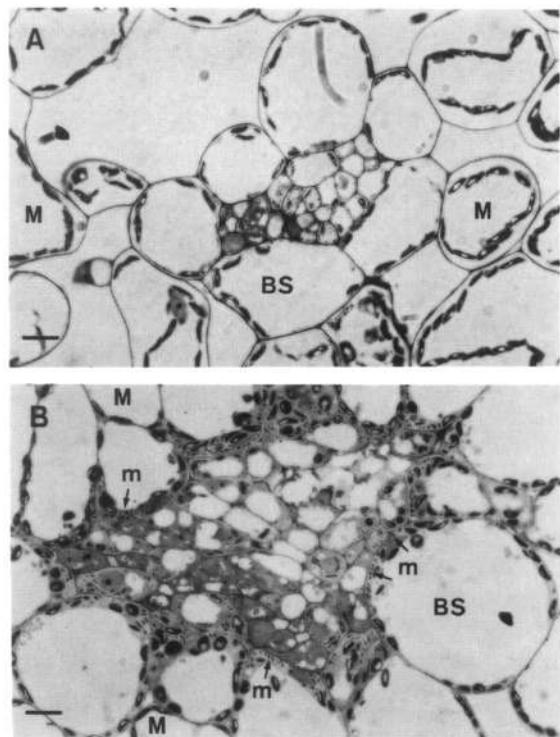


Fig. 1. Photomicrographs of sections through fully-expanded leaves of (a) the C₃ species *Brassica napus* and (b) the related C₃-C₄ intermediate species *Moricandia arvensis*. The prominent development of the bundle-sheath cells (BS) in the C₃-C₄ intermediate species is clearly seen, particularly the numerous mitochondria (m) on the centripetal faces of the bundle-sheath cells. The anatomy of the mesophyll (M) cells in both species are comparable. Scale bar = 20 μ m. (Micrographs kindly provided by Ms C. M. O'Neill, John Innes Centre, Norwich)

abundant per unit cell area than in adjacent mesophyll cells and that these mitochondria have twice the profile area of those in the mesophyll (Hylton et al., 1988; Brown and Hattersley, 1989). Recent work (E. L. Rylott and S. Rawsthorne, unpublished) has revealed that this anatomical development of the bundle-sheath cells in the C₃-C₄ intermediate *Moricandia arvensis* occurs from a C₃ default state. In other words, very early in the development of C₃-C₄ intermediate leaves the size and number of organelles in the bundle-sheath cells are comparable to those found in closely related C₃ species.

A further anatomical feature of leaves of C₃-C₄ intermediate species, at least in the genus *Panicum*, is revealed by a study of plasmodesmatal frequency between the bundle-sheath and mesophyll cells (Brown et al., 1983). C₃-C₄ intermediate species have a density of plasmodesmata at the bundle-

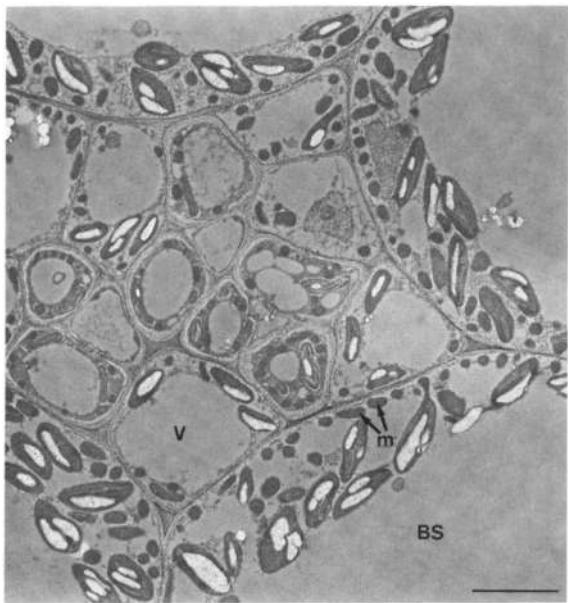


Fig. 2. Photomicrograph of a vascular bundle of the C_3 - C_4 species *Moricandia arvensis*. The presence of numerous mitochondria (m) on the innermost face of the bundle-sheath cells (BS) adjacent to the vascular cells (V) can be seen. Scale bar = 1.0 μm . (Micrograph kindly provided by Dr. E.L. Rylott, John Innes Centre, Norwich).

sheath/mesophyll interface which approaches that of C_4 species and is much greater than that of the C_3 species. In C_4 species, cellular anatomy is arranged so that the metabolism of the C_4 cycle occurs in adjacent bundle-sheath and mesophyll cells (Chapters 18, Furbank et al. and 19, Leegood). The exchange of metabolites between these cells occurs through diffusion down concentration gradients and is facilitated by the high density of shared plasmodesmatal connections (Chapter 19, Leegood). Based upon the present model for photorespiratory metabolism in C_3 - C_4 intermediate species (as discussed below), it is likely that the high density of plasmodesmata is required to facilitate metabolite exchange between cell types.

B. Photorespiratory Metabolism and the Importance of Glycine Decarboxylase

The leaves of C_3 - C_4 intermediate species in some genera, and notably *Flaveria*, are known to have activities of C_4 cycle enzymes that are elevated above those in related C_3 species. Given that C_4 photosynthesis is a mechanism that reduces photorespiration, expression of this pathway in C_3 - C_4 intermediate

species could be a means to decrease photorespiratory CO_2 loss. The extent to which C_4 metabolism contributes to the gas exchange phenotype of C_3 - C_4 intermediate species in those plants which express it, is unclear as discussed below. However, the role of cell-specific expression of glycine decarboxylase (GDC), the enzyme that catalyzes the release of CO_2 during photorespiration (Chapter 5, Douce and Heldt), in determining the C_3 - C_4 intermediate gas exchange phenotype is now undisputed.

In those species that have been studied, the maximum catalytic activities for enzymes involved in the photorespiratory pathway are broadly comparable in leaves of congeneric C_3 and C_3 - C_4 intermediate species. However, there are clear differences in the compartmentation of photorespiratory reactions within the leaves of C_3 - C_4 intermediate species of *Alternanthera*, *Flaveria*, *Moricandia*, *Mollugo*, *Panicum*, and *Parthenium*, compared to that in C_3 species. In the leaves of C_3 species all photorespiratory enzymes are present in all of the photosynthetic cells (Hylton et al., 1988; Rawsthorne et al., 1988ab; Tobin et al., 1989). This is not the case for C_3 - C_4 species. On the basis of immunogold localization studies and measurements of enzyme activity in protoplast fractions enriched in either bundle-sheath or mesophyll cells (Hylton et al., 1988; Moore et al., 1988; Rawsthorne et al., 1988ab; Devi et al., 1995) GDC is confined to the bundle-sheath cells of C_3 - C_4 species. Serine hydroxymethyltransferase (SHMT) activity is also enriched in bundle-sheath cells (Rawsthorne et al., 1988b). These two enzymes together catalyze the formation of serine, ammonia and CO_2 from two molecules of glycine in the mitochondria (Chapter 5, Douce and Heldt).

The early immunogold localization studies which addressed the distribution of GDC within the leaf used antibodies raised against the P protein of the complex. This protein subunit catalyses the release of CO_2 from glycine during the GDC reaction. More recent studies have shown that in the leaves of C_3 - C_4 intermediate species of *Panicum* and *Flaveria* the T, H, and L proteins of the GDC complex are also confined to bundle-sheath cells (Morgan et al., 1993). In leaves of *Moricandia arvensis* it is surprising that these three subunit proteins are still present in the mitochondria of the mesophyll cells (Morgan et al., 1993). The L protein, dihydrolipoamide dehydrogenase, is also a subunit (known as E3) of the mitochondrial pyruvate dehydrogenase complex

(Bourguignon et al., 1996) and its presence would therefore be expected in the mitochondria of all cells. However, studies have shown that the amount of the dihydrolipoamide dehydrogenase in the mitochondria which is not associated with GDC is small and the abundance of L protein in the mesophyll cell mitochondria of *M. arvensis* is not explained by this association with other multienzyme complexes (Bourguignon et al., 1988, 1996; Turner et al., 1992). Based on present knowledge, the T and H proteins only catalyze reactions that form parts of the overall activity of GDC. In the absence of P protein glycine decarboxylation will not occur. This means that the T, H, and L proteins would be redundant in the mesophyll cells of *M. arvensis*.

In further studies of *M. arvensis*, it is apparent that the ontogenetic development of GDC subunits is not necessarily coordinated with the development of C₃-C₄-like bundle-sheath tissue (Rylott et al. 1998). The GDC H-protein content of leaves increases from the earliest stages of leaf development, independent of developmental pattern in bundle-sheath. The increase in GDC P-protein, however, is synchronous with the ontogenetic development of mitochondria in the bundle sheath. This occurs when leaf length is between 6 and 12 mm, and there is a bundle sheath-specific, four-fold increase in the number of mitochondria followed by a doubling of their individual profile area. The P-protein is confined to bundle-sheath mitochondria throughout leaf development and its content in individual mitochondria increases prior to

the anatomical development of other aspects of the bundle sheath. In leaves and cotyledons that develop in the dark the expression of the P-protein and the organellar development is reduced, but the bundle-sheath cell-specificity is retained.

On the basis of the discovery that GDC activity is largely, if not completely, confined to the bundle-sheath cells of C₃-C₄ intermediate species, Rawsthorne et al. (1988a) proposed a model for photorespiratory metabolism in this group of plants which developed an earlier suggestion by Monson et al. (1984). The current model describing photorespiration in the C₃-C₄ intermediate species *M. arvensis* is shown in Fig. 3. Carbon dioxide is released in the bundle-sheath cells during photorespiration and the CO₂ released by the mitochondria must therefore pass out through the overlying chloroplasts in order to exit the leaf. The close association of mitochondria and the chloroplasts in the bundle-sheath cells is proposed to improve the extent of CO₂ recapture relative to that in C₃ species. In a C₃ leaf about 50% of the released CO₂ is recaptured, whereas in the leaf of a C₃-C₄ intermediate species this value can be as high as 75% (e.g. Hunt et al., 1987).

IV. C₃-C₄ Gas-Exchange Patterns as a Result of the Unique Compartmentation of Photorespiratory Metabolism

One of the unique traits of C₃-C₄ intermediates is the

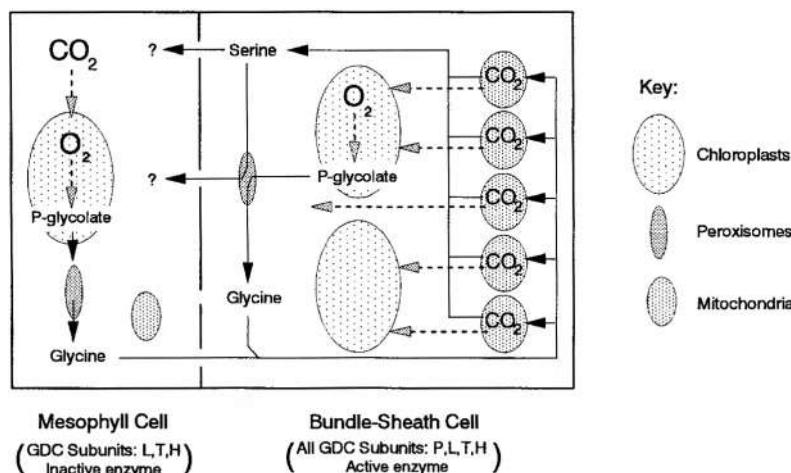


Fig. 3. A model for photorespiratory metabolism in the leaves of the C₃-C₄ intermediate species *Moricandia arvensis* (after Rawsthorne et al., 1988a). The relationship between the metabolism of bundle-sheath and mesophyll cells and the cell-specificity of expression of the subunits of glycine decarboxylase is indicated.

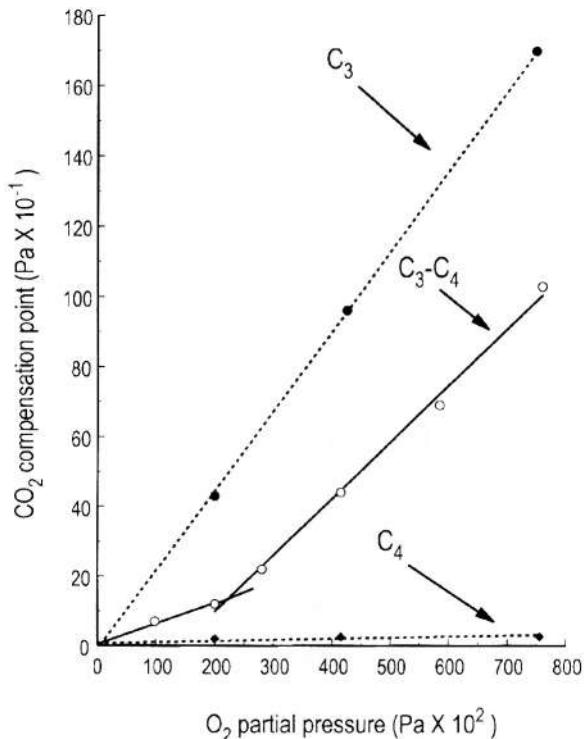


Fig. 4. Response of the CO_2 compensation point to ambient O_2 partial pressure in the C_3 species *Neurachne queenslandica*, the $\text{C}_3\text{-}\text{C}_4$ intermediate species *Panicum milioides*, and the C_4 species *Neurachne munroi*. Note the biphasic response of the $\text{C}_3\text{-}\text{C}_4$ intermediate species. Redrawn from Hattersley et al. (1986).

biphasic response of the CO_2 compensation point (Γ) to atmospheric O_2 concentration (Keck and Ogren, 1976; Quebedeaux and Chollet, 1977; Apel, 1980; Brown and Morgan, 1980; Holaday et al., 1982; Hattersley et al., 1986; Hunt et al., 1987). In the face of increased O_2 , Γ of C_3 plants increases linearly, a response that can be explained on the basis of Rubisco kinetics (Laing et al., 1974). In contrast, C_4 plants exhibit little response to changes in O_2 concentration. The biphasic response in $\text{C}_3\text{-}\text{C}_4$ intermediate plants is evident as a weaker response to low O_2 concentrations compared to higher O_2 concentrations (Fig. 4). The breakpoint between these responses is quite variable within and among $\text{C}_3\text{-}\text{C}_4$ species, and in some cases is not readily apparent (Hattersley et al., 1986). The biphasic response of Γ is of critical importance to explaining any mechanism of $\text{C}_3\text{-}\text{C}_4$ intermediate function (Monson et al., 1984; Peisker and Bauwe, 1984; Hattersley et al., 1986).

Three explanations of the biphasic response have been offered in past studies. Keck and Ogren (1976) proposed the presence of two carboxylases in the

same cell, one with a higher affinity towards inorganic carbon than the other (e.g., PEP carboxylase versus RuBP carboxylase). At low O_2 concentrations, it was hypothesized that the high-affinity carboxylase recycled photorespired CO_2 , lowering Γ . At higher O_2 concentrations, it was thought that this recycling system would become CO_2 -saturated as the rate of photorespiration increased, allowing Γ to increase according to a different linear relationship. Peisker and Bauwe (1984) modified this model to be consistent with data at the time, which suggested the presence of a limited, but complete, C_4 cycle in the most well-studied $\text{C}_3\text{-}\text{C}_4$ intermediate, *Panicum milioides* (Rathnam and Chollet, 1979). In the Peisker and Bauwe model, the C_4 cycle effectively recycles photorespired CO_2 at low O_2 concentrations, but becomes constrained by low PEP regeneration rates, at higher O_2 concentrations, resulting in higher CO_2 losses and a different linear response for Γ . This model lost support, however, when it was definitively shown that there was no functional C_4 cycle in *Panicum milioides* (Edwards et al., 1982).

Using the qualitative model proposed by Rawsthorne et al. (1988a) (Fig. 3), von Caemmerer (1989) developed a quantitative model of CO_2 flux rate in $\text{C}_3\text{-}\text{C}_4$ intermediate species. The model assumes the glycine shuttle and CO_2 recycling mechanism as discussed above. Using this model, von Caemmerer (1989) showed that the biphasic response of Γ could be explained with a variety of assumptions (Fig. 5). In essence, at low O_2 the recycling of photorespired CO_2 in the bundle-sheath cells is highly efficient and Γ is relatively low. As O_2 concentration increases, this system becomes overwhelmed and more photorespired CO_2 leaks out, increasing Γ . The predicted response is more curvilinear than linearly biphasic, but as pointed out by von Caemmerer (1989) the empirical results that have been collected to date are not of high enough quality to confidently discern details of the shape this response. Using the glycine shuttle model, it can be shown that as the fraction of total leaf Rubisco partitioned to the bundle-sheath cells increases, the extent of the low- O_2 phase becomes greater. This is because there is more Rubisco in the bundle-sheath cells to recycle photorespired CO_2 . Likewise, the low O_2 phase can be extended by assuming lower conductances to CO_2 leakage from the bundle-sheath cells (Fig. 5).

Recycling photorespired CO_2 through a glycine shuttle has the potential to increase the light-saturated CO_2 assimilation rate per unit of leaf area (A_{\max}).

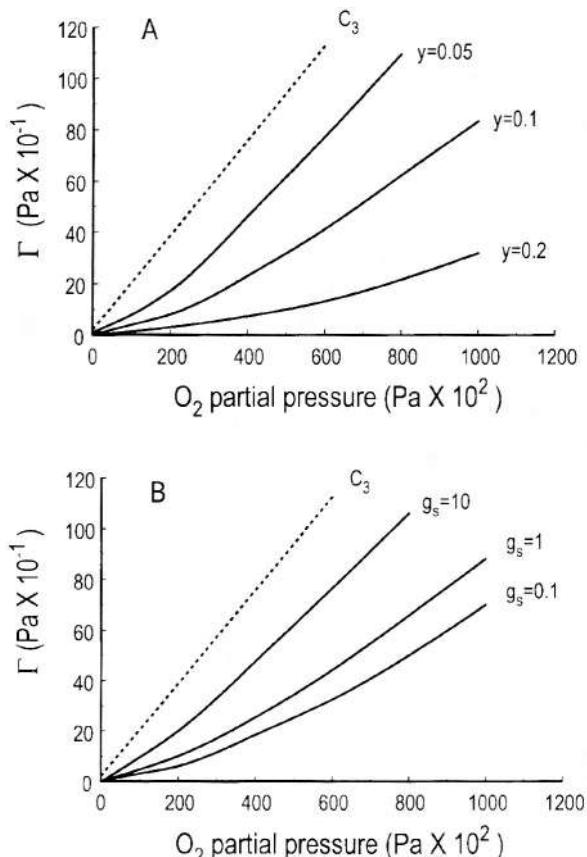


Fig. 5. Modeled responses of the CO_2 compensation point (Γ) to ambient O_2 partial pressure using a model of the glycine shuttle. In Panel A model results are provided for the C_3 state and three different values for the fraction of leaf Rubisco allocated to bundle-sheath cells (y). For this simulation, bundle sheath conductance to CO_2 was assumed to be $1 \text{ mmol m}^{-2} \text{ s}^{-1}$. In Panel B model results are presented for the C_3 state and three different values for bundle-sheath cell conductance to CO_2 diffusion (g_s ; in units of $\text{mmol m}^{-2} \text{ s}^{-1}$). In this simulation the fraction of Rubisco allocated to the bundle sheath was assumed to be 0.1. Redrawn from von Caemmerer (1989).

Because the bundle-sheath tissue of C_3 plants tends to exhibit low photosynthetic activity, increasing the capacity of this tissue for CO_2 assimilation in $C_3\text{-}C_4$ intermediates would increase the potential carboxylation rate per unit leaf area. This assumes that increasing the capacity for CO_2 assimilation of the bundle-sheath tissue occurs with no cost to CO_2 assimilation capacity of the mesophyll. In essence, the $C_3\text{-}C_4$ intermediate state allows for more photosynthetic machinery to be packed into each unit of leaf area. Even without a change in the total carboxylation capacity of the leaf, however, the glycine shuttle can potentially result in increased

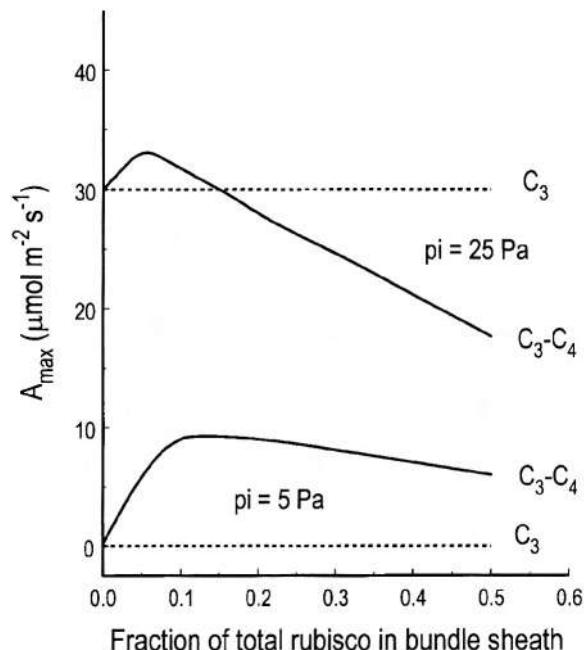


Fig. 6. Modeled responses of the maximum CO_2 assimilation rate per unit leaf area (A_{\max}) to the fraction of Rubisco allocated to bundle-sheath cells in a hypothetical $C_3\text{-}C_4$ intermediate species possessing the glycine shuttle. The simulation was conducted for two different mesophyll cell CO_2 concentrations, 25 and 5 Pa. Redrawn from von Caemmerer (1989).

A_{\max} . Using the model proposed by von Caemmerer (1989), it can be shown that with the proper balance between glycine decarboxylation rate and Rubisco carboxylation rate in the bundle sheath, CO_2 partial pressures in the bundle sheath can be an order of magnitude higher than in the intercellular air spaces. By allocating a small fraction of the leaf's Rubisco to the bundle sheath, as opposed to the mesophyll, it operates in an environment that reduces competitive inhibition by the oxygenase reaction and increases the rate of carboxylation (Fig. 6). As is evident in the data of Fig. 6, the advantage to A_{\max} only occurs when a small fraction (e.g., <15%) of the leaf's Rubisco is allocated to the bundle sheath. At higher fractions, A_{\max} is inhibited because (1) as more Rubisco is sequestered in the bundle sheath there is less oxygenase activity in the mesophyll and thus less glycine shuttled to the bundle sheath, and (2) as the amount of Rubisco in the bundle sheath increases it overwhelms the capacity of the glycine shuttle to provide CO_2 , and given limited access to intercellular CO_2 , the carboxylation rate of Rubisco decreases (von Caemmerer, 1989).

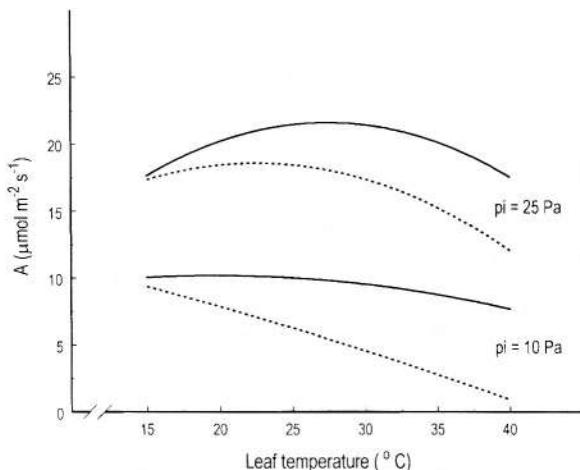


Fig. 7. The modeled response of CO_2 assimilation rate (A) to leaf temperature using the model of von Caemmerer (1989) for a glycine shuttle in the $\text{C}_3\text{-}\text{C}_4$ intermediate species, *Flaveria floridana*. In this case, the influence of C_4 -like biochemistry has been ignored. The simulation was conducted for two different intercellular CO_2 concentrations, 25 and 10 Pa. Redrawn from Schuster and Monson (1990).

On the basis of the simulation presented in Fig. 6, one would predict only small improvements (e.g., 10%) in A_{\max} due to the presence of a glycine shuttle. However, it is important to note that this simulation was conducted at 25 °C. At higher temperatures, the ratio of oxygenase to carboxylase activity increases, and there is greater potential for the glycine shuttle to increase A_{\max} . At intercellular CO_2 partial pressures (p_i) typical of C_3 and $\text{C}_3\text{-}\text{C}_4$ species (e.g., 25 Pa) A_{\max} is predicted to be 23–32% higher in $\text{C}_3\text{-}\text{C}_4$ species at leaf temperatures between 35–40 °C, compared to C_3 species (Fig. 7). At lower intercellular CO_2 partial pressures, this difference increases to reflect a 71–87% improvement in $\text{C}_3\text{-}\text{C}_4$ species, compared to C_3 species. The interactions among leaf temperature, p_i , and mode of carbon assimilation can be further seen in the data presented in Fig. 8. In this case, light-saturated photosynthesis rates for the $\text{C}_3\text{-}\text{C}_4$ species are predicted to be higher than those for the C_3 species at all values for p_i , a difference that increases at 35 °C, relative to 25 °C.

Improvements in A_{\max} through the $\text{C}_3\text{-}\text{C}_4$ intermediate mechanism occur without a need for increasing p_i through increased stomatal conductance. As discussed above, internally-generated, photorespired CO_2 is re-assimilated. Such a mechanism would be advantageous in that improvements should occur in photosynthetic water-use efficiency (PWUE;

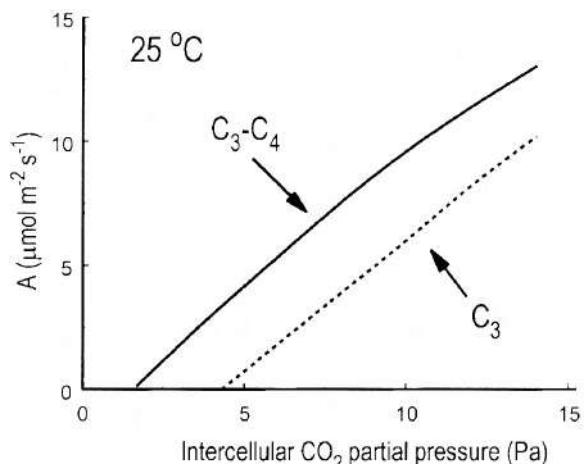


Fig. 8. The modeled response of CO_2 assimilation rate (A) to intercellular CO_2 partial pressure using the model of von Caemmerer (1989) for a glycine shuttle in the $\text{C}_3\text{-}\text{C}_4$ intermediate *Flaveria floridana*. The simulation was conducted for two different leaf temperatures, 25 and 35 °C. Redrawn from Schuster and Monson (1990).

CO_2 assimilated per unit of H_2O transpired). Once again, given that the enhancement of A_{\max} depends upon re-cycling of photorespired CO_2 , improvements in PWUE should be greatest at warm leaf temperatures (Fig. 9). As an alternative to variation in p_i , A_{\max} in C_3 plants is influenced by the fraction of leaf nitrogen allocated to Rubisco. Greater allocation to Rubisco would increase the potential rate of carboxylation per unit of leaf area, but unless there are concomitant increases in stomatal conductance, p_i will decrease and the increased A_{\max} will occur at a cost to photosynthetic nitrogen-use efficiency (PNUE; CO_2 assimilated per unit of N allocated to Rubisco). As discussed above, it is likely that a fraction of the

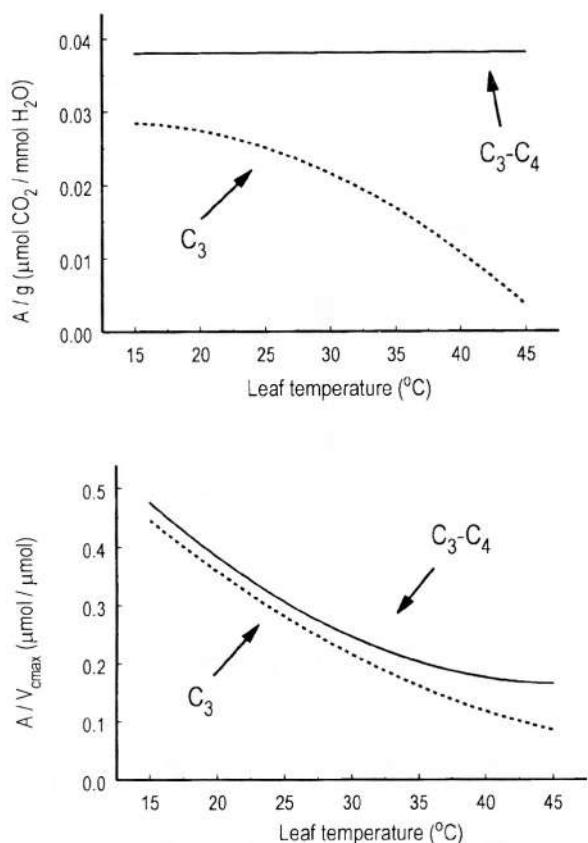


Fig. 9. The modeled response of leaf photosynthetic water-use efficiency (estimated as the ratio of CO₂ assimilation rate, A, to leaf conductance to H₂O, g), and leaf photosynthetic nitrogen-use efficiency (estimated as the ratio of A to the maximum carboxylation capacity V_{cmax}), as a function of leaf temperature. The response was modeled assuming the presence of glycine shuttle and parameterized for the C₃-C₄ intermediate species, *Flaveria floridana*. Redrawn from Schuster and Monson (1990).

Rubisco in C₃-C₄ intermediates operates with greater carboxylation efficiency, and thus greater PNUE, than that for C₃ species. This enhanced carboxylation efficiency is dependent on the re-cycling of photorespired CO₂, and thus any improvements in PNUE should be greatest at warm leaf temperatures. This can be demonstrated using the von Caemmerer (1989) model of C₃-C₄ intermediates (Fig. 9).

At this time it is not entirely clear as to the adaptive advantages, if any, imparted by C₃-C₄ intermediate patterns of CO₂ assimilation. However, as demonstrated by the model simulations described above, it is possible that the re-cycling of photorespired CO₂ provides advantages in terms of A_{max}, PWUE, and PNUE. In gas-exchange studies of different C₃-C₄ intermediate species, advantages of these types have

been noted at low p_i values (Brown and Simmons, 1979; Brown, 1980; Hattersley et al., 1986). To date, however, the advantages in terms of enhanced A_{max} at the p_i values characteristic of normal atmospheric CO₂ conditions (e.g., p_i = 25 Pa and p_a = 36 Pa), have not been observed for C₃-C₄ species, compared to related C₃ species. The model of von Caemmerer (1989) can be used to predict a 12–25% enhancement in A_{max} at 25 °C, depending upon specific parameterization values. Measurements have generally revealed no significant differences in A_{max} between C₃-C₄ and C₃ congeners in this temperature range. At present it is not clear whether the model reflects an unrealistic situation, or whether congeneric differences other than the mode of CO₂ assimilation might offset the predicted differences in A_{max}.

V. The Biochemical Intermediacy of *Flaveria* C₃-C₄ Intermediates and its Relationship to Reductions in Photorespiration

There is clear evidence that C₃-C₄ intermediates in the genera *Alternanthera*, *Moricandia*, *Panicum* and *Parthenium* do not have a C₄ cycle which could account for their low rates of photorespiration. It is assumed that the C₃-C₄ intermediate traits associated with these species are due to the glycine shuttle described above. Evidence against the existence of C₄ photosynthesis in C₃-C₄ intermediates of these genera comes from studies of the initial products of ¹⁴CO₂ assimilation by leaves, and of the activities and cellular locations of C₄-cycle enzymes (as discussed in Rawsthorne et al., 1992). Label from ¹⁴CO₂ is not transferred from C₄ compounds to Calvin cycle intermediates during photosynthesis. Activities of PEP carboxylase and the C₄ cycle decarboxylases are far lower than in C₄ leaves, and RuBP carboxylase and PEP carboxylase are both present in mesophyll and bundle-sheath cells. Early work on *Mollugo verticillata* suggested that limited C₄ metabolism might operate in this species (Sayre et al., 1979). However, more recent assessment of this data (Edwards and Ku, 1987) suggests that C₄ photosynthesis is unlikely to account for the C₃-C₄ intermediate gas exchange traits of this species.

C₃-C₄ species in the genus *Flaveria* exhibit patterns of ¹⁴CO₂ assimilation that reflect considerable C₄-cycle activity (Monson et al., 1986). However, in most of these *Flaveria* species the presence of the C₄ cycle is ineffective at reducing the level of O₂

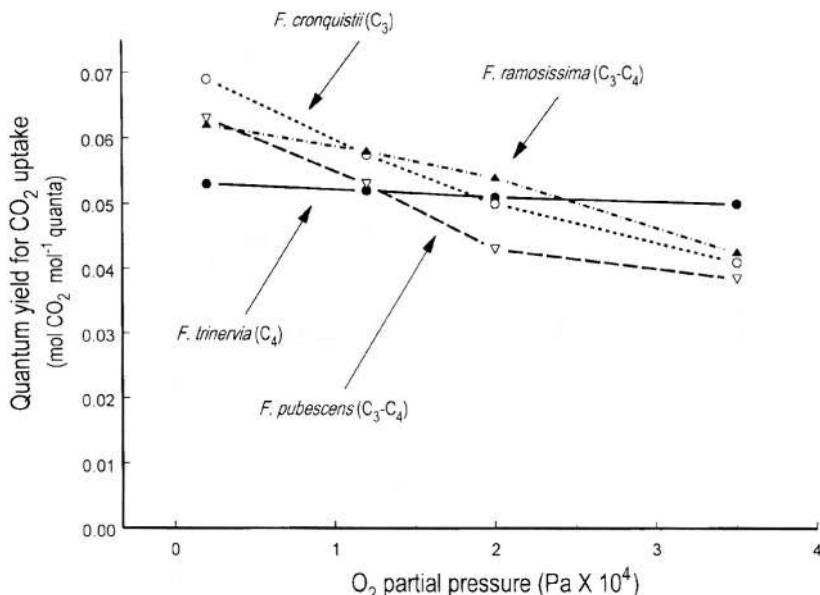


Fig. 10. Quantum yield measurements in response to ambient O_2 partial pressure in various *Flaveria* species. The low quantum yield values at $2 \times 10^3 \text{ Pa}$ (2% O_2) for the C_3-C_4 intermediates *F. ramosissima* and *F. pubescens*, compared to the C_3 species *F. cronquistii*, presumably reflect the extra energy requirement of the partial C_4 cycle in the intermediate species. The lower quantum yield value for *F. pubescens* at normal O_2 ($2.1 \times 10^4 \text{ Pa}$ or 21%), compared to the C_3 or C_4 species presumably reflects inefficient operation of the C_4 cycle with no concomitant reduction in O_2 inhibition of carboxylation. The fact that the quantum yield for the C_3-C_4 intermediate *F. ramosissima* is higher at $2 \times 10^4 \text{ Pa}$ O_2 suggests that the energy required to operate its C_4 cycle is offset by reductions in photorespiratory energy consumption through reduced O_2 inhibition of carboxylation. Redrawn from Monson et al. (1986).

inhibition of A_{\max} and on the basis of quantum-yield analyses, there is evidence of futile cycling of CO_2 between the C_3 and C_4 cycles and ineffective CO_2^- -concentrating activity (Fig. 10). Thus the C_4 cycle, while being present in these intermediate species, functions inefficiently. At this time, reductions in photorespiration in most C_3-C_4 *Flaveria* species cannot be ascribed to reductions in RuBP oxygenase activity through a CO_2 concentrating mechanism. In addition to the presence of C_4 -like biochemistry, C_3-C_4 *Flaveria* species possess the glycine shuttle described above (Hylton et al., 1988). It is possible that the reduced photorespiration rates of these biochemically-intermediate species are due to the glycine shuttle, and have no relationship to the presence of C_4 -like biochemistry. Further studies of this issue are warranted.

In some C_3-C_4 *Flaveria* species, there is evidence for reduced RuBP oxygenase activity, possibly due to the presence of inefficient, though effective, C_4 -like biochemistry. In *Flaveria ramosissima*, 50–60% of the atmospheric CO_2 assimilation occurs through the C_4 pathway (Rumpho et al., 1984; Monson et al., 1986). Gas-exchange measurements with this species

also reveal evidence for reductions in the inhibition of A_{\max} and the quantum yield for CO_2 uptake by 21 kPa O_2 (Rumpho et al., 1984; Monson et al., 1986). However, this species also exhibits differential compartmentation of GDC (Moore et al., 1988). Thus, while a reduction in the O_2 inhibition of photosynthesis is likely to be due to the presence of C_4 -like CO_2 -concentrating activity, the potential role of a glycine shuttle cannot be excluded. Moreover, the C_4 -like CO_2 concentrating activity may not completely account for the observed low CO_2 compensation point. A similar case can be made for the so-called C_4 -like C_3-C_4 intermediate *Flaveria* species (Monson et al., 1987; Moore et al., 1989). In these species 80–90% of the atmospheric CO_2 assimilation occurs through the C_4 pathway. Additionally, one can observe evidence for significant suppression of RuBP oxygenation in 21 kPa O_2 . Once again, it is likely that the C_4 -like CO_2 -concentrating activity is responsible for a fraction of the lower CO_2 compensation points in these species. However, the precise contribution of the C_4 cycle relative to the glycine shuttle is not known.

There are anomalous metabolic patterns associated

with the assimilation of CO₂ in C₃-C₄ *Flaveria* species that remain to be explained. For example, in contrast to C₃ and C₄ species, significant quantities of ¹⁴CO₂ are assimilated into glycine, serine and, rather unusually, fumarate in leaves of C₃-C₄ *Flaveria* species (Monson et al., 1986). The appearance of ¹⁴C in fumarate is likely to be due to the presence of the large fumarate pool which is present in their leaves (Rawsthorne et al., 1992). The maximum catalytic activities of enzymes involved in C₄ photosynthesis (PEP carboxylase, NADP-malate dehydrogenase, NADP-malic enzyme and pyruvate, Pi dikinase (PPDK) are reported to be greater in a number of C₃-C₄ *Flaveria* species than in C₃ species or C₃-C₄ species from other genera (Ku et al., 1983). However, these activities are only 10–15% of those extracted from *Flaveria* species which have been classified as C₄ or C₄-like (Ku et al., 1991). In addition, compartmentation between bundle-sheath and mesophyll cells of the enzymes involved in the carboxylation and decarboxylation stages of the C₄ cycle is not complete or does not occur in leaves of *F. ramosissima* (C₃-C₄) or *F. brownii* (C₄-like) as judged from protoplast fractionation experiments. The latter species has maximum catalytic activities of PEP carboxylase, PPDK and NADP-ME which approach those in true C₄ or other C₄-like *Flaveria* species which have more limited (5–10 %) direct fixation of CO₂ by Rubisco (Moore et al., 1989). There is no marked differential distribution of Rubisco or PEP carboxylase between mesophyll and bundle-sheath in any C₃-C₄ *Flaveria* species examined to date (Bauwe, 1984; Reed and Chollet, 1985). Enrichment of Rubisco in the bundle-sheath relative to PEP carboxylase and vice versa for the mesophyll has been reported for *F. brownii*. However, without complete separation of the enzymes involved in the carboxylation and decarboxylation phases of the C₄ pathway futile cycling of CO₂ through C₄ acids and hence extra energy consumption may occur.

In terms of gas-exchange, the C₃-C₄ *Flaveria* species exhibit many of the same advantages reported for species with only a glycine shuttle. In a study of C₃-C₄ intermediate *Flaveria* species, Monson (1989b) noted improvements in PWUE at low p_i, but no improvements in PNUE in two out of the three species studied, when compared to a C₃ congener. The one C₃-C₄ species that exhibited improved PNUE is *F. ramosissima*, a species with reduced O₂ inhibition of photosynthesis, and presumably a somewhat effective CO₂-concentrating mechanism. In the C₃-

C₄ intermediate *Flaveria floridana*, improvements in A_{max} at warm leaf temperatures have been observed, even at normal p_i values (Schuster and Monson, 1990; Monson and Jaeger, 1991). It is not clear whether these improvements are due to the glycine shuttle system, or a C₄-like CO₂-concentrating mechanism.

VI. Intercellular Metabolite Movement in C₃-C₄ Leaves

The partitioning of photorespiratory metabolism between bundle-sheath and mesophyll cells in the leaves of C₃-C₄ species has a number of implications for the movement of metabolites between the two cell types. The first relates to how the metabolites are transported between the cells. Studies of leaf anatomy in C₃-C₄ intermediate species (Brown and Hattersley, 1989) reveal that glycine produced during photorespiration in mesophyll cells may have to move over several cell distances in order to be decarboxylated in bundle-sheath cells. How this glycine shuttle is facilitated is not known but exchange between bundle-sheath and mesophyll is likely to be via plasmodesmatal interconnections as described above. Rawsthorne and Hylton (1991) have speculated that glycine may move down a concentration gradient from the mesophyll to the bundle-sheath based upon measurements of glycine pool size during light/dark transitions and on the localization of GDC to the bundle-sheath cells. The change in glycine content of C₃-C₄ leaves during the immediate post-illumination period and of the labeling of glycine during pulse-chase experiments with ¹⁴CO₂, indicate that the glycine pool is a result of photorespiration and is much larger in C₃-C₄ than in C₃ leaves (Holaday and Chollet, 1983; Monson et al., 1986; Rawsthorne and Hylton, 1991).

A second implication of the C₃-C₄ glycine shuttle (Fig. 3) is that leaf nitrogen metabolism must be different from that in C₃ species. The GDC reaction produces ammonia which would need to be reassimilated. In a C₃ leaf this assimilation occurs in all photorespiring cells. In a C₃-C₄ leaf, however, the same rate of reassimilation occurs in a much smaller proportion of the leaf cells (i.e. in the bundle-sheath). This would require that nitrogen be returned to the mesophyll cells. To date there is no definitive evidence that the activity of enzymes required for the reassimilation of ammonia are elevated in bundle-

sheath, as compared to mesophyll, cells in the leaves of C₃-C₄ species. However, where reported, the activities would be high enough to account for the ammonia flux in vivo which can be estimated from the rate of photorespiratory CO₂ release (Rawsthorne et al., 1988b).

The production of serine by GDC/SHMT, and the requirement to reassimilate ammonia, in the bundle-sheath cells are clear consequences of the metabolic scheme proposed in Fig. 3. However, it is unclear in what form the carbon and nitrogen that is lost from the mesophyll cells, in the form of glycine efflux, is returned. Rawsthorne et al. (1992) have argued that return of carbon to the mesophyll is essential if the plant is to maintain flux through the photosynthetic carbon reduction cycle. It is possible that serine produced in the bundle-sheath could diffuse back to the mesophyll and so return one of the two nitrogens and three of the four carbon atoms derived from the original two molecules of glycine. How or whether the nitrogen balance between mesophyll and bundle-sheath is maintained is not known.

VII. Carbon Isotope Discrimination Patterns in C₃-C₄ Intermediates

The recycling of photorespired CO₂ should cause a shift in the carbon isotope composition of assimilated carbon. This is because Rubisco in the bundle sheath cells of C₃-C₄ intermediates has the opportunity to discriminate against ¹³CO₂ that has been released through decarboxylation of glycine that already carries a 'light' δ¹³C value relative to the atmosphere. Thus, the total discrimination against ¹³C should be slightly higher in C₃-C₄ intermediate plants, compared to C₃ plants. The level of isotopic discrimination should increase at low p_i values, since the fraction of recycled CO₂ to total assimilated CO₂ will increase (von Caemmerer, 1989). These predictions have been tested using on-line, isotopic fractionation measurements. When measured with leaves of *Panicum milioides*, a C₃-C₄ intermediate species that possesses the glycine shuttle, short-term discrimination against ¹³C was not significantly different from C₃ species at normal, ambient CO₂, but was substantially higher at low CO₂ (Fig. 11). It is not clear why the presence of the glycine shuttle does not affect the discrimination signature at normal, ambient CO₂. However, it is consistent with the fact that modeled influences of the glycine shuttle, whether they relate to predictions

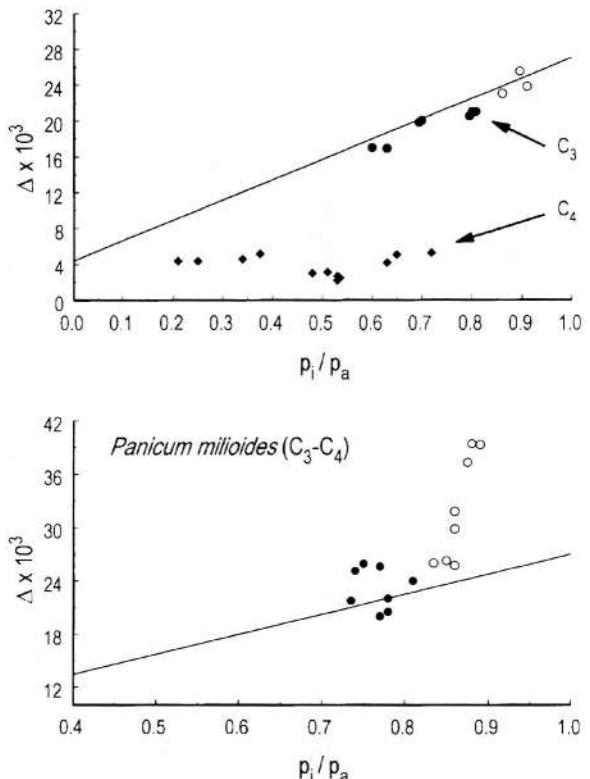


Fig. 11. In the upper panel is presented the measured response of discrimination against ¹³CO₂ (Δ) to the ratio of intercellular and ambient CO₂ partial pressures (p_i/p_a) for two C₃ species (data pooled for *Phaseolus vulgaris* and *Flaveria pringlei*) at p_a = 330 μbar (closed symbols) or p_a = 100 μbar (open symbols), and a C₄ species (data pooled from several experiments with *Amaranthus edulis*). The solid line represents the theoretical relationship between these variables as predicted from the model of Farquhar et al. (1982). In the lower panel is presented the measured response of Δ to p_i/p_a for the C₃-C₄ intermediate species, *Panicum milioides*. Values in the lower panel represent measurements made at p_a = 330 μbar (closed symbols) or p_a = 100 μbar (open symbols). Redrawn from von Caemmerer and Hubick (1989).

of A_{max} or discrimination against ¹³C, are difficult to observe at normal, ambient CO₂.

In those C₃-C₄ intermediate species that possess C₄-like biochemistry, discrimination against ¹³C is influenced, not only by the presence of the glycine shuttle, but also by the degree to which the C₄ and C₃ cycles are integrated (Monson et al., 1988). An apparent enigma occurs in that C₃-C₄ *Flaveria* species with considerable capacity to assimilate atmospheric CO₂ through the C₄ pathway, exhibit C₃-like carbon isotope ratios (expressed as δ¹³C values). In an early analysis, Peisker (1985) explained this pattern by invoking a PEP-regeneration constraint to the activity of PEP carboxylase, and minimizing assimilation of

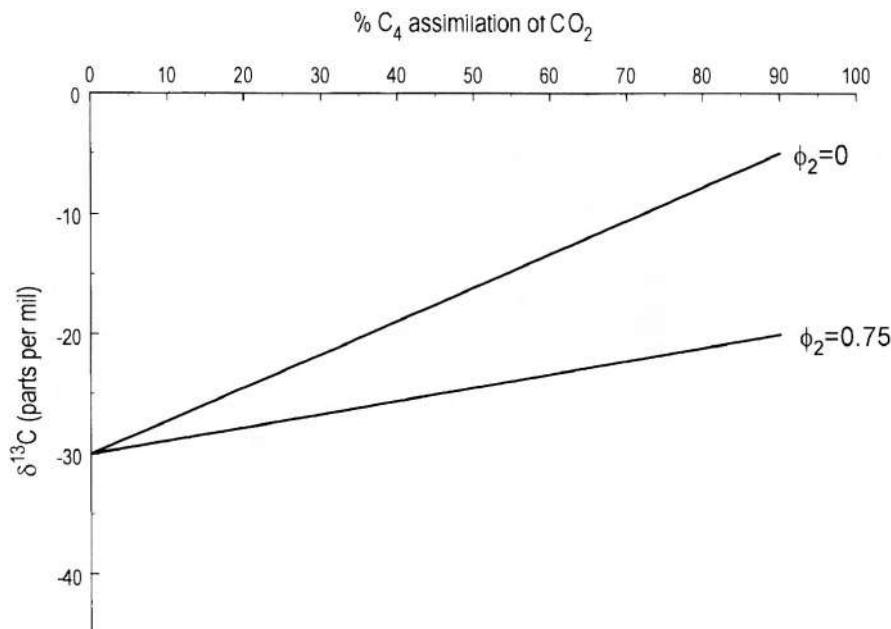


Fig. 12. The modeled relationship between the % of CO₂ assimilated by the C₄ pathway and the expected leaf tissue carbon isotope ratio (expressed as $\delta^{13}\text{C}$) at two different values for bundle-sheath CO₂ leakage (ϕ). The simulation was conducted according to the models described in Monson et al. (1988) and Farquhar (1983). The relationship demonstrates that as the proportion of CO₂ assimilated by the C₄ pathway increases in C₃-C₄ intermediate species, discrimination against ¹³C should decrease, and this will be dependent on the capacity for CO₂ to leak from the bundle-sheath compartment.

atmospheric CO₂ through the C₄ pathway. With this model, C₃-like $\delta^{13}\text{C}$ values are the result of C₄ photosynthetic activity that is too low to significantly affect the carbon isotope signature.

In an alternative approach, Monson et al. (1988) allowed for significant rates of CO₂ assimilation through the C₄ pathway, but invoked a low degree of coordination between the C₄ and C₃ cycles. Thus, the C₄ system is ‘leaky’. In other words, CO₂ that is assimilated and then released through decarboxylation by the C₄ pathway has a limited potential to accumulate at the site of RuBP carboxylation. High leakage of CO₂ from the site of RuBP carboxylation provides RuBP carboxylase the opportunity for discrimination against ¹³C, and shifts the predicted $\delta^{13}\text{C}$ value toward the C₃-like extreme (Fig. 12). Using a model based on the calculation of CO₂ leakage rates from measured quantum yields, Monson et al. (1988) predicted only small shifts in the $\delta^{13}\text{C}$ value of C₃-C₄ intermediate *Flaveria* species from the C₃ extreme, even for those species that assimilate up to 50% of their carbon through the C₄ pathway. In *Flaveria*, effective integration of the C₃ and C₄ cycles only occurs in species that assimilate more than 60% of their carbon through the C₄ pathway (Fig. 13).

This suggests that there is an upper limit beyond which the evolution of increased activity of an inefficient, poorly integrated C₄ cycle becomes too costly. In *Flaveria*, this limit appears to be the point at which more than 50% of the assimilated carbon occurs through the C₄ pathway. Above this limit, selection tends to improve integration between the C₄ and C₃ pathways at the same time that the fractional magnitude of C₄ assimilation increases.

VIII. C₃-C₄ Intermediates and the Evolution of C₄ Photosynthesis

There has been much speculation as to the significance of C₃-C intermediates in the evolutionary transition from the C₃ to C₄ state (e.g. Monson and Moore, 1989; Monson 1996, 1999). As discussed above, CO₂ assimilation through the C₃-C₄ pathway can give rise to improved photosynthetic performance at warm temperatures when comparisons are made with C₃ plants (Schuster and Monson, 1990). Improved gas exchange characteristics at such temperatures could translate into improved fitness and so provide the basis for the evolution of more C₄-like traits

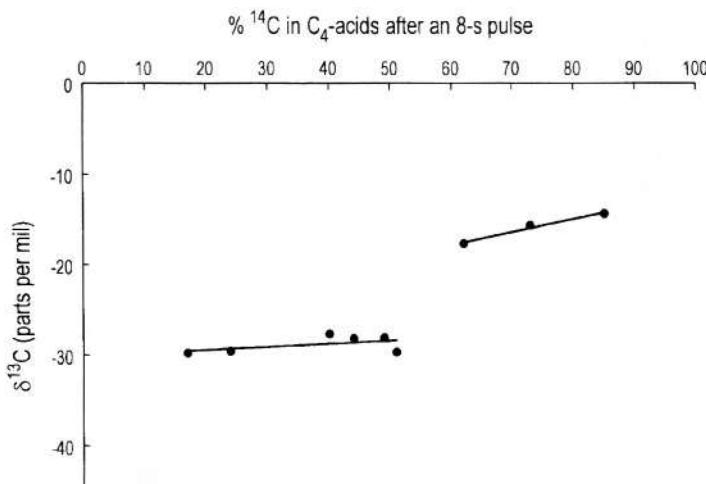


Fig. 13. The measured relationship between the amount of ambient $^{14}\text{CO}_2$ assimilated into C_4 organic acids following an 8-s exposure and the leaf carbon isotope ratio (expressed as $\delta^{13}\text{C}$). The data were derived from pooled measurements on different *Flaveria* species as described in Monson et al. (1988). Each point represents the average of measurements taken from a single species. Different points represent measurements taken from different species.

(Monson and Jaeger, 1991). In recognizing this possibility one cannot be certain of the driving force behind such an evolutionary event, nor can one be certain of the steps in which such evolution occurred. The evolution of $\text{C}_3\text{-C}_4$ intermediate traits could have improved water-use-efficiency, reduced photorespiratory CO_2 loss, or both, and thereby contributed to adaptive advantages in comparison to C_3 progenitors. This would have been particularly true if, as predicted in paleoclimate models, atmospheric CO_2 concentrations have decreased since the Miocene, thereby increasing photorespiration rates relative to photosynthesis rates (Ehleringer et al., 1991).

Based upon our knowledge of metabolism and gas exchange in present day C_3 , $\text{C}_3\text{-C}_4$, and C_4 species across and within genera it is reasonable to speculate upon how C_4 photosynthesis might have evolved from the older C_3 mechanism. By drawing upon the spectrum of $\text{C}_3\text{-C}_4$ phenotypes which exist, a sequence of discrete steps is apparent, each representing a progressive change towards C_4 photosynthesis (Fig. 14). Whether such steps actually occurred we do not know, but the fact that they are drawn from across the higher plant phylogenetic tree suggests that the polyphyletic evolution of C_4 photosynthesis may have had common mechanistic origins.

The similar anatomical development of bundle-sheath cells and the consistent absence of GDC from mesophyll cells in all $\text{C}_3\text{-C}_4$ species studied to date indicates that the differential distribution of GDC could have been a primary event in the evolution of

C_4 photosynthesis. In *Panicum laxum* the partial loss of GDC from the mesophyll (Hylton et al., 1988) and partial development of the bundle-sheath cells (Brown et al., 1983) are suggestive of this species representing an early step in this process. This step could have been followed by total loss of at least one of the GDC proteins from the mesophyll, as in *M. arvensis* followed by more complete loss of GDC proteins as seen in other $\text{C}_3\text{-C}_4$ species. Consistent with this hypothesis, GDC in C_4 species is, as in $\text{C}_3\text{-C}_4$ species, confined to bundle-sheath cells (Ohnishi and Kanai, 1983; Hylton et al., 1988).

How the evolution of true C_4 metabolism, as may be represented in the genus *Flaveria* (Fig. 13), occurred upon this base of partitioning of photorespiratory metabolism is unclear. The common requirements of the C_4 and $\text{C}_3\text{-C}_4$ intermediate pathways for intercellular metabolite exchange represent a possible link between the two mechanisms. Perhaps C_4 metabolism evolved initially as a consequence of the partitioning of photorespiratory metabolism and the consequences for C and N exchange between the bundle-sheath and mesophyll. Recruitment of existing ' C_3 genes' and changes in their expression in C_4 species is certainly evident (Ku et al., 1996; Monson, 1999). The studies of genes encoding C_4 enzymes have revealed that in some instances the expression of a single orthologous gene present in C_3 , $\text{C}_3\text{-C}_4$ and C_4 species has been increased and targeted to a specific cell type in the C_4 species. In other cases the ancestral ' C_3 gene' has

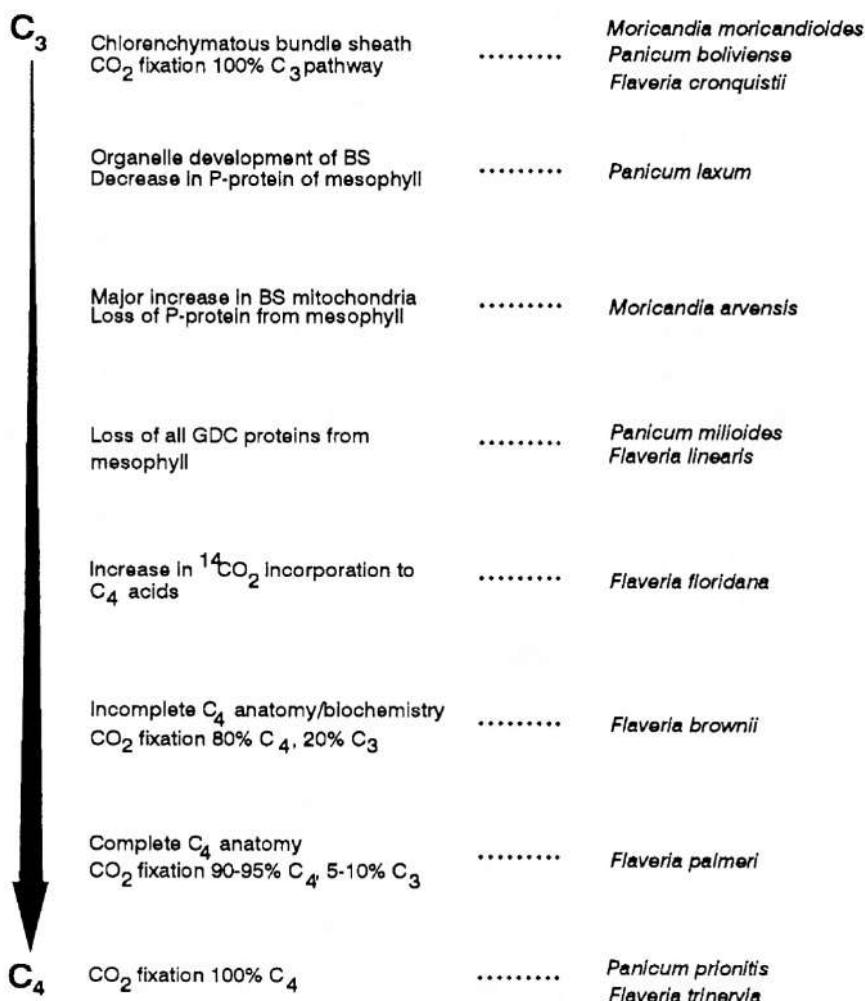


Fig. 14. A possible scheme for the evolution of C₄ photosynthesis from the C₃ mechanism. Discrete steps are identified by drawing upon observations from present day C₃, C₃-C₄, C₄-like, and C₄ species from the genera *Moricandia*, *Panicum* and *Flaveria*.

been replicated and the expression of the replicated gene has then been modified to provide a C₄-type expression pattern (see Ku et al., 1996 for details). Recent studies of the *Flaveria* species have, however, provided the first convincing evidence that C₃-C₄ plants do indeed represent intermediates in the evolution between C₃ and C₄ photosynthesis. One of the GDC subunits, the moderately conserved H-protein, has now been used as molecular marker to examine phylogenetic relationships within the genus (Kopriva et al., 1996). A phylogenetic tree of these sequences comprises three main clusters: (i) the C₃ species *F. pringlei* and *F. cronquistii*, (ii) all C₃-C₄ species including the C₄-like *F. brownii*, and (iii) the advanced C₄ species *F. australasica*, *F. trinervia*, *F. bidentis*, and *F. palmeri* (Kopriva et al., 1996). The

ancient position of C₃ photosynthesis in the genus is clearly reflected, as well as a twofold and independent evolution of C₄ photosynthesis in different lineages. Perhaps more importantly these data provide direct evidence for the phylogenetically intermediate position of C₃-C₄ intermediates.

IX. Concluding Statement

Knowledge about the metabolic details of C₃-C₄ intermediates has crystallized as a result of research over the past decade and a half. It is now clear that many of the C₃-C₄ intermediate traits can be explained on the basis of the glycine shuttle, originally elucidated by Rawsthorne et al. (1988a). This

mechanism appears to be ubiquitous among C_3 - C_4 intermediate species, at least those of terrestrial origin. (Some aquatic plants have been shown to exhibit C_3 - C_4 intermediate traits, but the underlying mechanisms appear to differ with respect to the glycine shuttle described above; e.g., see Bowes this volume.) There are still significant uncertainties that must be resolved before a complete understanding of the glycine-shuttle mechanism is understood. For example, it is still unclear as to whether the CO_2 concentration of bundle-sheath cells in C_3 - C_4 plants is increased during light-dependent CO_2 assimilation. It is also unclear as to why the predicted influences of a glycine shuttle on leaf-level CO_2 assimilation rates, and water- and nitrogen-use efficiency, are not observed when actual measurements are made at normal, ambient CO_2 concentrations.

The functional significance of the C_4 -like biochemistry found in C_3 - C_4 *Flaveria* intermediates is also uncertain. It is possible that traits such as a low CO_2 compensation point and the biphasic response of the CO_2 compensation point to changes in ambient O_2 concentration are due to the glycine shuttle in the *Flaveria* intermediates. Other traits such as reduced O_2 inhibition of photosynthesis cannot be readily explained by the glycine shuttle and are likely due to the limited CO_2 -concentrating capacity of the C_4 -like biochemistry (Moore et al., 1987). However, the precise nature of any CO_2 -concentrating activity in C_3 - C_4 *Flaveria* species is still equivocal. Likewise, it is unclear as to how intracellular CO_2 concentrations can be increased given the lack of C_4 -like partitioning of the C_4 and C_3 cycles between mesophyll and bundle-sheath cells.

Finally, many of the uncertainties involved with the evolutionary status of C_3 - C_4 intermediate plants can now be resolved using phylogenetic reconstruction based on molecular sequence data from specific proteins (e.g., Kopriva et al., 1996). This type of insight will be of critical importance as we attempt to (1) reconstruct specific metabolic paths to C_4 photosynthesis, (2) place the evolution of C_4 photosynthesis into the context of past ecological and biogeochemical events (e.g., global-level dynamics in atmospheric concentrations and Neogene expansion of global grasslands), and (3) attempt to identify the significant selective forces that have driven plants to this alternative mode of carbon assimilation.

References

- Apel P (1980) CO_2 compensation concentration and its O_2 dependence in *Moricandia spinosa* and *Moricandia moricandioides* (Cruciferae). Biochem Physiol Pflanzen 175: 386–388
- Bauwe H (1984) Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of C_3 , C_4 , and C_3 - C_4 intermediate species of *Flaveria* (Asteraeae). Biochem Physiol Pflanzen 179: 253–268
- Bourguignon J, Neuberger, M and Douce R. (1988) Resolution and characterization of the glycine-cleavage reaction in pea leaf mitochondria. Biochem J 255: 169–178
- Bourguignon J, Merand V, Rawsthorne S, Forest E and Douce R (1996) The glycine decarboxylase and pyruvate dehydrogenase complexes share the same dihydrolipoamide dehydrogenase in pea leaf mitochondria. Mass spectrometry and primary structure analysis. Biochem J 313: 229–234.
- Brown RH (1980) Photosynthesis of grass species differing in carbon dioxide fixation pathways. IV. Analysis of reduced oxygen response in *Panicum milioides* and *Panicum schenckii*. Plant Physiol 65: 346–349
- Brown RH and Bouton JH (1993) Physiology and genetics of interspecific hybrids between photosynthetic types. Annu Rev Plant Physiol Plant Molec Biol 44: 435–456
- Brown RH and Hattersley PW (1989) Leaf anatomy of C_3 - C_4 species as related to evolution of C_4 photosynthesis. Plant Physiol 91: 1543–1550
- Brown RH and Morgan JA (1980) Photosynthesis of grass species differing in carbon dioxide fixation pathways. VI. Differential effects of temperature and light intensity on photorespiration in C_3 , C_4 , and intermediate species. Plant Physiol 66: 541–544
- Brown RH and Simmons RE (1979) Photosynthesis of grass species differing in CO_2 fixation pathways. I. Water-use efficiency. Crop Sci: 19: 375–379
- Brown RH, Bouton JH, Rigby L and Rigler M (1983) Photosynthesis of grass species differing in carbon dioxide fixation pathways. VIII. Ultrastructural characteristics of *Panicum* species in the Laxa group. Plant Physiol 71: 425–431
- Devi T, Rajagopalan AV and Raghavendra AS (1995) Predominant localization of mitochondria enriched with glycine-decarboxylating enzymes in bundle sheath cells of *Alternanthera tenella*, a C_3 - C_4 intermediate species. Plant Cell Environ 18: 589–594
- Edwards GE and Ku MSB (1987) Biochemistry of C_3 - C_4 intermediates. In: Hatch MD and Boardman NK (eds) The Biochemistry of Plants, Vol 10, pp 275–325. Academic Press, London
- Edwards GE, Ku MSB, Hatch MD (1982) Photosynthesis in *Panicum milioides*, a species with reduced photorespiration. Plant Cell Physiol 23: 1185–1195
- Ehleringer JR, Sage RF, Flanagan LB and Pearcy RW (1991) Climate change and the evolution of C_4 photosynthesis. Trends Ecol Evol 6: 95–99
- Farquhar GD (1983) On the nature of carbon-isotope discrimination in C_4 species. Austr J Plant Physiol 13: 205–226
- Farquhar GD, O'Leary MH and Berry, JA (1982) On the relationship between carbon-isotope discrimination and the

- intercellular carbon dioxide concentration in leaves. *Austr J Plant Physiol* 9: 121–137
- Hattersley PW, Wong S-C, Perry S, Roksandic Z (1986) Comparative ultrastructure and gas exchange characteristics of the C₃-C₄ intermediate *Neurachne minor* S.T. Blake (Poaceae). *Plant Cell Environ* 9: 217–233
- Holaday AS and Chollet R (1983) Photosynthetic/photorespiratory carbon metabolism in the C₃-C₄ intermediate species, *Moricandia arvensis* and *Panicum miliooides*. *Plant Physiol* 73: 740–745
- Holaday AS, Harrison AT and Chollet R (1982) Photosynthetic/photorespiratory CO₂ exchange characteristics of the C₃-C₄ intermediate species, *Moricandia arvensis*. *Plant Sci Lett* 27: 181–189
- Hunt S, Smith AM and Woolhouse HW (1987) Evidence for a light-dependent system for reassimilation of photorespiratory CO₂, which does not include a C₄ cycle, in the C₃-C₄ intermediate species *Moricandia arvensis*. *Planta* 171: 227–234
- Hylton CM, Rawsthorne S, Smith AM, Jones DA and Woolhouse HW (1988) Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C₃-C₄ intermediate species. *Planta* 175: 452–459
- Keck RW and Ogren WL (1976) Differential oxygen response of photosynthesis in soybean and *Panicum miliooides*. *Plant Physiol* 58: 552–555
- Kennedy RA and Laetsch WM (1974) Plant species intermediate for C₃, C₄ photosynthesis. *Science* 184: 1087–1089
- Kopriva S, Chu CC and Bauwe H (1996) Molecular phylogeny of *Flaveria* as deduced from the analysis of nucleotide sequences encoding H-protein of the glycine cleavage system. *Plant Cell Environ* 19: 1028–1036
- Ku MSB, Monson RK, Littlejohn RO, Nakamoto H, Fisher DB and Edwards GE (1983) Photosynthetic characteristics of C₃-C₄ intermediate *Flaveria* species. I. Leaf anatomy, photosynthetic responses to CO₂ and O₂, and activities of key enzymes in the C₃ and C₄ pathways. *Plant Physiol* 71: 944–948
- Ku MSB, Wu J, Dai Z, Scott RA, Chu C and Edwards GE (1991) Photosynthetic and photorespiratory characteristics of *Flaveria* species. *Plant Physiol* 96: 518–528
- Ku MSB, Kano-Murakami Y and Matsuoka M (1996) Evolution and expression of C₄ photosynthetic genes. *Plant Physiol* 111: 949–957
- Laetsch WM (1971) Chloroplast structural relationships in leaves of C₄ plants. In: Hatch MD, Osmond CB and Slatyer RO (eds) *Photosynthesis and Photorespiration*, pp 323–352. Wiley-Interscience, New York
- Laing WA, Ogren WL, Hageman RH (1974) Regulation of soybean net photosynthetic CO₂ fixation by the interaction of CO₂, O₂, and ribulose-1,5-bisphosphate carboxylase. *Plant Physiol* 54: 678–685
- Monson RK (1989a) On the evolutionary pathways resulting in C₄ photosynthesis and Crassulacean acid metabolism. *Adv Ecol Res* 19: 57–110
- Monson RK (1989b) The relative contributions of reduced photorespiration, and improved water- and nitrogen-use efficiencies, to the advantages of C₃-C₄ intermediate photosynthesis in *Flaveria*. *Oecologia* 80: 215–221
- Monson RK (1996) The use of phylogenetic perspective in comparative plant physiology and developmental biology. Ann Missouri Botanical Garden 83: 3–16
- Monson RK (1999) The origins of C₄ genes and evolutionary pattern in the C₄ metabolic phenotype. In: Sage R and Monson RK (eds) *C₄ Plant Biology*, pp 377–410. Academic Press, San Diego
- Monson RK and Jaeger CH (1991) Photosynthetic characteristics of C₃-C₄ intermediate *Flaveria floridana* (Asteraceae) in natural habitats: Evidence of advantages to C₃-C₄ photosynthesis at high leaf temperatures. *Amer J Botany* 78: 795–800
- Monson RK and Moore BD (1989) On the significance of C₃-C₄ intermediate photosynthesis to the evolution of C₄ photosynthesis. *Plant Cell Environ* 12: 689–699
- Monson RK, Edwards GE and Ku MSB (1984) C₃-C₄ intermediate photosynthesis in plants. *BioScience* 34: 563–574
- Monson RK, Moore BD, Ku MSB and Edwards GE (1986) Co-function of C₃- and C₄-photosynthetic pathways in C₃, C₄, and C₃-C₄ intermediate *Flaveria* species. *Planta* 168: 493–502
- Monson RK, Schuster W, and Ku MSB (1987) Photosynthesis in *Flaveria brownii* Powell. A C₄-like C₃-C₄ intermediate. *Plant Physiol* 85: 1063–1067
- Monson RK, Teeri J, Ku MSB, Gurevitch J, Mets L and Dudley S (1988) Carbon-isotope discrimination by leaves of *Flaveria* species exhibiting different amounts of C₃- and C₄-cycle co-function. *Planta* 174: 145–151
- Moore BD, Ku MSB and Edwards GE (1987) C₄ photosynthesis and light-dependent accumulation of inorganic carbon in leaves of C₃-C₄ and C₄ *Flaveria* species. *Austr J Plant Physiol* 14: 657–668
- Moore BD, Monson RK, Ku MSB and Edwards GE (1988) Activities of principal photosynthetic and photorespiratory enzymes in leaf mesophyll and bundle sheath protoplasts from the C₃-C₄ intermediate *Flaveria ramosissima*. *Plant Cell Physiol* 29: 999–1006
- Moore BD, Ku MSB and Edwards GE (1989) Expression of C₄-like photosynthesis in several species of *Flaveria*. *Plant Cell Environ* 12: 541–549
- Morgan CL, Turner SR and Rawsthorne S (1993) Coordination of the cell-specific distribution of the four subunits of glycine decarboxylase and serine hydroxymethyltransferase in leaves of C₃-C₄ intermediate species from different genera. *Planta* 190: 468 – 473
- Ogren WL (1984) Photorespiration: Pathways, regulation and modification. *Annu Rev Plant Physiol* 35: 415–442
- Ohnishi J and Kanai R (1983) Differentiation of photorespiratory activity between mesophyll and bundle sheath cells of C₄ plants. I. Glycine oxidation by mitochondria. *Plant Cell Physiol* 24: 1411–1420
- Peisker M (1985) Modelling carbon metabolism in C₃-C₄ intermediate species. 2. Carbon-isotope discrimination. *Photosynthetica* 19: 300–311
- Peisker M and Bauwe H (1984) Modelling carbon metabolism in C₃-C₄ intermediate species. I. CO₂ compensation concentration and its O₂ dependence. *Photosynthetica* 18: 9–19
- Quebedeaux B and Chollet R (1977) Comparative growth analyses of *Panicum* species with differing rates of photorespiration. *Plant Physiol* 59: 42–44
- Rathnam CKM and Chollet R (1979) Photosynthetic carbon metabolism in *Panicum miliooides*, a C₃-C₄ intermediate species: evidence for a limited C₄ dicarboxylic acid pathway of photosynthesis. *Biochim Biophys Acta* 548: 500–519
- Rawsthorne S and Bauwe H (1998) C₃-C₄ photosynthesis. In:

- Raghavendra AS (ed) Photosynthesis, A Comprehensive Treatise, pp 150–162. Cambridge University Press, Cambridge
- Rawsthorne S and Hylton CM (1991) The post-illumination CO_2 burst and glycine metabolism in leaves of C_3 and $\text{C}_3\text{-}\text{C}_4$ intermediate species of *Moricandia*. *Planta* 186: 122–126
- Rawsthorne S, Hylton CM, Smith AM and Woolhouse HW (1988a) Photorespiratory metabolism and immunogold localization of photorespiratory enzymes in leaves of C_3 and $\text{C}_3\text{-}\text{C}_4$ intermediate species of *Moricandia*. *Planta* 173: 298–308
- Rawsthorne S, Hylton CM, Smith AM and Woolhouse HW (1988b) Distribution of photorespiratory enzymes between bundle-sheath and mesophyll cells in leaves of the $\text{C}_3\text{-}\text{C}_4$ intermediate species *Moricandia arvensis* (L.) DC. *Planta* 176: 527–532
- Rawsthorne S, von Caemmerer S, Brooks A and Leegood RC (1992) Metabolic interactions in leaves of $\text{C}_3\text{-}\text{C}_4$ intermediate plants. In: Tobin AK (ed) Plant Organelles. Compartmentation of Metabolism in Photosynthetic Cells, SEB Seminar Series, Vol 50, pp. 113–139. Cambridge University Press, Cambridge
- Reed JE and Chollet R (1985) Immunofluorescent localization of phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase/oxygenase proteins in leaves of C_3 , C_4 , and $\text{C}_3\text{-}\text{C}_4$ *Flaveria* species. *Planta* 165: 439–445
- Rumpho M, Ku MSB, Cheng SH and Edwards GE (1984) Photosynthetic characteristics of $\text{C}_3\text{-}\text{C}_4$ intermediate *Flaveria* species. III. Reduction of photorespiration by a limited C_4 pathway of photosynthesis in *Flaveria ramosissima*. *Plant Physiol* 75: 993–996
- Rylott EL, Metzlaff K and Rawsthorne S (1998) Developmental and environmental effects on the expression of the $\text{C}_3\text{-}\text{C}_4$ intermediate phenotype in *Moricandia arvensis* (L.) DC. *Plant Physiol* 118: 1277–1284
- Sayre RT, Kennedy RA and Prignitz DJ (1979) Photosynthetic enzyme activities and localization in *Mollugo verticillata* populations differing in the levels of C_3 and C_4 cycle operation. *Plant Physiol* 64: 293–299
- Schuster WS, Monson RK (1990) An examination of the advantages of $\text{C}_3\text{-}\text{C}_4$ intermediate photosynthesis in warm environments. *Plant, Cell Environ* 13: 903–912
- Tobin AK, Thorpe JR, Hylton CM and Rawsthorne S (1989) Spatial and temporal influences on the cell-specific distribution of glycine decarboxylase in leaves of wheat (*Triticum aestivum* L.) and pea (*Pisum sativum* L.). *Plant Physiol* 91: 1219–1225
- Turner SR, Ireland RJ and Rawsthorne S (1992) Purification and primary amino acid sequence of the L subunit of glycine decarboxylase. Evidence for single lipoamide dehydrogenase in plant mitochondria. *J Biol Chem* 267: 7745–7750
- von Caemmerer S (1989) A model of photosynthetic CO_2 assimilation and carbon-isotope discrimination in leaves of certain $\text{C}_3\text{-}\text{C}_4$ intermediates. *Planta* 178: 463–474
- von Caemmerer S and Hubick KT (1989) Short-term carbon-isotope discrimination in $\text{C}_3\text{-}\text{C}_4$ intermediate species. *Planta* 178: 475–481

Chapter 23

Induction of Crassulacean Acid Metabolism— Molecular Aspects

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Summary

Crassulacean acid metabolism (CAM) represents a notable adaptation to photosynthetic CO_2 fixation that affords plants a competitive advantage in CO_2 - or water-limiting environments. The physiological and biochemical characteristics of CAM are well understood, as are the ecophysiological implications of the pathway. As our knowledge expands, it has become increasingly evident that the CAM adaptation exhibits an enormous degree of metabolic plasticity, especially in the extent to which CAM is influenced by environmental

and developmental factors. In many species, CAM is induced in response to environmental stress conditions or as part of a developmental progression towards maturity. Our current understanding of induction, relationship to environmental factors, and regulation of the CAM cycle at the molecular genetic level will be reviewed. Since 1989, with the characterization of the first genes from a CAM plant, mechanisms have been uncovered which reveal the dependence of CAM gene regulation on a host of factors including plant growth regulators, various abiotic stresses, light, and circadian rhythms. The regulation of CAM gene expression occurs predominantly at the transcriptional level; however, posttranscriptional, translational, and posttranslational regulatory events play additional roles in fine-tuning the expression of the pathway in response to environmental changes and in synchrony with plant development. One of the most captivating areas of CAM research, for the foreseeable future, is centered around understanding perception and signal transduction mechanisms that induce CAM and control circadian rhythm. Finally, development of genetic models and new approaches using transgenic CAM plants promise to enhance our understanding of the molecular basis of CAM induction in the near future.

I. Introduction

Two distinct metabolic pathways for sequentially assimilating and concentrating atmospheric CO₂ exist in approximately 10% of higher plants—C₄ photosynthesis and CAM. The driving forces responsible for the evolution of these photosynthetic adaptations may include limited CO₂ availability due to ancient decreases in global atmospheric CO₂ concentrations, high light and temperature conditions, lack of water, or a combination of these environmental stress factors. Under heat and water stress, CO₂ often becomes limiting when stomata close, which leads to increased photorespiration, a consequence of the incomplete discrimination of CO₂ over O₂ by Rubisco. C₄ and CAM plants can suppress photorespiration by utilizing a ‘CO₂ pump’ involving PEPC that elevates

intracellular CO₂ concentrations in the vicinity of Rubisco. In C₄ plants, this concentration occurs through the spatial separation of PEPC and Rubisco between two cell types (Furbank and Taylor, 1995; Ku et al., 1996). C₄ photosynthesis requires a developmental program by which cell-specific differentiation patterns form two distinct photosynthetic cell types, mesophyll and bundle sheath. The genetic modifications required for the generation of these cell types in the C₄ leaf are considered extensive (Ku et al., 1996; Chapter 20, Dengler and Taylor), or, at least, more substantial than the changes stipulated for the establishment of CAM carried out in mesophyll cells alone. This notion is supported by phylogenetic studies which suggest that CAM evolved earlier and is more widespread than C₄ photosynthesis (Ehleringer and Monson, 1993; Raven and Spicer, 1996; Smith and Winter, 1996). CAM plants perform both carboxylation reactions within mesophyll cells, but the reactions, which have been described as a sequence of four individual phases, are separated temporally (Osmond, 1978; Winter and Smith, 1996; Cushman and Bohnert, 1997).

Emergence of the CAM pattern also demanded numerous genetic modifications, but evolution favored alterations in diurnal or circadian regulation, modifications of intracellular partitioning of photosynthetic products, and changes in metabolic signaling for the regulation of the two competing carboxylation reactions within a single cell. Additionally, expression of the pathway can vary extensively during development, in response to environmental conditions, and among phylogenetically related CAM-performing species. The anatomical, phylogenetic, evolutionary, ecological, physiological, and biochemical aspects of CAM have

Abbreviations: ABA – abscisic acid; 6-BAP – 6-benzylaminopurine; CAM – Crassulacean Acid Metabolism; CaM – calmodulin; cGMP – cyclic guanosine 3'-5'-cyclic monophosphate; enolase – 2-phospho-D-glycerate hydrolase; EGTA – Ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; EMS – ethyl methane sulfonate; ER – endoplasmic reticulum; EST – expressed sequence tag; FBP – fructose-1, 6-bisphosphatase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; HMG – high mobility group; MAL – malate; MDH – malate dehydrogenase; IP₃ – inositol 1, 4, 5-triphosphate; MAPK – mitogen activated protein kinase; ME – malic enzyme; MJA – methyl jasmonate; MYB – myeloblastosis proto-oncogene product; NAD – nicotinamide adenine dinucleotide, oxidized; NADP – nicotinamide adenine dinucleotide phosphate, oxidized; OAA – oxaloacetate; Pi – inorganic orthophosphate; PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase; PEPCK – PEP carboxykinase; PGM – phosphoglyceromutase; PP1 – protein phosphatase 1; PP2A – protein phosphatase 2A; PP2B – protein phosphatase 2B (calcineurin); PPDK – pyruvate orthophosphate dikinase; RPK – receptor protein kinase; Rubisco – ribulose 1,5-bisphosphate carboxylase/oxygenase; T-DNA – transferred DNA; V-ATPase – V-type H⁺-translocating ATPase

been reviewed (Kluge and Ting, 1978; Osmond, 1978; Osmond and Holtum, 1981; Cockburn, 1985; Ting, 1985; Winter, 1985; Lüttge, 1987; Griffiths, 1988, 1989, 1992; Monson, 1989; Leegood and Osmond, 1990; Smith and Bryce, 1992; Winter and Smith, 1996). More recently, molecular genetic aspects of the CAM have been reviewed (Lüttge, 1993; Cushman and Bohnert, 1996; Cushman and Bohnert, 1997). In this chapter we summarize the developmental and environmental factors that regulate CAM induction. Our discussion includes biochemical, developmental, and molecular genetic aspects of this phenomenon, with emphasis on the signal recognition and transduction processes orchestrating gene expression changes which result in CO_2 assimilation patterns that improve competitiveness and survival in xeric or aquatic plant habitats.

II. Permutations and Metabolic Plasticity of CAM

A striking feature of CAM is the extreme physiological and metabolic plasticity of the pathway and the degree to which pathway expression can be influenced by developmental or environmental factors. The extent of malate cycling, carbohydrate storage, and the contribution of nocturnal CO_2 fixation to net carbon gain can vary substantially. Thus, different CAM species can range from nearly C_3 to obligate CAM, including facultative CAM species that swing into and out of CAM depending on changes in environmental conditions or developmental status (Lüttge, 1993; Edwards et al., 1996; Smirnoff, 1996; Ting et al., 1996). In some obligate CAM species, net CO_2 uptake occurs almost exclusively at night even under well watered conditions. Other CAM plants appear nearly- C_3 and usually exhibit net CO_2 uptake only during the day. This mode of CAM, referred to as CAM cycling, is characterized by the appearance of diurnal fluctuations in C_4 acid levels in the absence of daytime stomatal closure and little or no net nocturnal carbon assimilation (Ting, 1985). By refixing respiratory CO_2 at night, plants that perform CAM-cycling are thought to be poised to enter full CAM rapidly when called for by prevailing environmental conditions. Another variation, called CAM-idling, occurs under extreme drought stress conditions when stomata remain closed day and night, yet diurnal fluctuations in organic acids continue as a result of refixation of respiratory CO_2 .

(Ting, 1985, 1987; Patel and Ting, 1987; Bastide et al., 1993). CAM-idling may help preserve the activities of photosynthetic enzymes until favorable growth conditions return. Between the two extremes of CAM-cycling and CAM-idling are found various patterns of gas exchange and C_4 acid fluctuation that fall within a continuum of the four phases of CAM (Osmond, 1978; Leegood and Osmond, 1990; Smith and Bryce, 1992) as dictated by a complex interplay of environmental and developmental parameters (Borland and Griffiths, 1996).

III. Control of CAM Induction

Facultative CAM plants shift from C_3 to CAM or different modes of CAM (e.g., CAM-cycling or CAM-idling) in response to developmental cues or environmental signals, such as photoperiod length, high salinity, or water deficit, and plant growth regulators. The ability to shift into CAM is present in a wide variety of species from diverse families. The inducible pathway is predominantly found among the *Aizoaceae*, *Clusiaceae*, *Crassulaceae*, *Vitaceae*, *Piperaceae*, and *Portulacaceae* (Winter and Smith, 1996). Inducible CAM-like behavior has also been reported in the C_4 genus *Portulaca* (Mazen, 1996; Kraybill and Martin, 1996). CAM has also been recognized in several genera of aquatic plants including *Isoetes*, *Crassula*, *Littorella*, and *Sagittaria* (Keeley, 1996). In aquatic environments, CAM enhances inorganic carbon acquisition under conditions of limited CO_2 availability (Raven and Spicer, 1996). In *Littorella uniflora* (shoreweed), which grows partially inundated along the margins of lakes, CAM is induced by rehydration of the plants which perform C_3 photosynthesis when not immersed in water (Robe and Griffiths, 1992). In agreement with these observations, the ratio of PEPC:Rubisco activity was found to be consistently higher under submerged rather than emergent conditions (Keeley, 1996). This behavior is in contrast to the usual induction of CAM by water-limiting conditions. The exact nature of the signals that regulate CAM activity in aquatic environments remains an open question and requires further attention.

In this section, we will focus on the role that development, environmental stress, and plant growth regulators have on CAM induction in three different model CAM genera: *Peperomia* ssp., *Kalanchoë*,

and *Mesembryanthemum*. Developmental aspects of CAM induction have been in the foreground of studies using *Peperomia* ssp. (Ting et al., 1996), whereas photoperiodic induction and circadian rhythms of CAM has been analyzed most comprehensively in *Kalanchoë* (Brulfert et al., 1985, 1988; Wilkins, 1992). Since the initial discovery that high salinity can serve as a reliable trigger to switch *M. crystallinum* from C_3 photosynthesis into CAM (Winter and von Willert, 1972), this plant has become an important model for studying how CAM is induced (Cushman and Bohnert, 1997) and other aspects of ecological adaptation to environmental stress (Adams et al., 1998).

A. Development

Developmental status plays an important role in modulating the timing and extent to which CAM is expressed (Kluge and Ting, 1978; Winter, 1985). An age-dependent shift from C_3 photosynthesis to CAM has been documented for many *Peperomia* species, which provide an excellent model for the study of CAM ontogeny since, during normal development, leaves show a progressive expression of CAM through the sequence: $C_3 \rightarrow$ CAM cycling \rightarrow CAM (Sipes and Ting, 1985; Holthe et al., 1987, 1992; Ting et al. 1996). In *Peperomia scandens* and *P. campotricha*, leaves are arranged in successive false whorls from young leaves lacking CAM attributes to mature leaves with CAM (Ting et al., 1996). As leaves age, diurnal fluctuations of acidity increased in parallel with PEPC activity, PEPC protein and mRNA accumulation (Ting et al., 1993, 1996). Increased CAM correlated with increased leaf thickness attributable mainly to a thickening and expansion of the spongy mesophyll and multiple epidermis. Increased air spaces within these tissues evidently increase the potential for gas exchange, thus facilitating CO_2 fixation in older leaves (Nobel, 1988). In contrast, palisade mesophyll cells, the site of predominantly C_3 activity, showed no significant changes in size. In older leaves, water stress can stimulate PEPC mRNA accumulation and activity. Upon rewetting, however, PEPC activities return to amounts typical of the constitutive CAM state prior to the imposition of water stress suggesting that the PEPC response to water stress is fully reversible (Ting et al., 1996). Studies exploring the relationship between leaf aging, photoperiod and the induction of CAM in *K. blossfeldiana* and *K. velutina* have also demonstrated that

leaf aging promoted CAM under both short- and long-day conditions (Queiroz and Brulfert, 1982; Brulfert et al., 1982b).

In *M. crystallinum*, immature leaves or young plants are less able to induce CAM and CAM-specific genes such as *Ppc1* or *Ppd1* (see Table 1) following environmental stress suggesting that a critical developmental threshold dictates the onset or inducibility of CAM or CAM-specific genes (von Willert et al., 1976a; Ostrem et al., 1987; Cushman et al., 1990; Cheng and Edwards, 1991; Herppich et al., 1992; Bohnert et al., 1994; Fißlthaler et al., 1995). However, the timing of CAM induction or CAM-specific gene expression can vary considerably depending on photoperiod length, temperature, nutrient supply, or volume of the rooting medium (Cheng and Edwards, 1991; Schmitt and Piepenbrock 1992a; Piepenbrock et al., 1994) suggesting that environmental factors rather than development dictate CAM induction. Reductions in cell and leaf turgor pressure and water content occur even in well-watered plants with progressing age (Heun et al., 1981; Rygol et al., 1986; Winter and Gademann, 1991) indicating that incremental development of low levels of CAM activity in such plants seems to arise from water stress rather than from a developmental program (Schmitt and Piepenbrock 1992a; Piepenbrock et al., 1994). This is in agreement with earlier observations by Winter (1973b) who documented conditions which maintained high leaf water contents (moderate light intensity and high relative humidity) and effectively postponed the onset of CAM. More recently, split roots experiments have demonstrated roots can perceive water stress and convey this information to leaves triggering a switch from C_3 to CAM photosynthesis without detectable reductions in leaf turgor (Eastmond and Ross, 1997). As in *Peperomia*, stress-mediated induction of PEPC activity, protein or mRNA is reversed in *M. crystallinum* plants or excised leaves following removal of stress (von Willert et al., 1976b; Vernon et al., 1988; Schmitt, 1990; Piepenbrock and Schmitt, 1991; Schmitt and Piepenbrock, 1992b). Whatever their relative contribution to CAM, it is obvious that, at least in certain CAM plants, a combination of developmental and environmental factors influences CAM induction.

B. Environmental Factors

Many different environmental factors can influence the extent of nocturnal CO_2 fixation in CAM plants.

Table 1. Enzymes and genes involved in Crassulacean acid metabolism

Enzyme	Gene	Organism	Subcellular Location	Inducers	Accession number or Citation ¹
Carboxylases:					
Phosphoenolpyruvate carboxylase	<i>Ppc1</i>	<i>M. crystallinum</i>	CYT	NaCl, ABA, drought, BAP, light	X13660, X14587, X63774
	<i>Ppc2</i>	<i>M. crystallinum</i>	CYT	–	X14588
	<i>Ppc1;1...2</i>	<i>K. blossfeldiana</i>	CYT	ABA, drought, short day length	X87818, X87819
	<i>Ppc2;1...2</i>	<i>K. blossfeldiana</i>	CYT	–	X87820, X87821
	<i>Ppc1;1...2</i>	<i>V. planifolia</i>	CYT	?	X87148, X87149
	<i>Ppc1</i>	<i>P. aculeata</i>	CYT	?	X95860
	<i>Ppc1</i>	<i>W. mirabilis</i>	CYT	?	X91404
	<i>Ppc1</i>	<i>A. arboreascens</i>	CYT	?	D83052
	<i>Ppc1</i>	<i>T. usneoides</i>	CYT	?	X91406
	<i>Ppc1</i>	<i>N. ampullacea</i>	CYT	?	X95861
	<i>Ppc1</i>	<i>I. histrix</i>	CYT	?	X95854
	<i>Ppc1</i>	<i>I. duriei</i>	CYT	?	X95859
Rubisco – large subunit	<i>rbcL</i>	<i>M. crystallinum</i>	CP	–	M98515
	<i>rbcL</i>	<i>K. daigremontiana</i>	CP	–	L11189
	<i>rbcL</i>	<i>Peperomia sp.</i>	CP	–	L12661
	<i>rbcL</i>	<i>C. marnieriana</i>	CP	–	L01899
	<i>rbcL</i>	<i>S. rubrotinctum</i>	CP	–	L01956
	<i>rbcL</i>	<i>A. procera</i>	CP	–	M62563
	<i>rbcL</i>	<i>A. comosus</i>	CP	–	L19977
	<i>rbcL</i>	<i>A. vera</i>	CP	–	L05029
	<i>rbcL</i>	<i>A. bakeri</i>	CP	–	Z73680
Rubisco – small subunit	<i>RbcS1...6</i>	<i>M. crystallinum</i>	CP	–	L10212; M38316, L10214; M38317, M38318, L10215; M31640; L10213
Decarboxylases:					
NADP-Malic enzyme	<i>Mod1</i>	<i>M. crystallinum</i>	CYT	NaCl	X64424
NAD-Malic enzyme	<i>Mod2</i>	<i>M. crystallinum</i>	MT	NaCl	Holtum and Winter, 1982
PEP carboxykinase	?	<i>T. fasciculata,</i> <i>T. utricularia,</i> <i>N. fulgens, A. aristata,</i> <i>H. camosa</i>	CYT	?	Walker and Leegood, 1996
Malate metabolism enzymes:					
NADP-Malate dehydrogenase	<i>Mdh1</i>	<i>M. crystallinum</i>	CP	NaCl	X63727
NAD-Malate dehydrogenase	<i>Mdh2</i>	<i>M. crystallinum</i>	CYT	NaCl	X96539

Table I. Continued

Enzyme	Gene	Organism	Subcellular Location	Inducers	Accession number or Citation ¹
Glycolytic/gluconeogenic enzymes:					
Pyruvate orthophosphate dikinase	<i>Ppdk1</i>	<i>M. crystallinum</i>	CP	NaCl	X78347, X82489
Enolase	<i>Pgh1;1...2</i>	<i>M. crystallinum</i>	CYT	NaCl, drought, cold, hypoxia, ABA, BAP	U09194
Phosphoglyceromutase	<i>Pgm1</i>	<i>M. crystallinum</i>	CYT	NaCl, drought, ABA, BAP	U16021
Phosphoglyceratekinase	<i>Pgk1</i>	<i>M. crystallinum</i>	CYT	NaCl	N. Forsthoefel, C. Michalowski, unpublished data
Phosphoglyceratekinase	?	<i>M. crystallinum</i>	CP	NaCl	Winter et al., 1982
NAD-Glyceraldehyde 3-phosphate dehydrogenase	<i>GapC1</i>	<i>M. crystallinum</i>	CYT	NaCl	M29956
NADP-Glyceraldehyde 3-phosphate dehydrogenase	?	<i>M. crystallinum</i>	CP	NaCl	Holtum and Winter, 1982
Phosphoglucomutase	<i>Pglm1</i>	<i>M. crystallinum</i>	CYT	?	U84888
Fructose 1,6-bisphosphatase	?	<i>M. crystallinum</i>	CP	NaCl	Holtum and Winter, 1982
Phosphofructokinase	?	<i>M. crystallinum</i>	CP	—	Holtum and Winter, 1982
Pyruvate kinase	?	<i>M. crystallinum</i>	?	—	Holtum and Winter, 1982
Hexokinase	?	<i>M. crystallinum</i>	CYT	—	Holtum and Winter, 1982
Glucose-6-phosphate dehydrogenase	?	<i>M. crystallinum</i>	?	—	Holtum and Winter, 1982
Phosphohexose isomerase	?	<i>M. crystallinum</i>	CYT/CP	NaCl	Holtum and Winter, 1982
Alpha-amylase	?	<i>M. crystallinum</i>	CP/Cyt?	NaCl	Paul et al., 1993
Beta-amylase	?	<i>M. crystallinum</i>	Cyt?	NaCl	Paul et al., 1993
D-enzyme	?	<i>M. crystallinum</i>	CP/Cyt?	NaCl	Paul et al., 1993
R-enzyme	?	<i>M. crystallinum</i>	CP/Cyt?	NaCl	Paul et al., 1993
Starch phosphorylase	?	<i>M. crystallinum</i>	CP/Cyt	NaCl, diurnal rhythm	Paul et al., 1993
Photorespiration Enzymes:					
Glycollate oxidase	?	<i>M. crystallinum</i>	PER	?	U80071
Glycine cleavage protein H	?	<i>M. crystallinum</i>	PER	?	U79768
Glycine cleavage protein T	?	<i>M. crystallinum</i>	PER	?	U79769
Protein Kinases:					
PEPC kinase	?	<i>M. crystallinum</i> , <i>B. fedtschenkoi</i>	CYT	Circadian rhythm	Li and Chollet, 1994; Hartwell et al., 1996
SNF1 kinase	<i>MK1</i>	<i>M. crystallinum</i>	CYT	?	J.C. Cushman, unpublished data

Table I. Continued

Enzyme	Gene	Organism	Subcellular Location	Inducers	Accession number or Citation ¹
Protein Kinases: (Continued)					
SNF1 kinase	<i>MK9</i>	<i>M. crystallinum</i>	CYT	NaCl, Drought, ABA, 6-BAP	Z226846
'AGC' kinase	<i>MK6</i>	<i>M. crystallinum</i>	CYT	NaCl, diurnal rhythm	Z30329
'AGC' kinase	<i>MK8</i>	<i>M. crystallinum</i>	CYT	?	Z30331
'AGC' kinase	<i>MK10</i>	<i>M. crystallinum</i>	CYT	?	Z30333
CDPK	<i>CPK1</i>	<i>M. crystallinum</i>	CYT	?	T. Taybi and J.C. Cushman, unpublished data
Tonoplast Enzymes:					
H ⁺ -ATPase, A subunit	<i>AtpvA</i>	<i>M. crystallinum</i>	TP	NaCl	Löw et al., 1996
H ⁺ -ATPase, B subunit	<i>AtpvB</i>	<i>M. crystallinum</i>	TP	NaCl	Löw et al., 1996
H ⁺ -ATPase, E subunit	<i>AtpvE</i>	<i>M. crystallinum</i>	TP	NaCl	X92118
H ⁺ -ATPase, c subunit	<i>Atpvc</i>	<i>M. crystallinum</i> , <i>K. daigremontiana</i>	TP	NaCl, ABA, Light	X94999, U51563, U16244
Tonoplast intrinsic protein	<i>MipF</i>	<i>M. crystallinum</i>	TP	?	U43291
Pyrophosphatase	?	<i>M. crystallinum</i> , <i>K. daigremontiana</i>	TP	—	Becker et al., 1995
Miscellaneous:					
Phosphoribulokinase	<i>Prk1</i>	<i>M. crystallinum</i>	CP	—	M73707
Ferredoxin-NADP ⁺ reductase	<i>Fnr1</i>	<i>M. crystallinum</i>	CP	—	X13884
Thioredoxin H	<i>Trd1</i>	<i>M. crystallinum</i>	CP	?	U87141
Sulphydryl endopeptidase	<i>Sep1</i>	<i>M. crystallinum</i>	VAC?	NaCl, drought, ABA, 6-BAP, MJA	U30322
TATA binding protein	<i>Tbp1</i>	<i>M. crystallinum</i>	NC	—	U25275
14-3-3 protein	?	<i>M. crystallinum</i>	—	?	U80070
GTP-binding protein	?	<i>M. crystallinum</i>	CYT	?	U87142
GTP-binding protein	?	<i>M. crystallinum</i>	CYT	?	U87143
RNA-binding protein	?	<i>M. crystallinum</i>	CP	—	L15080
Ribosome inactivating protein	<i>Rip1</i>	<i>M. crystallinum</i>	CYT	NaCl, diurnal rhythm	U80072
Glutamate dehydrogenase	?	<i>M. crystallinum</i>	—	NaCl	Whitehouse et al., 1991
Aspartate aminotransferase	?	<i>M. crystallinum</i>	—	NaCl	Holtum and Winter, 1982
Alanine aminotransferase	?	<i>M. crystallinum</i>	—	NaCl	Holtum and Winter, 1982

¹ Citations provided where accession numbers unavailable. Dashes (—) indicate gene is not induced. Question marks (?) indicate information not available. CYT, cytoplasm; CP, chloroplast; MT, mitochondria; PER, peroxisome; TP, tonoplast; NC, nucleus; VAC, vacuole.

Some acute factors, such as drought, salinity and high temperature stress result in rapid, reversible CAM induction, whereas factors such as photoperiod initiate a slow, irreversible induction sometimes coordinated with developmental events such as flowering. Environmental stress induction of CAM occurs in a wide variety of facultative CAM or C₃/CAM intermediate genera including *Mesembryanthemum* (Edwards et al., 1996; Cushman and Bohnert, 1996; Schmitt et al., 1996), *Clusia* (Lüttge, 1996; Zotz and Winter, 1996), *Kalanchoë* (Brulfert et al., 1993; Taybi et al., 1995; Kluge and Brulfert, 1996), *Calandrinia* (Winter et al., 1981), *Cissus* (De Santo and Bartoli, 1996), *Sedum* (Smirnoff, 1996), *Peperomia* (Ting et al., 1996), and *Plectranthus* (Herppich et al., 1998).

K. blossfeldiana has been the model for investigating the influence of light intensity, light quality, and duration on the regulation of CAM induction (Brulfert et al., 1988). This species was the first identified as an inducible CAM plant resulting from changes in photoperiod length (Gregory et al., 1954; Queiroz, 1968). Since this discovery, *Kalanchoë* species have been used to study the effects of light on the diurnal or circadian rhythmicity of CAM induction (Brulfert et al., 1982a,b; Brulfert and Quieroz, 1982). When *Kalanchoë* plants grown under long-day photoperiods are transferred to short day photoperiods, CAM is induced within seven days, but full expression of the pathway may take 40–60 short days (Brulfert et al., 1975, 1982b). Control by short daylength was also observed in green callus tissue cultures from *K. blossfeldiana*, however, a lag time of 30–40 days preceded the onset of CAM (Brulfert et al., 1987; Kluge et al., 1987). Short-day induction can be effectively inhibited by interrupting the dark period by exposure to red light (Gregory et al., 1954; Mricha et al., 1990) a process controlled by phytochrome in intact plants (Brulfert et al., 1975, 1985) and green callus tissue of *K. blossfeldiana* (Mricha et al., 1990). The coupling of photoperiod to CAM induction may provide a means for the plant to measure the advance of the season and thus may be an effective way of establishing the metabolic machinery for CAM prior to the onset of seasonal environmental conditions such as drought (Brulfert et al., 1982b; Queiroz and Brulfert, 1982). In contrast to the rapid induction by salinity or drought stress, the slow photoperiodic induction of CAM in *K. blossfeldiana* appears to be irreversible (Queiroz and Brulfert, 1982). This observation suggests that

the mechanisms by which photoperiodism controls CAM induction may differ fundamentally from those mediating salinity or drought stress induction.

M. crystallinum, the best-studied inducible CAM model, exhibits rapid CAM induction in response to high salinity (Winter and von Willert, 1972; Winter 1973a) as well as other conditions such as drought, high light combined with low humidity, exposure of roots to low temperature, or root anoxia (Winter 1973b, 1985). The induction is associated with large increases in the extractable activity of enzymes associated with malate formation and decarboxylation, glycolysis and gluconeogenesis, and starch synthesis and degradation (Holtum and Winter, 1982, Winter et al., 1983; Paul et al., 1993; see also Table 1). Notably, the activities of some C-6 compound processing enzymes (Holtum and Winter, 1982) or photorespiratory enzymes (Whitehouse et al., 1991) are not induced.

In *M. crystallinum*, long photoperiods, high light intensity, and phytochrome are associated with accelerating the life cycle of the plant, stimulating malate anion accumulation, and inducing the activity of CAM enzymes such as NADP-malic enzyme and PEPC (Cheng and Edwards, 1991). Although plants exposed to constant illumination accumulated high amounts of malate and key CAM enzymes, they did not exhibit diurnal fluctuations in malate or titratable acidity suggesting that CAM requires a dark period of sufficient length to effect the regulation of enzymes. High light intensity and quality can act synergistically with NaCl or ABA to enhance the expression of the CAM-specific form of PEPC in *M. crystallinum* (McElwain et al., 1992). Expression is further improved by incandescent light (McElwain et al., 1992) or a low ratio of red:far red light suggesting that phytochrome mediates the enhancement (Cockburn et al., 1996). Plants grown under low red:far red light ratios flowered earlier, exhibited longer internode lengths leading to a closed canopy, and accumulated greater amounts of the osmoprotectant pinitol than plants grown under high red:far red ratios. These responses, along with CAM induction, are thought to be components of a stress response syndrome associated with adaptation to arid conditions (Cockburn et al., 1996).

C. Plant Growth Regulators

Many stresses can lead to pronounced changes in the absolute amount of endogenous plant growth

regulators and in the ratios of different regulators. Thus, various plant growth regulators have been used to study CAM induction. Distinguishing the effects of individual regulators from other environmental factors, such as light intensity, or gauging their interplay, however, remains a challenge. ABA has long been recognized for its important role in CAM induction. In *Portulacaria afra* treated with ABA, CAM and PEPC activity increase in response to water stress (Ting, 1981). Following salinity or drought stress treatment, endogenous amounts of ABA increase markedly in both root and leaf tissues within a few hours (Sipes and Ting, 1985; Thomas et al., 1992b; Taybi et al., 1995). Endogenous pools of ABA increase during short-day triggering of CAM induction in *K. blossfeldiana* (Brulfert et al., 1982a; Taybi et al., 1995). Increases in ABA accumulation precede CAM gene activation, whether brought about by drought stress or short-day regimes (Taybi et al., 1995). The concept of assigning a central role to ABA has been reinforced by the observation that exogenous application of ABA can induce CAM (Ting, 1985; Taybi et al., 1995), elevate activities (Chu et al., 1990; Dai et al., 1994; Taybi et al., 1995) and mRNA amounts of CAM enzymes including PEPC (Cushman et al., 1993; Taybi et al., 1995), enolase (Forsthoefel et al., 1995a), PGM (Forsthoefel et al., 1995a), and V-ATPase subunit c (Tsiantis et al., 1996). An analog of ABA and a potent antitranspirant, farnesol, can also trigger CAM induction (Dai et al., 1994). ABA distribution within tissues as well as the absolute content of ABA or ABA conjugates may also be important for CAM induction (Dai et al., 1994). Despite these observations, CAM induction by NaCl stress can also occur in leaves in which ABA biosynthesis has been suppressed (Thomas et al., 1992b). Furthermore, the efficacy of CAM induction by ABA is influenced by light intensity (McElwain et al., 1992). Modulation of CAM gene expression by ABA may be mediated directly through ABA-responsive elements (ABREs), present in the 5' flanking region of *Ppc1*, *GapCI*, and *Ppd1* genes (Cushman and Bohnert, 1992; Schmitt et al., 1996).

Cytokinins have been documented to either suppress or mimic stress-induced CAM induction depending on the organ to which the growth regulator is applied. When applied to shoots or detached leaves, cytokinin appears to suppress PEPC transcript, enzyme activity, and CAM induction by salt or dehydration. It also inhibits PEPC transcript accumulation in leaves of well-watered plants, and

accelerates the net decrease in PEPC transcripts in excised leaves during dehydration recovery (Schmitt and Piepenbrock, 1992b; Dai et al., 1994; Schmitt et al., 1996). In detached leaves, the suppression of PEPC transcripts or activity is rapid (within 8 h) and occurs in both well-watered and stressed leaves in a dose-responsive manner (Schmitt and Piepenbrock, 1992b; Dai et al., 1994). Cytokinin accumulation is also negatively correlated with PEPC transcript accumulation suggesting that leaf cytokinins act as negative effectors of CAM expression (Peters et al., 1997). It has also been postulated that water stress limits the supply of cytokinin from the roots via the transpiration stream resulting in derepression of PEPC expression (Schmitt and Piepenbrock, 1992b). In contrast, when applied to the rooting medium of soil or hydroponically grown *M. crystallinum*, cytokinin can mimic environmental stress by increasing the accumulation of PEPC transcripts, protein, and enzyme activity (Thomas et al., 1992b; Dai et al., 1994; Peters et al., 1997). Enolase and PGM transcripts have been shown to be similarly affected (Forsthoefel et al., 1995a,b). Unlike ABA, however, cytokinin accumulation in *M. crystallinum* leaves declines during salinity stress (Peters et al., 1997). Furthermore, induction occurs slowly over a period of one to seven days and is associated with other salt-induced physiological reactions such as accumulation of proline, polyols, and osmotin (Thomas et al., 1992b; Thomas and Bohnert, 1993; Peters et al., 1997). While cytokinin is known to directly modulate many changes in stress-related gene expression (Harding and Smigocki, 1994; Thomas et al., 1995), its effect may be secondary in nature, possibly resulting from water deficits caused by inhibition of root growth (Stenlid, 1982; Su and Howell, 1992). The opposing effects of the same growth regulator on CAM induction may also depend on the location of cytokinin application and the time of analysis following treatment (Schmitt et al., 1996; Peters et al., 1997). For example, application of cytokinin to leaves suppresses PEPC transcript accumulation stimulated by its simultaneous application to roots (Peters et al., 1997).

The plant growth regulator MJA is an important signal molecule which influences gene expression in a range of processes such as wounding or pathogen attack, osmotic or dehydration stress, and leaf senescence (Sembdner and Parthier, 1993; Reinbothe et al., 1994). MJA represses the expression of genes involved in photosynthetic carbon fixation. It has

also been implicated in modulating CAM induction by down-regulating PEPC expression and CAM. Exogenous application of MJA to leaves of *M. crystallinum* for several days inhibited the accumulation of PEPC transcripts, enzyme activity and CAM induction in salt stressed plants. MJA accelerated the decline in PEPC transcript amounts in excised leaves undergoing stress recovery (Schmitt et al., 1996) and inhibited PEPC accumulation in well-watered plants (Dai et al., 1994). When applied together, MJA and 6-BAP have an additive effect on the attenuation of PEPC and CAM induction. Consistent with a role in leaf senescence, treatment of *M. crystallinum* roots with MJA resulted in a strong and rapid induction of sulphhydryl endopeptidase expression in leaves (Forsthoefel et al., 1998). This proteinase may function to recycle amino acids for de novo synthesis of enzymes associated with stress adaptation. MJA also causes global changes in translational activity in response to environmental stress (Reinbothe et al., 1993a, 1994). Such changes are thought to be mediated, at least in part, through post-transcriptional modification of transcripts (Reinbothe et al., 1993a,b) and by stress-induced increases in ribosome inactivating proteins (RIPs) implicated in programmed senescence and stress adaptation (Stipe et al., 1996; Rippmann et al., 1997). The interplay of development, environmental stress, and growth regulators involved in the signal perception and transduction pathways that results in CAM induction is considered in more detail in Section VI.

IV. Genes, Transcripts, and Proteins

The enzymatic machinery required for CAM is present in all higher plants. In CAM plants, however, the key enzymes responsible for nocturnal CO_2 fixation, malate metabolism, glycolysis, and gluconeogenesis are expressed more abundantly than in C_3 plants. For example, during the shift from C_3 to CAM in *M. crystallinum* increased enzyme activities for less than 15 enzymes have been documented (Holtum and Winter, 1982; Winter et al., 1982). However, it has been estimated that the expression of several hundred genes is altered during this transition with about 100 genes being up-regulated (Meyer et al., 1990). As the discovery of genes from *M. crystallinum* and other CAM plants continues

(Table 1), more than 20 genes have been characterized whose expression is enhanced following environmental stress conditions known to induce CAM. In addition, many of these genes and enzymes are tightly regulated on a diurnal or circadian basis. The characterization of CAM-specific genes, gene families, and their expression patterns in facultative CAM plants has begun to shed light on the molecular mechanisms underlying the evolution and expression of genes responsible for CAM induction.

Two classes of CAM-related genes are beginning to emerge from molecular genetic analyses of gene structure and expression patterns. One class of genes, exemplified by the PEPC gene families in *M. crystallinum* and *K. blossfeldiana*, are encoded as small multigene families of which only one or two CAM-specific isoforms become highly induced during CAM induction to accommodate high rates of nocturnal CO_2 fixation (Cushman et al., 1989; Gehrig et al., 1995). In contrast, the expression of the C_3 isoforms, which fulfill various anaplerotic 'house-keeping' or tissue-specific functional roles, remains essentially unchanged. Multigene families having at least one CAM-specific isogene have also been reported for NADP-ME, NADP-MDH, enolase and PGM in *M. crystallinum* (Cushman, 1992, 1993; Forsthoefel et al., 1995a,b) and for the V-ATPase *c* subunit genes in *M. crystallinum* (Tsiantis et al., 1996) and *K. daigremontiana* (Bartholomew et al., 1996). Induction of V-ATPase likely takes place to sustain adequate rates of malate transport into the vacuole (Ratajczak et al., 1994; Barkla et al., 1995). Although the exact mechanism of gene recruitment to fulfill CAM-specific functions remains unknown at present, one scenario is gene duplication followed by gradual alterations in the 5' and 3' flanking regions and in coding regions which result, ultimately, in the creation of C_3 or CAM isoforms, each with distinct expression patterns. Alternatively, recruitment of a specific gene family member may have evolved through recombination or transposon-induced translocation events (Kloeckener-Gruisse and Freeling, 1995). The second class of inducible transcripts is represented by single genes which can apparently play roles in both C_3 photosynthesis and CAM as is the case of NAD-GAPDH (Ostrem et al., 1990) and PPDK (Fißlthaler et al., 1995). For these genes, transcriptional activation under stress conditions seems to occur through the action of stress-responsive enhancer elements.

For enzymes encoded by multigene families, the relative contribution to CAM of gene products arising from specific family members needs to be determined, particularly when these proteins reside in different subcellular compartments. For example, both mitochondrial NAD-ME, and cytosolic NADP-ME are represented by multiple isoforms (Saitou et al., 1994, 1995; Cook et al., 1995) and enzymes from both compartments are coordinately induced to support daytime malate decarboxylation (Holtum and Winter, 1982; Cushman, 1992; Ingram and Smith, 1995). Likewise, a cytosolic NAD-MDH and a chloroplastic NADP-MDH participate in nocturnal malic acid formation (Holtum and Winter, 1982; Cushman, 1993). Comparative studies of the expression, structure and kinetic properties of isozymes are needed to resolve the role that individual enzymes play in CAM.

V. Regulation of CAM Gene Expression

The switch from C₃ photosynthesis to CAM serves as an important paradigm for complex adaptations of photosynthetic carbon fixation to environmental stress. The kinetics of CAM induction depend on the type and severity of the stress and the developmental status of the plant (Cushman et al., 1990; Piepenbrock and Schmitt, 1991; Herppich et al., 1992; Schmitt and Piepenbrock, 1992a). Considering the correlation between stress severity and CAM induction, the buildup of many CAM-related enzyme activities during the transition (Holtum and Winter, 1982; Winter et al., 1982) is directly tied to increases in enzyme protein levels (Winter et al., 1982; Michalowski et al. 1989a; Schmitt et al., 1989) arising from de novo protein synthesis (Foster et al., 1982; Höfner et al., 1987). Transport processes at the tonoplast are enhanced during the CAM switch as a result of increased expression of V-ATPase subunits (Lüttege et al., 1995; Dietz and Arbinger, 1996; Löw et al., 1996; Tsiantis et al., 1996). Light-dependent chloroplast pyruvate transport is also induced in *M. crystallinum* (Koreeda et al., 1996), presumably as a result of increased synthesis of the pyruvate transporter. Chloroplastic starch degradation enzymes (Paul et al., 1993) and presumably triose and hexose phosphate transporters are also enhanced during CAM (Neuhaus and Schulte, 1996). Transcriptional, post-transcriptional, and translational controls are

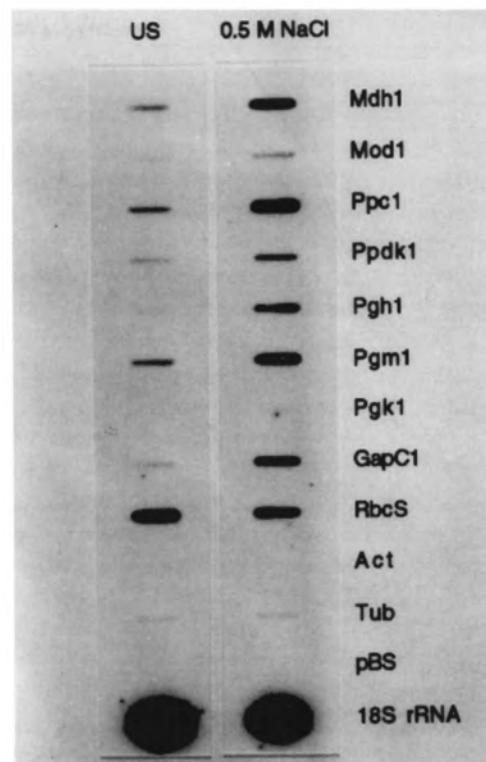


Fig. 1. In vitro transcription run-on assays of CAM specific genes. Cloned gene-specific probes for enzymes involved in malate metabolism and glycolysis/gluconeogenesis were blotted onto nitrocellulose and hybridized with transcripts radiolabeled in vitro by isolated nuclei from leaves of plants that were well watered (unstressed, US) or stressed for 5 d with 0.5 M NaCl (0.5 M NaCl). Transcription rates increased anywhere from two- to five-fold for: *Mdh1*, NADP⁺ malate dehydrogenase; *Mod1*, NAD(P)⁺ malic enzyme; *Ppc1*, phosphoenolpyruvate carboxylase; *Ppdk1*, pyruvate orthophosphate dikinase; *Pgh1*, enolase; *Pgm1*, phosphoglycerate mutase; *Pgk1*, phosphoglycerate kinase; *GapC1*, glyceraldehyde 3-phosphate dehydrogenase. Those genes not induced by salt stress include: *rbcS*, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; *Act*, actin, *Tub*, tubulin, 18S rRNA, 18S ribosomal RNA from soybean. Negative control, pBS, Bluescript SK+.

primarily responsible for the build-up of CAM-related enzymes, whereas post-translational controls mediate circadian regulation of enzyme activities during the day-night cycle.

A. Transcriptional Regulation

The expression of CAM-specific genes is regulated primarily at the level of transcription. PEPC mRNA accumulation occurs within 2–3 hours following

salinity or dehydration stress of detached leaves of *M. crystallinum* (Schmitt, 1990) or *K. blossfeldiana* (Brulfert et al., 1993; Taybi et al., 1995). Protein accumulation begins eight to ten hours after stress. Water stress increases PEPC activity, protein and mRNA accumulation even in the youngest leaves of *P. camptotricha* and *P. scandens*, (Ting et al., 1996) and PEPC transcripts in *M. crystallinum* leaves as young as one week old (Schmitt et al., 1996). Run-on transcription assays with nuclei isolated from leaves of *M. crystallinum* have shown that increased mRNA accumulation of many genes is a direct consequence of increased transcription rates (Fig. 1). Transcription rates increase from two- to six-fold in response to salinity stress depending on the gene in question (Cushman et al., 1989; Cushman, 1992, 1993; Vernon et al., 1993; Forsthoefel et al., 1995a,b). Similar increases in transcription rates of a CAM-specific isogene of PEPC have been found in *K. blossfeldiana* induced by short-day photoperiods (N. Richard and J. Brulfert, unpublished).

Transcriptional activation of CAM-specific genes is likely to be mediated by the action of transcriptional activator and repressor proteins through interactions in the promoter regions of these genes. Nuclear extracts from *M. crystallinum* contain multiple DNA-binding proteins which interact with the 5' flanking region of *Ppc1* to form DNA-protein complexes in vitro over a region of about 1200 nucleotides (Cushman and Bohnert, 1992). One particularly abundant complex, designated PC AT-1, interacts with AT-rich sequences between -205 to 1-28 of the *Ppc1* promoter and displays either increased abundance or tighter DNA binding affinity in nuclear extracts from CAM-induced, salt-stressed plants (Cushman and Bohnert, 1992). Although the exact role of PCAT-1 is unclear, it shares characteristics of HMG-like proteins suggesting it may play an architectural role in the assembly of active transcription complexes during CAM induction (Grasser, 1995). Less abundant DNA-binding complexes, detected in nuclear extracts prepared from roots and leaves of well-watered plants, may function as transcriptional repressors (Cushman and Bohnert, 1992). Other complexes interacting with 'salt-enhancer' regions of the *Ppc1* and *GapC1* promoters are more abundant following CAM-induction (Schaeffer et al., 1995). These salt-inducible DNA-binding activities resemble dehydration-inducible MYB-like factors in *Arabidopsis* (Urao et al., 1993) or the ABA-inducible complexes in nuclear

extracts from the resurrection plant, *C. plantagineum* (Nelson et al., 1994). Further analysis of DNA-binding proteins and their binding characteristics is required to elucidate the mechanisms by which they activate transcription during CAM induction.

The coordinate transcriptional activation of a set of CAM-specific genes (Fig. 1) suggests that common *cis*-acting regulatory elements mediate stress-inducible expression patterns. To test this hypothesis requires identification of specific regulatory elements in facultative CAM species such as *M. crystallinum*. A common strategy involves fusing regulatory sequences to a reporter gene, such as β -glucuronidase (GUS), and analyzing the resulting expression patterns either in stably transformed plants or in a transient transformation system. A transient expression system based on microprojectile bombardment of detached *M. crystallinum* leaves has been developed to identify *cis*-acting regulatory elements in the 5' flanking region of stress-inducible CAM genes (Schaeffer et al., 1995). Fusion constructs consisting of either the *Ppc1* (-977 to -679) or the *GapC1* (-735 to -549) 'salt-enhancer' region to their respective non-salt-responsive, 'minimal' promoters (-119 or -108, respectively) confirm that these regions are sufficient to confer salt-inducible gene expression. In fact, these regions in isolation are more effective than the intact promoters in conferring salt-induced gene expression, confirming the presence of silencing elements elsewhere in the 5' flanking regions. Fusion of the *Ppc1* -977 to -6-79 region to a truncated 'minimal' (-45) *Ppc1* promoter, however, completely abolished salt-inducibility supporting the requirement for cooperation between at least two *cis* elements for salt-responsive transcription (H. J. Schaeffer and J. C. Cushman, unpublished). Cooperation of two or more *cis* elements for regulation of gene transcription has been documented for promoters responsive to gibberellic acid (Rogers et al., 1994), ABA (Shen and Ho, 1995), light (Terzaghi and Cashmore, 1995), and ethylene (Xu et al., 1996). Comparison of the *Ppc1* and *GapC1* distal regions sufficient for salt-inducible gene expression revealed common MYB consensus binding sites suggesting that MYB-related transcription factors may be involved in controlling transcriptional activation events during CAM induction (Schaeffer et al., 1995). The participation of *myb*-related homologs from *Arabidopsis* (Urao et al., 1993) and maize (Hattori et al., 1992) in transcriptional responses to ABA, salt and dehydration

stress, suggests that related factors in *M. crystallinum* may also play a role in the expression of inducible CAM gene expression.

In addition to osmo-responsive elements, the promoter regions of CAM-specific PEPC, NAD-GAPDH, and PPDK genes contain GT motifs (BoxII) which may function in light-responsive gene expression and consensus ABA response elements which may participate in ABA-mediated gene expression events (Cushman et al., 1993; Schmitt et al., 1996). Other transcriptional control elements and factors conferring tissue-specific, diurnal or circadian, developmental, dehydration-, light-, and hormone-responsive gene expression must also be present.

B. Posttranscriptional and Translational Regulation

Posttranscriptional and translational mechanisms also play a role in regulating CAM expression, however, only indirect evidence for these control mechanisms is presently available. A comparison of transcription rates and transcript accumulation in *M. crystallinum* undergoing CAM induction at different ages showed that *Ppc1* mRNA stability is enhanced in older plants (Cushman et al., 1990). In contrast, *RbcS* transcripts encoding the small subunit of Rubisco decline rapidly upon salt stress (DeRocher and Bohnert, 1993; Bohnert et al., 1997). Transcription rates and mRNA accumulation for enolase increase in response to salinity stress without a corresponding increase in protein amounts suggesting that posttranscriptional/translational control factors participate in the expression of this glycolytic enzyme (Forsthoefel et al., 1995a). An increase in chloroplast RNA-binding proteins that may function to stabilize specific transcripts has been shown to accompany CAM induction (Breiteneder et al., 1994). While changes in mRNA populations following stress have been well documented (Ostrem et al., 1987), changes in mRNA utilization and translational efficiency are likely to contribute significantly to CAM-related expression patterns. During the transition from C₃ to CAM, total protein synthesis declines, whereas the synthesis of specific proteins, such as PEPC, increases (Höfner et al., 1987). Altered translational activity is correlated with changes in mRNA distribution on polysomes (Bohnert et al., 1999) and enhanced expression of a ribosome-inactivating protein (RIP)

in *M. crystallinum* (Rippmann et al., 1997). RIPs may alter translation through the turnover of ribosomes or sub-populations of ribosomes in response to stress.

C. Posttranslational Regulation

Posttranslational control mechanisms regulate diurnal or circadian activities of several key CAM enzymes. Most studies have focused on the reversible day/night regulation of PEPC which must be tightly controlled in order to avoid futile cycles of carboxylation and decarboxylation. The activity of this enzyme is influenced allosterically by both positive (glucose 6-P, triose-P) and negative (L-malate) effectors (Chollet et al., 1996; Vidal and Chollet, 1997). In addition, control of PEPC occurs by covalent modification through reversible phosphorylation by PEPC kinase first described in *Bryophyllum fedtschenkoi* and *Kalanchoë blossfeldiana* (Nimmo et al., 1984, 1986; Brulfert et al., 1986) and more recently in *M. crystallinum* (Baur et al., 1992; Li and Chollet, 1994; Weigend, 1994) as well as in numerous C₃ and C₄ species (Chollet et al., 1996; Vidal and Chollet, 1997). In CAM plants, the dephosphorylated 'day form' of the enzyme is more sensitive to malate inhibition. The more active, phosphorylated 'night form' has higher affinity for PEP and is more sensitive to glucose 6-P and triose-P (positive effectors), but less sensitive to L-malate (negative effector) (Nimmo et al., 1984; Nimmo et al., 1986; Jiao and Chollet, 1991). The phosphorylation site responsible for night activation of the CAM enzyme has been located at a highly conserved serine residue present in the N-terminal region of higher plant polypeptides which is absent from bacterial or cyanobacterial enzymes (Vidal and Chollet, 1997), although additional phosphorylation sites may be present. PEPC kinase activity is dependent on mRNA and protein synthesis and is controlled in a circadian fashion (Carter et al., 1991; 1996). In contrast, protein synthesis-dependent dephosphorylation of PEPC by a protein phosphatase 2A (PP2A) does not appear to be regulated by an endogenous rhythm (Carter et al., 1990). PEPC kinase activity is regulated at the level of translatable mRNA in *B. fedtschenkoi* (Hartwell et al., 1996) with PEPC kinase mRNA being approximately 20 times more abundant at night than during the day. PEPC kinase activity disappears within three hours of the decline in translatable mRNA indicating

rapid protein turnover (Hartwell et al., 1996). Inhibition of translation also blocks the appearance of translatable PEPC kinase mRNA indicating that upstream signaling events, such as the operation of the circadian clock, also require protein synthesis (Hartwell et al., 1996). These observations confirm that transcriptional regulatory events can not only govern the production of many CAM enzymes, but also control their post-translational regulation.

PEPC kinase is induced coincidentally with its target substrate by salt stress in *M. crystallinum* (Li and Chollet, 1994). Coordinate induction of PEPC and its regulatory kinase by ABA has been documented in detached leaves of *K. blossfeldiana*. Kinase accumulation is slightly delayed with respect to PEPC synthesis (T. Taybi and J. Brulfert, personal communication). Although the factors controlling PEPC kinase expression or activity are not understood, fluctuations in cytosolic malate levels or the processes that control malate movement into and out of the vacuole appear to play a role in the generation and regulation of its circadian rhythm (Anderson and Wilkins, 1989c). In C₄ plants, however, PEPC kinase appears to be regulated by phosphorylation via an upstream Ca²⁺-dependent protein kinase (Giglioli-Guivarac'h et al., 1996). Molecular cloning of the gene encoding PEPC kinase should greatly facilitate identification of the circadian oscillator and the intracellular signaling mechanisms by which it is regulated (see next section). Progress towards this goal has been made through the partial purification of a Ca²⁺-independent kinase identified as two polypeptides (39 and 32 kDa) capable of phosphorylating purified PEPC from C₃, C₄, and CAM species. Whether the two proteins represent two isoforms or are the result of proteolytic processing of a single protein remains unclear (Li and Chollet, 1994).

In addition to PEPC, several other CAM enzymes undergo post-translational regulatory phosphorylation events. PEPCK is phosphorylated in C₃, C₄, and CAM plants (Walker and Leegood, 1996). In PEPCK-type CAM plants, this decarboxylase should only be active during the day to avoid a futile carboxylation cycle between PEP and OAA since both PEPC and PEPCK are cytosolic. In *Tillandsia fasciculata*, PEPCK is phosphorylated at night and dephosphorylated during the day (Walker and Leegood, 1996). This phosphorylation pattern is likely to modulate the activity of the enzyme over the course of the diurnal cycle, however, it is not known how this occurs or whether PEPCK is regulated by light

or in response to a circadian rhythm.

Diurnal and circadian regulation of nitrate reductase in *B. fedtshchenkoi* is likely to involve multi-step post-translational regulation (Huber et al., 1996; Lillo et al., 1996). Covalent modification of NAD-ME may also be responsible for diurnal changes in the kinetic properties of this enzyme (Cook et al., 1995). Post-transcriptional control of enolase activity by reversible phosphorylation has been suggested for enolase from *M. crystallinum* (Forsthoefel et al., 1995a). Likewise, the activities of many chloroplastic and possibly cytosolic enzymes such as NAD-GAPDH, NADP-MDH, FBP, and enolase are light-regulated through covalent redox-modification of the ferredoxin-thioredoxin system which acts at redox-sensitive cysteine residues that participate in disulfide bridge formation (Anderson et al., 1995).

D. Circadian Rhythms

The circadian rhythm of photosynthetic gene expression in plants has provided important insights in the mechanisms of circadian clocks (Anderson and Kay, 1996). One of the most extensively studied circadian rhythms includes the rhythm of CO₂ metabolism in *Bryophyllum (Kalanchoë) fedtschenkoi* (Wilkins, 1992) and *K. daigremontiana* (Lütteg and Beck, 1992, 1996; Lütteg et al., 1996). Studies of CAM behavior in *K. blossfeldiana* (Queiroz and Morel, 1974), *K. fedtshenkoi* (Wilkins, 1984), and *K. diagremontiana* (Buchanan-Bollig and Smith, 1984a,b) have shown that plants kept under constant environmental conditions exhibit a persistent circadian rhythm of CO₂ exchange that is directly related to the circadian rhythm of PEPC activity and properties (Nimmo et al., 1984). Changes in enzyme properties parallel the in vitro and in vivo phosphorylation state of PEPC and PEPC kinase activity (Nimmo et al., 1986, 1987; Carter et al., 1991).

The reversible day/night pattern of PEPC phosphorylation is not simply controlled by light as PEPC kinase activity appeared several hours after the onset of darkness and disappeared a few hours before the end of darkness (Nimmo et al., 1984; Carter et al., 1991). Furthermore, the phosphorylation state of PEPC exhibits a persistent circadian rhythm under conditions of continuous illumination or darkness (Nimmo et al., 1987; Kusumi et al., 1994). Extremes in light intensity and temperature perturb circadian rhythms of CO₂ fixation (Anderson and

Wilkins, 1989a,b,c; Lüttge and Beck, 1992) through modifications not only of PEPC kinase activity (Carter et al., 1995a,b; 1996), but also through changes in malate concentrations in the vacuole and cytoplasm as controlled by permeability or transport at the tonoplast (Grams et al., 1996, 1997). These observations suggest that malate also plays a role in regulating PEPC kinase activity and/or PEPC activity. PEPC kinase activity can also be disrupted by treating detached leaves with inhibitors of transcription (cordycepin) and translation (cycloheximide or puromycin) (Carter et al., 1991; Nimmo, 1993; Hartwell et al., 1996). Despite these observations, the origin and the nature of the circadian oscillator controlling PEPC kinase activity remain elusive. Recent observations that PEPC kinase expression exhibits a circadian rhythm controlled at the level of transcription and possibly also at the level of translation (Hartwell et al., 1996), strongly suggest that PEPC kinase is an important component of the circadian oscillator. Protein phosphatase inhibitors are known to disrupt circadian oscillator function (Comolli et al., 1996). This observation provides further support for the role of protein phosphorylation in controlling the circadian rhythm of CAM. Once the gene for PEPC kinase is cloned, it should be possible to resolve whether this kinase is generating oscillations or controlling them. Entrainment studies using *Bryophyllum* leaves have confirmed that phytochrome is likely to serve as the only photoreceptor for the regulation of the circadian rhythm (Harris and Willkins, 1978 a,b). The input and output pathways of many phytochrome regulated circadian clocks utilize Ca^{2+} as a second messenger (Johnson et al., 1995; Anderson and Kay, 1996). Knowledge of the biochemical components of the circadian clock should lead to insights into the signaling events controlling clock entrainment and the induction of phase shifts.

E. Tissue-Specific Regulation

Traditionally, CAM was thought to be present in all chloroplast-containing tissues and absent in hydrenchymous tissues without developed chloroplasts as indicated by differences in CAM enzyme distribution between photosynthetically active and water-storing tissues (Kluge and Ting, 1978; Earnshaw et al., 1987; Winter, 1987; Springer and Outlaw, 1988). In general, chloroplast-containing cells fit the classical CAM enzyme spectrum, whereas chlorophyll-deficient tissues express CAM weakly

suggesting they serve in a 'CO₂ storage' capacity (Springer and Outlaw, 1988). However, recent analyses using histochemical techniques have established distinct spatial separation of CAM components within tissues. The most exhaustive studies of cell specialization within tissues have used *Peperomia camptotricha* (Nishio and Ting, 1987). In this species the leaf is organized into four cell layers: an upper multiple epidermis, a middle one- to two-layered palisade mesophyll, a lower spongy mesophyll, and an abaxial epidermis. Physical separation of these tissues indicated that CAM enzyme activity (PEPC and ME) and nocturnal CO₂ fixation predominates in the spongy mesophyll and to a lesser extent to the multiple epidermis (Nishio and Ting, 1987). Tissue printing of *Peperomia* leaves confirmed PEPC protein and transcript expression localized preferentially to these two layers (Ting et al., 1994). In contrast, enzymes characteristic of light-dependent C₃ photosynthesis and Rubisco activity are more abundant in palisade mesophyll (Nishio and Ting, 1987, 1993). Rubisco protein is distributed throughout the leaf whereas *rbcS* mRNA is most abundant in the palisade layer (Ting et al., 1994). *Peperomia* species may be unique in the degree of tissue-specific distributions of CAM and C₃ enzymes, however, in view of the morphological complexity of leaves in many CAM plants, a variety of tissue-specific expression patterns of C₃ and CAM enzymes may exist.

Tissue differentiation determines the extent to which CAM is induced. Photoperiodic (short-day) induction of CAM is more effective in leaves than in callus tissue from *K. blossfeldiana* (Brulfert et al., 1987) with the degree of induction being correlated positively with the degree of tissue organization (Kluge et al., 1987). Likewise, salinity stress has little effect on malate accumulation and PEPC activity or expression in heterotrophic cell suspension cultures (Treichel et al., 1988; Thomas et al., 1992) or green callus tissues from *M. crystallinum* (Yen et al. 1995) compared to the inductive effects observed in intact plants. The basis for the apparent dependence of the CAM syndrome on organized tissues is not fully understood.

The inability to induce CAM in cell cultures may be due to the lack of a functional photosynthetic apparatus (Thomas et al., 1992a). Recent studies with photomixotrophic cell cultures of *M. crystallinum* grown on starch showed only a weak (four-fold) induction of PEPC activity (Yen et al., 1995), although this was greater than that observed for

heterotrophically grown cell suspensions. These results suggest that the development of a photosynthetic machinery is paralleled by an increase in CAM-related gene expression. However, photoautotrophic cell cultures from *M. crystallinum*, grown with CO₂ as the sole carbon source, were unable to fully develop CAM (Willenbrink and Huesemann, 1995). These cells showed significantly increased rates of CO₂ fixation in the light, but when salt stressed, did not display CAM-related fluctuations or net nocturnal accumulations of malic acid. Nonetheless, they exhibited slight increases (1.5- to 4-fold) in extractable PEPC, NADP-ME, NAD- and NADP-MDH, and PPDK activities (Willenbrink and Huesemann, 1995). These increases could signal partial CAM induction in response to salinity at the cellular level without the corresponding fluctuations in C₄ acid or starch accumulation. Such fluctuations may depend on regulatory signals that are disrupted in the suspension cell cultures which may lack fully functional chloroplasts. One of the most important factors governing photosynthetic gene expression is the metabolic flux of carbohydrates and other metabolites, specifically hexoses, within sink and source leaves (Koch, 1996). In isolated maize protoplasts, the expression of several light-regulated photosynthetic genes, including PEPC and other chloroplast components such as the phosphate/triose-phosphate translocator is repressed by feedback inhibition of hexose sugars (Sheen, 1990, 1994). Hexokinases may serve as sensors and signaling molecules for sugar repression of photosynthetic genes (Jang and Sheen, 1997; Jang et al., 1997). Thus, it is likely that the signaling mechanisms associated with carbohydrate metabolism are disrupted under tissue culture conditions preventing appropriate expression and regulation of CAM-specific enzymes.

VI. Signal Transduction

The mechanisms by which stimuli are transduced into the activation of CAM gene expression and other stress-adaptive responses are poorly understood. Factors modulating gene expression in response to development, environmental stress, and plant growth regulator homeostasis reveal complex signal-transduction pathways for CAM induction. Following the initial perception of external stimuli by sensor/receptor complexes, signaling molecules (e.g., ABA)

or second messengers (e.g., Ca²⁺) form a complex web of parallel and converging pathways (Shinozaki and Yamaguchi-Shinozaki, 1996). Glimpses into the complexity of this web have already been obtained in *M. crystallinum* indicating multiple signal transduction pathways leading to CAM induction and other stress adaptations (Fig. 2) (Vernon et al., 1993; Tsiantis, 1996).

Among the well-characterized genes and proteins for stress-responsive pathways that assure survival for *M. crystallinum* by metabolic adaptations are three genes in different pathways. *Ppc1*, the gene encoding an induced isoform of PEPC, is strictly related to CAM biochemistry (Cushman et al., 1989), whereas *Vmac1* (*Atpvc*), which encodes the small c subunit of the vacuolar H⁺-ATPase, is responsible for pH and proton gradient maintenance across the tonoplast membrane (Löw et al., 1996; Tsiantis et al., 1996). The third gene is *Imt1*, which encodes inositol-O-methyltransferase, an enzyme involved in biosynthesis of ononitol (Vernon and Bohnert, 1992; Ishitani et al., 1996). Although all three genes are induced by salinity, they exhibit very different expression patterns in response to other types of environmental stress, reflecting distinctive aspects of their specific roles in stress adaptation (Vernon et al., 1993; Bohnert and Jensen, 1996; Tsiantis, 1996; Tsiantis et al., 1996). *Ppc1* mRNA accumulation can be readily induced by drought and treatment with 6-BAP, but not by low temperature. In contrast, *Imt1* mRNA fails to accumulate in response to drought and 6-BAP treatment, but does accumulate in response to low temperature (Vernon et al., 1993). Salinity induction of *Vmac1* expression appears to rely specifically on ionic stress, but not on osmotic stress components as neither mannitol nor drought are particularly effective inducers in mature plants (Tsiantis, 1996). Additional complexity is introduced by development because *Ppc1* and *Vmac1* cannot be induced in very young plants, whereas *Imt1* is only salt-inducible in mature plants, but is equally drought and salt-inducible in cotyledons, and strongly salt- and marginally drought-inducible in seedlings and very young plants (Tsiantis, 1996). Crosstalk between different signal-transduction chains can give rise to signal amplification and a wide variety of complex responses.

A. Photoreception

Phytochrome plays a central role in the initiation of

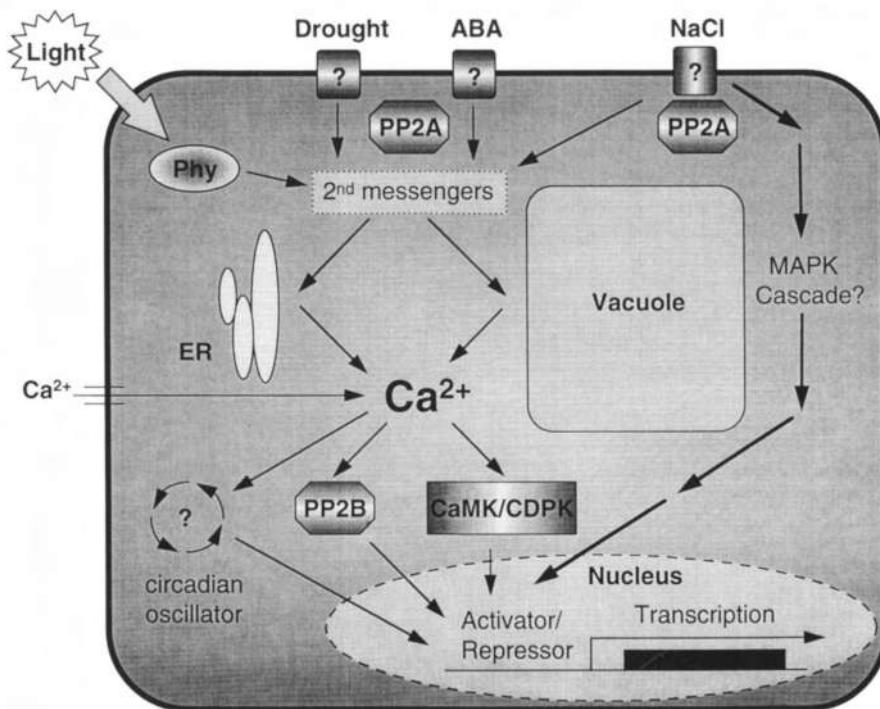


Fig. 2. Model of signal transduction events involved in CAM induction in *Mesembryanthemum crystallinum*. Perception of environmental stimuli from light, drought, salinity (NaCl), and ABA is mediated by phytochrome (Phy) or other sensors or receptors. Signals are transduced via PP2A and second messengers other than Ca²⁺. Second messengers, such as IP₃, alter uptake or release of Ca²⁺ from external or intracellular (ER and vacuole) stores. Cross-talk between signal transduction pathways is expected to occur. Increased [Ca²⁺] activates the circadian oscillator, protein phosphatases (PP2B), or calmodulin/Ca²⁺-dependent protein kinases (CaMK/CDPK) which ultimately result in the activation or repression of transcription. A MAPK kinase cascade (not shown) may also participate in NaCl signal transduction.

light-sensing signal transduction pathways (Quail et al., 1995). Phytochrome participates not only in the photoperiodic induction of CAM in *K. blossfeldiana* (Gregory et al., 1954; Brulfert et al., 1975; Brulfert et al., 1985) and the appearance of a CAM-specific isoform of PEPC (Brulfert et al., 1982a,c), but also regulates the circadian rhythm of CO₂ metabolism in *Bryophyllum* (Harris and Wilkins, 1978a,b). In *M. crystallinum*, long photoperiods, high light intensity, and red light enhanced NADP-ME and PEPC expression (Cheng and Edwards, 1991; McElwain et al., 1992; Cockburn et al., 1996). A low, but not a high, ratio of red:far red light stimulated PEPC expression and malate accumulation (Cockburn et al., 1996). These effects, which were amplified in salt stressed plants, suggested that phytochrome and NaCl or ABA may act along the same or parallel signal transduction pathways to promote CAM induction (McElwain et al., 1992; Cockburn et al., 1996). Recent studies based on microinjection of

putative signaling molecules or inhibitors indicated that heterotrimeric G proteins participate in three distinct phytochrome signal transduction pathways (Neuhaus et al., 1993; Bowler et al., 1994a,b). Phytochrome A activates G proteins leading to increases in cGMP levels or cellular levels of Ca²⁺ and the activation of calmodulin (Millar et al., 1994). The same signaling pathways are likely to both activate and repress different responses (Neuhaus et al., 1997). We expect that similar phytochrome-mediated signal transduction mechanisms participate in the activation or repression of CAM gene expression (see Fig. 3).

During the transition from C₃ to CAM, stomatal behavior is reversed, relative to stomatal rhythms in C₃ and C₄ plants. Red and/or blue light fail to induce stomatal opening in *P. atra* plants performing CAM (Lee and Assmann, 1992). In well-watered *M. crystallinum*, blue light is more effective than red light in opening stomata. When in CAM mode, however,

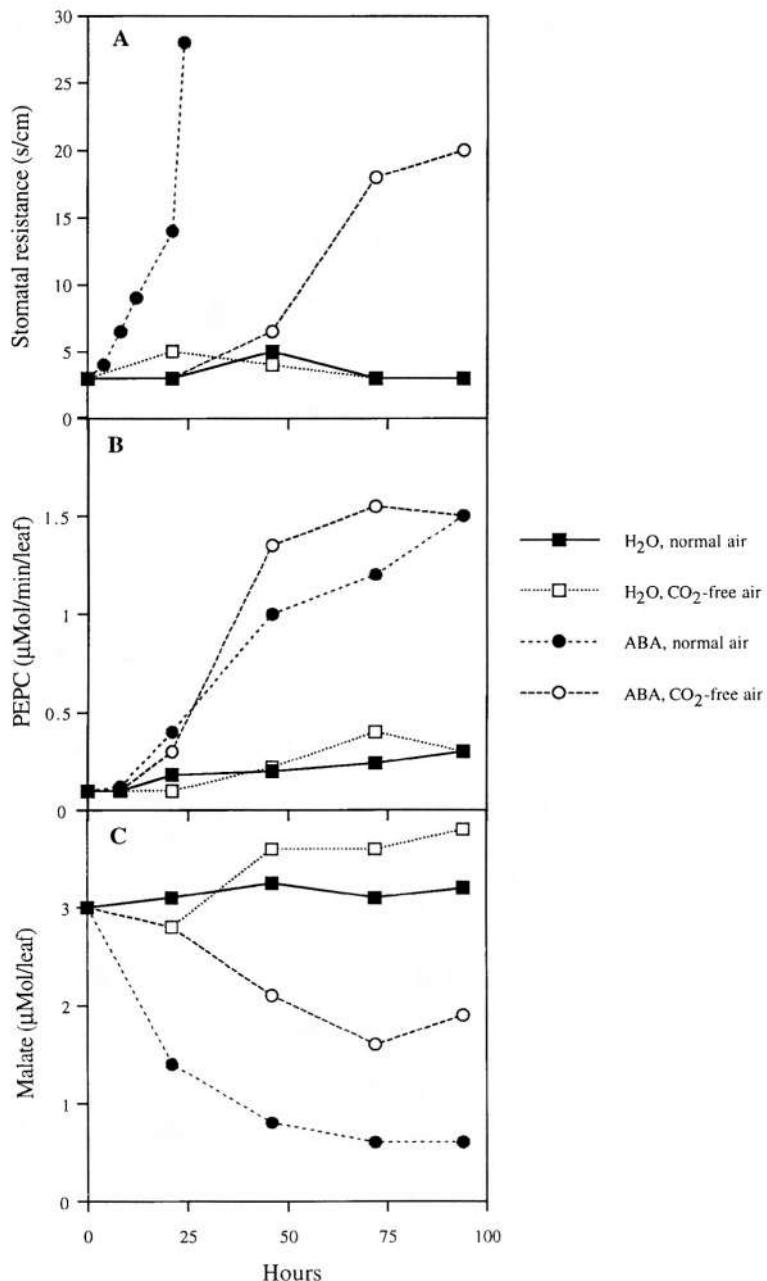


Fig. 3. Effects of CO_2 and ABA on CAM induction in detached leaves of *Kalanchoe blossfeldiana*. Stomatal resistance (A), PEPC activity (B) and malate content (C) in detached leaves immersed in H_2O or a solution of 10 μM ABA under normal air or CO_2 -free air.

illumination also failed to open stomata suggesting that the blue- and red-light photoreceptors become inoperable (Mawson and Zaugg, 1994). Treatment of epidermal tissues from plants performing CAM with fusicoccin, a fungal toxin that activates the plasmalemma H^+ -ATPase, failed to cause an increase in stomatal opening, whereas ABA-induced stomatal

closure was still functional. Inactivation of guard-cell photoreceptors may allow other factors, such as ABA or changes in $[\text{CO}_2]$, to dictate stomatal movements (Mawson and Zaugg, 1994). The nature and role of photoreceptors and other components in the signal transduction pathway(s) operating in response to light in CAM plants remain uncertain at

present. However, recent evidence suggests that CAM induction by NaCl stress abolished both the white- and blue-light-stimulated stomatal opening and light-dependent zeaxanthin formation (Tallman et al., 1997). These results suggest that the inhibition of light-dependent zeaxanthin formation in guard-cell chloroplasts maybe one of the important components that regulates the shift from diurnal to nocturnal stomatal opening during CAM induction. Other components such as Ca^{2+} - or CaM-dependent protein kinases may also be involved in maintaining stomatal closure (Cotelle et al., 1996; Li et al., 1998).

B. Intracellular Calcium

In higher plants, intracellular Ca^{2+} acts as an important second messenger for signal transduction associated with a wide range of stimuli and plant growth regulators (Poovaiah and Reddy, 1993; Bethke et al., 1995; Bush, 1995). The concentration of Ca^{2+} within various subcellular compartments is tightly controlled by a series of channels and pumps which are in turn regulated by extra- or intracellular signals. For example, ABA-, stretch-, or voltage-dependent Ca^{2+} channels at the plasma membrane, inositol 1,4,5-triphosphate (IP_3), cyclic ADP-ribose, and voltage-dependent channels at the tonoplast, and voltage-gated (presumably mechanosensitive) channels at the endoplasmic reticulum have been characterized along with Ca^{2+} exchangers and Ca^{2+} ATPases (Bethke et al., 1995; Bush, 1995; Barbier-Bryggo et al., 1997). A variety of signals cause transient, sustained or oscillating changes in Ca^{2+} in different subcellular locations (Bethke et al., 1995; Johnson et al., 1995; Knight et al., 1996). External signals (light, NaCl) and hormones (ABA) involved in CAM, as well as the circadian clock that regulates the daily CAM rhythm, are likely to act through fluctuations of intracellular Ca^{2+} . However, direct evidence for the participation of Ca^{2+} signaling in CAM induction is lacking.

Recent experiments with detached *M. crystallinum* leaves provided indirect evidence for the role of intracellular $[\text{Ca}^{2+}]$ in CAM induction. Treatment of detached leaves with the Ca^{2+} ionophore, ionomycin, and thapsigargin, a specific inhibitor of endomembrane Ca^{2+} -ATPases and stimulator of intracellular Ca^{2+} release, resulted in increased *Ppc1* and *Vmac1* mRNA accumulation (Tsiantis, 1996). The effect of thapsigargin on *Ppc1* expression was time-dependent, dose-responsive, and synergistic with NaCl treatment

(Taybi and Cushman 1998). These results are confirmed by the observation that the calcium chelator, EGTA, completely abolished *Ppc1* mRNA induction by thapsigargin, salinity, drought, and ABA treatments (Taybi and Cushman, 1998). In contrast, thapsigargin and ionomycin treatments did not induce *Imt1*, suggesting that expression of this gene is regulated by a distinct signal transduction pathway (Tsiantis, 1996).

C. Protein Kinases and Phosphatases

Protein phosphorylation and dephosphorylation participate in a wide range of signal transduction pathways (Stone and Walker, 1995; Smith and Walker, 1996). Signal perception is mediated by transmembrane receptors through the activation of a kinase domain intrinsic to receptor protein kinases (RPKs) (Braun and Walker, 1996). Recently, an RPK from *Arabidopsis* was isolated that is induced by dehydration, high salt, low temperature and ABA treatment suggesting that receptor kinases participate in the transmission of ABA and environmental stress signals (Hong et al., 1997). Downstream targets for RPK-mediated signaling have been identified as type 2C protein phosphatases which interact in a phosphorylation-dependent manner with specific subsets of RPKs (Braun et al., 1997). In *Arabidopsis*, water-stress-responsive gene expression is mediated by at least four independent signal transduction pathways with some pathways being ABA-dependent, whereas others are ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 1996). In addition, various signal transduction components such as MAP kinases, two-component histidine kinases, calcium-dependent protein kinase, and phospholipase C are stress-induced suggesting that both $\text{IP}_3\text{-Ca}^{2+}$ and MAP kinase cascades are involved (Jonak et al., 1996; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997; Mizoguchi et al., 1997). These observations suggest that water stress stimuli that lead to CAM induction are mediated by multiple, independent signal transduction pathways involving protein phosphorylation.

Specific inhibitors of kinases and phosphatases have provided a powerful approach to initially assess the role of protein phosphorylation in controlling the cellular events leading to CAM induction. Okadaic acid, a toxin that inhibits PP2A and to a lesser extent PP1, inhibited the salt-responsive induction of *Ppc1* and *Imt1* transcripts, whereas it was not effective in

abolishing the induction of *Vmac1* (Tsiantis, 1996). Okadaic acid and the PP2A inhibitor, cantharidic acid, were also effective inhibitors of *Ppc1* transcript accumulation induced by dehydration and ABA. These results implicate PP2A (and PP1) or related phosphatases in signaling leading to CAM induction, however, such experimental results must be viewed with extreme caution given the nonspecific effect of these inhibitors. Cyclosporin A, which specifically targets Ca^{2+} /calmodulin-activated PP2B (calcineurin), blocked the salt-induction of *Imt1*, but did not negatively affect *Ppc1* or *Vmac1* expression (Tsiantis, 1996). Cyclosporin A, in fact, resulted in increased *Ppc1* transcript accumulation during stress or ABA treatments of detached leaves (Taybi and Cushman, 1998). Thus, PP2B (calcineurin) activity seems to participate in negative or positive regulation of both ionic and dehydration stress signaling depending on the specific gene in question.

Although pharmacological studies cannot rigorously identify specific kinases or phosphatases, they clearly indicate that multiple independent, yet interrelated signal transduction pathways lead to CAM gene expression (Fig. 2). An example of convergent signaling pathways was revealed by treating detached *M. crystallinum* leaves with W7, a specific inhibitor of Ca^{2+} /calmodulin-dependent protein kinases, which blocked *Ppc1* transcript accumulation in response to ionic, osmotic, and dehydration stress (Taybi and Cushman, 1998). This result suggests that Ca^{2+} /CaM protein kinases play a role in stress-induced increases in *Ppc1* expression. In contrast, the effect on CAM induction in *M. crystallinum* was enhanced by cyclosporin A pretreatment presumably through the opposing activities of PP2B and Ca^{2+} /CaM kinase. Interestingly, both activities can be influenced by intracellular $[\text{Ca}^{2+}]$. Differential sensitivity to changing intracellular $[\text{Ca}^{2+}]$ through different Ca^{2+} -dependent kinases and phosphatases each having unique Ca^{2+} -binding properties and kinetics could explain selective activation of different signaling pathways in response to various stimuli. Definitive identification of the specific kinases and phosphatases and branchpoints associated with these different pathways will require a combination of biochemical and genetic approaches.

Characterization of sensors and transducers will be essential to advance our understanding of the various signaling pathways leading to CAM induction. Such analyses are presently underway in

M. crystallinum from which a number of protein kinase genes have been cloned. One ser/thr protein kinase cDNA termed MK9 (Baur et al., 1994) shares similarity to an ABA-, dehydration-, cold-, and osmotic stress-inducible protein kinase from wheat (Hoolaap and Walker-Simmons, 1995). MK9 also shares sequence homology with SNF1 ('sucrose nonfermenting'), a protein kinase from yeast required for derepression of glucose-repressed genes (Jiang and Carlson, 1996) that may act downstream of hexokinase in sugar signaling (Jang and Sheen, 1997). MK9 expression is enhanced by drought stress, ABA and 6-BAP treatment (J. Cushman, unpublished) suggesting it may play roles in either sugar-responsive or stress-responsive signaling or crosstalk between these pathways. A second ser/thr protein kinase cDNA (MK6) has been described which shows preferential expression at night in *M. crystallinum* plants performing CAM (B. Baur, personal communication). This protein kinase may play a role in controlling the diurnal or circadian expression of CAM enzymes. Additional protein kinases related to cyclic-nucleotide-dependent (PKA and PKG) or Ca^{2+} -phospholipid-dependent kinases (PKC) have also been isolated from *M. crystallinum* ('AGC' kinases, Table 1). This group of kinases is likely to act downstream of second messengers in signal transduction cascades (Stone and Walker, 1995). Other signal transducing components such as Ca^{2+} -dependent kinases, G-proteins and 14-3-3 proteins have also been characterized in *M. crystallinum* (Table 1).

D. Plant Growth Regulators

Induction of CAM gene expression has been associated with the action of several plant growth regulators, although their precise function remains unclear. ABA acts through a complex network of signaling pathways (Giraudat, 1995). Endogenous increases in ABA or exogenous ABA application correlate with CAM induction and CAM gene expression (Sipes and Ting, 1985; Ting, 1985, 1987; Chu et al., 1990; Thomas et al., 1992b; Thomas and Bohnert, 1993; Dai et al., 1994; Taybi et al., 1995). The duration of ABA application dictates the extent of CAM induction (Taybi et al., 1995). However, ABA has never been shown to induce *Imt1*, whereas it will induce *Vmac1*, and *Ppc1* (depending on age) transcript accumulation (Tsiantis, 1996).

To determine if ABA exerts its action directly or indirectly through reduced CO_2 concentrations, detached *K. blossfeldiana* leaves were exposed to ABA under normal air or CO_2 -free conditions (Fig. 3A). A rapid increase in stomatal resistance in air was observed, however, stomatal closure was delayed significantly in the absence of CO_2 (Fig. 3A). ABA elicited an increase in PEPC activity (Fig. 3B) and malate consumption (Fig. 3C) which was counteracted by low CO_2 . ABA and CO_2 deficit do not act synergistically with respect to changes in PEPCase activity or malate accumulation. Furthermore, CO_2 deficit does not trigger CAM induction brought about by either exogenous ABA exposure or environmental stress. Conversely, when detached leaves are supplied with ABA in the presence of saturating CO_2 (5%), a typical CAM pattern of CO_2 exchange is observed at night, confirming that ABA control of stomatal behavior is not governed by $[\text{CO}_2]$ (Taybi, 1995).

Cytokinins regulate various developmental processes, however, little is known about their role in signal transduction (Binns, 1994). Cytokinins, like ABA, induce a variety of CAM-related transcripts (Table 1), mimic salinity- or drought-induced CAM induction (Thomas and Bohnert, 1993; Dai et al., 1994), and mimic salt-induced physiological reactions such as proline, polyol, and osmotin accumulation when applied to roots (Thomas and Bohnert, 1993; Dai et al., 1994). However, cytokinins can also have an inhibitory effect on salinity- or dehydration-induced PEPC expression when supplied to detached leaves (Schmitt and Piepenbrock, 1992b; Dai et al., 1994). Recently, a histidine RPK (Kakimoto, 1996) and cytokinin-responsive SNF1-like kinases have been identified (Sano and Youssefian, 1994). Characterization of related components in CAM plants should lead to a greater understanding of cytokinin signaling mechanisms during CAM induction.

VII. Future Prospects

CAM is a complex adaptation to CO_2 - or water-limited environments requiring the coordination of gene expression, biochemistry, physiology, and anatomical features that distinguish the pathway from C_3 photosynthesis. Recent work with facultative CAM species has begun to yield substantial insights into how developmental and environmental signals initiate

CAM gene expression and maintain circadian oscillations of the cycle. To further advance our knowledge of the molecular basis of CAM induction, a combination of biochemical, genetic and molecular approaches will be needed.

A. Genetic and Molecular Genetic Analysis of CAM

Mutant collections in genetic model plants such as maize (Neuffer et al., 1996) and *Arabidopsis* (Meyerowitz and Somerville, 1994) have provided plant biologists with essential tools for understanding plant structure, development, and metabolism. To our knowledge, however, there are no reports of mutants defective in CAM or of mutants in any CAM species. Facultative CAM species, such as *K. blossfeldiana* or *M. crystallinum*, will be particularly useful if they can be developed into genetic models. *M. crystallinum* is a logical choice since it represents the best-studied CAM plant at the molecular level (see Table 1). It is a self-fertile species with a relatively small genome, twice that of *Arabidopsis*, and nine chromosomes ($2N = 18$), and it shows developmentally regulated polyploidy (DeRocher et al., 1990; Adams et al., 1997). The normal life cycle of *M. crystallinum* takes five months to complete in its natural habitat (Winter et al., 1978). However, the ability to grow the plants under long photoperiods, which shortens the life cycle to only seven weeks, miniaturizes the plants, and accelerates CAM induction (Cheng and Edwards, 1991), will expedite genetic studies.

Strategies which have been used to isolate photosynthetic carbon metabolism mutants (Somerville, 1986) can be applied to detect mutants in CAM species. One simple strategy involves screening of mutagenized plants for the presence or absence of starch (Caspar et al., 1985) which, for example, led to the detection of a mutant that lacked the chloroplast isozyme of phosphoglucomutase. Mutations in Rubisco activase and Rubisco enzymes have been obtained by growing mutagenized plants at elevated CO_2 concentrations (0.7–1 %) followed by transfer to a low CO_2 atmosphere (Somerville, 1984; Blackwell et al., 1988; Lea and Forde, 1994). Mutants lacking C_4 photosynthetic pathway enzymes, such as PEPC, NAD-ME, and glycine decarboxylase have been described (Dever et al., 1995). In addition, several maize C_4 photosynthetic mutants have been

characterized that are defective in bundle sheath and mesophyll cell differentiation (Langdale and Kidner, 1994; Langdale, 1995; Roth et al., 1996).

To begin genetic studies in a CAM species, mutant collections have been established in *M. crystallinum* following irradiation with fast neutrons and exposure to chemical mutagens (H. J. Bohnert and J. C. Cushman, unpublished). Pilot experiments using both X-ray- and EMS-generated mutants have resulted in the isolation of phenotypes that should be meaningful for the genetic dissection of CAM. However, T-DNA (Feldman, 1991) or transposon mutagenesis strategies (Altmann et al., 1995) and promoter and exon trapping approaches (Smith and Fedoroff, 1995) should also be explored. Selection schemes for screening putative CAM mutants that rely on detecting plants that fail to conduct nocturnal acidification and/or daytime starch accumulation have also been developed (Fig. 4). A concerted effort by several laboratories for generating, characterizing, and maintaining CAM mutant populations should provide an important genetic resource that will aid in the identification and characterization of genes important to CAM.

Another strategy that will allow rapid identification of novel genes from CAM plants is partial or complete sequencing of cDNAs for expressed sequences. Such expressed sequence tags (ESTs) complement genetic

analyses and provide markers for genomic mapping and gene expression studies. Analysis of large numbers of randomly selected cDNAs from rice (Uchimiya et al., 1992; Sasaki et al., 1994), maize (Keith et al., 1993), *Brassica napus* (Park et al., 1993), and *Arabidopsis* (Cooke et al., 1996) provided an invaluable resource for plant molecular biologists and for genome sequencing efforts. As a prelude to the analysis of the *M. crystallinum* genome, two EST projects have been initiated: one targeting leaf tissue of unstressed and stressed plants (J. C. Cushman, unpublished), and the other targeting transcripts specific for epidermal bladder cells (D. E. Nelson and H. J. Bohnert, unpublished) that seem to be essential for ion homeostasis under salt stress conditions. Rather than representing a duplication of ongoing EST sequencing programs in other species, the judicious sequencing of cDNAs in a CAM species is justified because CAM is a unique adaptation absent from C₃ or C₄ model plants.

B. Transgenic Analysis

Central to a long-term strategy for developing any CAM plant into a useful genetic model system is the ability to easily and rapidly transform the species. Several species exhibit susceptibility to Agro-

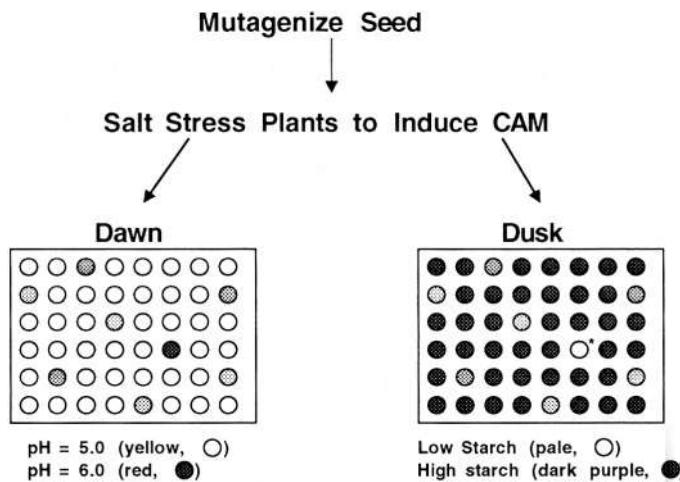


Fig. 4. *M. crystallinum* plants from mutagenized by EMS or fast neutron bombardment are planted in soil and screened after salinity stress treatment for the ability to perform nocturnal acidification or daytime starch accumulation. Duplicate leaf disc punches are collected at dawn or at dusk in a 48-well microtiter plate. At dawn, leaf discs are assayed for pH using chlorophenol red, a pH color indicator for the pH range spanning 5.0 (yellow, indicated by white circles) to 6.0 (red, indicated by black circles) to monitor C₄ acid accumulation. Intermediate levels of acidification are indicated by gray circles. At dusk, leaf discs are screened for starch accumulation using a mixture of iodine and potassium iodine. Wildtype plants that accumulate normal levels of starch show intense iodine staining (indicated by dark circle). Mutants deficient in daytime starch accumulation show weak iodine staining (indicated by white circles). Intermediate levels of starch accumulation are indicated by gray circles. Plants failing one or both tests are scored as putative CAM mutants (indicated by asterisk) and are selected for further characterization.

bacterium infection including *K. daigremontiana* (Garfinkel and Nester, 1980; Boulanger et al., 1986) and *M. crystallinum*. Successful *Agrobacterium*-mediated transformation of CAM plants has only been reported for a few species including *K. laciniata* (Jia et al., 1989) and *K. blossfeldiana* (Aida and Shibata, 1996). In *M. crystallinum*, transgenic hairy-root cultures (Andolfatto et al., 1994) or callus tissue (J. C. Thomas, T. Wulan and J. C. Cushman, unpublished) have been obtained following transformation by *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively. Even though fertile *M. crystallinum* plants can be regenerated via organogenesis from tissue explants (Meiners et al., 1991) and callus tissue (Wang and Lüttge, 1994) with low efficiency, no transgenic plants have been reported. Several reporter genes encoding β -glucuronidase (GUS), luciferase (LUC), and synthetic green fluorescence protein (GFP), are expressed in transient expression assays of bombarded *M. crystallinum* leaves and stably transformed tissues (Cushman et al., 1993; Schaeffer et al., 1995; T. Wulan, J. C. Cushman and D. E. Nelson, unpublished), indicating that these proteins provide suitable reporters for transgenic analyses. The ability to efficiently transform model CAM species will allow genetic manipulation of the CAM pathway and should facilitate investigations into the expression, structure, and function of enzymes and other key regulatory components.

Transgenic CAM plants will be essential for analyzing gene expression patterns that are distinct from those found in C_3 or C_4 plants. Several *M. crystallinum* promoters, including those for *Ppc1* (Cushman and Bohnert, 1993), *MipA*, *MipB*, *Vmac1*, *Imt1*, and *Inps1* (S. Yamada, M. Tsiantis and D. Nelson, unpublished), function in transgenic tobacco and *Arabidopsis* leading to strong expression of reporter proteins. However, in some cases, as with the *Ppc1* promoter, the salt-inducible expression pattern observed in *M. crystallinum* is not retained in transgenic tobacco suggesting that this C_3 plant lacks the necessary regulatory machinery (via activation or repression) to confer salinity-stress inducible expression (Cushman and Bohnert, 1993). The expression of other CAM-gene promoters in C_3 plants and the comparative analysis of the constructs in transgenic CAM plants, will provide information about the regulation, inducibility, and cell-specificity of CAM-specific control elements, whose existence, at present, can only be inferred.

VIII. Conclusions

The ability of some CAM plants to adapt to environmental stress by altering their ability to conduct CAM represents an important paradigm in photosynthesis research. Over the last eight years, our knowledge of the molecular genetics of CAM has increased tremendously, especially from research with facultative CAM plants. The picture of CAM induction that emerges is complex with gene expression changes being controlled at many different levels and multiple signal transduction pathways participating in the perception of diverse environmental factors. While a handful of stress-induced genes in the CAM pathway have now been characterized, it is anticipated that hundreds of genes and their corresponding gene products will be directly or indirectly involved. New initiatives such as large-scale comparative sequencing efforts of expressed genes and genomic DNA will provide a wealth of new information for molecular phylogenetic studies. The development of CAM mutants and efficient transformation systems promise to usher in a new era of research on CAM. These approaches, coupled with the addition of suitable cell biological systems, based on cell suspension cultures, protoplasts, or microinjection of single cells, will allow a thorough dissection of CAM regulation and signal transduction processes, and will open the door to new and exciting insights into the molecular mechanisms governing CAM induction.

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References

- Adams P, Nelson DE, Yamada S, Chmara W, Jensen RG, Griffiths H and Bohnert HJ (1998) Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). *New Phytol* 138: 171–190
- Aida R and Shibata M (1996) Transformation of *Kalanchoë blossfeldiana* mediated by *Agrobacterium tumefaciens* and transgene silencing. *Plant Sci* 121: 175–185
- Altmann T, Felix G, Jessop A, Kauschman A, Uwer U, Penacortes H and Willmitzer L (1995) Ac/Ds transposon mutagenesis in *Arabidopsis thaliana*: Mutant spectrum and frequency of Ds insertion mutants. *Mol Genet* 247: 646–652
- Anderson CM and Wilkins MB (1989a) Period and phase control by temperatures in the circadian rhythm of CO_2 exchange in illuminated leaves of *Bryophyllum fedtschenkoi*. *Planta* 177: 456–69
- Anderson CM and Wilkins MB (1989b) Control of the circadian rhythm of carbon dioxide assimilation in *Bryophyllum* leaves by exposure to darkness and high carbon dioxide concentrations. *Planta* 177: 401–408
- Anderson CM and Wilkins MB (1989c) Phase resetting of the circadian rhythm of carbon dioxide assimilation *Bryophyllum fedtschenkoi* leaves in relation to their malate content following brief exposure to high and low temperatures, darkness, and 5% carbon dioxide. *Planta* 180: 61–73
- Anderson LE, Li D, Parakeet N and Stevens FJ (1995) Identification of potential redox-sensitive cysteines in cytosolic forms of fructosebisphosphatase and glyceraldehyde-3-phosphate dehydrogenase. *Planta* 196: 118–124
- Anderson SL and Kay SA (1996) Illuminating the mechanism of the circadian clock in plants. *Trends Plant Sci* 1: 51–57
- Andolfatto R, Bornhäuser A, Bohnert HJ and Thomas JC (1994) Transformed hairy rootsof *Mesembryanthemum crystallinum*: Gene expression patterns upon salt stress. *Physiol Plant* 90: 708–714
- Barbier-Brygoo H, Joyard J, Pugin A and Ranjeva R (1997) Intracellular compartmentation and plant cell signalling. *Trends Plant Sci* 2: 214–222
- Barkla BJ, Zingarelli L, Blumwald E and Smith JAC (1995) Tonoplast Na^+/H^+ antiport activity and its energization by the vacuolar H^+ -ATPase in the halophytic plant *Mesembryanthemum crystallinum* L. *Plant Physiol* 109: 549–556
- Bartholomew DM, Rees DJG, Rambaut A and Smith JAC (1996) Isolation and sequence analysis of a cDNA encoding the c subunit of a vacuolar-type H^+ -ATPase from the CAM plant *Kalanchoë daigremontiana*. *Plant Molec Biol* 31: 435–442
- Bastide B, Sipes D, Hann J and Ting IP (1993) Effect of severe water stress on aspects of Crassulacean acid metabolism in *Xerosicyos*. *Plant Physiol* 103: 1089–1096
- Baur B, Dietz KJ and Winter K (1992) Regulatory protein phosphorylation of phosphoenolpyruvate carboxylase in the facultative Crassulacean-acid-metabolism plant *Mesembryanthemum crystallinum* L. *Eur J Biochem* 209: 95–101
- Baur B, Fisher K, Winter K and Dietz KJ (1994) cDNA sequences of a protein kinase from the halophyte *Mesembryanthemum crystallinum* L., encoding a SNF-1 homologue. *Plant Physiol* 106: 1225–1226
- Becker A, Canut H, Lütge U, Maeshima M, Marigo G and Ratajczak R (1995) Purification and immunological comparison of the tonoplast H^+ -pyrophosphatase from cells of *Catharanthus roseus* and leaves from *Mesembryanthemum crystallinum* performing C_3 -photosynthesis and the obligate CAM-plant *Kalanchoë daigremontiana*. *J Plant Physiol* 146: 88–94
- Bethke PC, Gilroy S and Jones RL (1995) Calcium and plant hormone action. In: Davies PJ (ed) *Plant Hormones: Physiology, Biochemistry and Molecular Biology*, pp 298–317. Kluwer Academic Publishers, Dordrecht
- Binns AN (1994) Cytokinin accumulation and action: Biochemical, genetic, and molecular approaches. *Annu Rev Plant Physiol Plant Mol Biol* 45: 173–196
- Blackwell RD, Murray AJS, Lea PJ, Kendall AC, Hall NP, Turner JC and Wallsgrove RM (1988) The value of mutants unable to carry out photorespiration. *Photosynth Res* 16: 155–176
- Bohnert HJ and Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. *Trends Biotech* 14: 89–97
- Bohnert HJ, Thomas JC, DeRocher EJ, Michalowski CB, Breiteneder H, Vernon DM, Deng W, Yamada S and Jensen RG (1994) Responses to salt stress in the halophyte *Mesembryanthemum crystallinum*. In: Cherry JH (ed) *Biochemical and Cellular Mechanisms of Stress Tolerance in Plants*, pp 415–428. Springer-Verlag, Berlin
- Bohnert HJ, DeRocher EJ, Michalowski CB and Jensen RG (1999) Environmental stress and chloroplast metabolism. In: Bansal KC (ed) *Recent Advances in Plant Molecular Biology*. Oxford and IBH Publishers, New Delhi, in press
- Borland AM and Griffiths H (1996) Variations in the phases of Crassulacean acid metabolism and regulation of carboxylation patterns determined by carbon-isotope-discrimination techniques. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 230–249. Springer-Verlag, Berlin
- Boulanger F, Berkalooff A and Richaud F (1986) Identification of hairy root loci in the T-regions of *Agrobacterium rhizogenes* Ri plasmids. *Plant Mol Biol* 6: 271–279
- Bowler C, Neuhaus G, Yamagata H and Chua NH (1994a) Cyclic GMP and calcium mediated phytochrome phototransduction. *Cell* 77: 73–81
- Bowler C, Yamagata H, Neuhaus G and Chua NH (1994b) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. *Genes Dev* 8: 2188–2202
- Braun DM and Walker JC (1996) Plant transmembrane receptors: New pieces in the signaling puzzle. *Trends Biochem Sci* 21: 70–73
- Braun DM, Stone JM and Walker JC (1997) Interaction of the maize and *Arabidopsis* kinase interaction domains with a subset of receptor-like protein kinases: Implications for transmembrane signaling in plants. *Plant J* 12: 83–95
- Bray (1997) Plant responses to water deficit. *Trends Plant Sci* 2: 48–54
- Breiteneder H, Michalowski CB and Bohnert HJ (1994) Environmental stress-mediated differential 3' end formation of chloroplast RNA-binding protein transcripts. *Plant Molec Biol* 26: 833–849
- Brulfert J, Guerrier D and Queiroz O (1975) Photoperiodism and enzyme rhythms: Kinetic characteristics of the photoperiodic induction of Crassulacean acid metabolism. *Planta* 125: 33–44
- Brulfert J, Müller D, Kluge M and Queiroz O (1982a) Photoperiodism and Crassulacean acid metabolism I. Immunological and kinetic evidences for different patterns of

- phosphoenolpyruvate carboxylase isoforms in photo-periodically inducible and non-inducible Crassulacean acid metabolism plants. *Planta* 154: 326–331
- Brulfert J, Guerrier D and Queiroz O (1982b) Photoperiodism and Crassulacean acid metabolism II. Relations between lead aging and photoperiod in Crassulacean acid metabolism induction. *Planta* 154: 332–338
- Brulfert J, Vidal J, Keryer E, Thomas M, Gadal P and Queiroz O (1985) Phytochrome control of phosphoenolpyruvate carboxylase synthesis and specific RNA level during photoperiodic induction in a CAM plant and during greening in a C_4 plant. *Physiol Vég* 23: 921–928
- Brulfert J, Vidal J, Le Marechal P, Gadal P, Queiroz Q, Kluge M and Krüger (1986) Phosphorylation-dephosphorylation process as a probable mechanism for the diurnal regulatory changes of phosphoenolpyruvate carboxylase in CAM plants. *Biochem Biophys Res Commun* 136: 151–159
- Brulfert J, Mricha A, Sossountzov L and Queiroz Q (1987) CAM induction by photoperiodism in green callus cultures from a CAM plant. *Plant Cell Environ* 10: 443–449
- Brulfert J, Kluge M, Güclü S and Queiroz O (1988) Interaction of photoperiod and drought as CAM inducing factors in *Kalanchoë blossfeldiana* Poelln., cv. Tom Thumb. *J Plant Physiol* 133: 222–227
- Brulfert J, Güclü S, Taybi T and Pierre JN (1993) Enzymatic responses to water stress in detached leaves of the CAM plant *Kalanchoë blossfeldiana* Poelln. *Plant Physiol Biochem* 31: 491–197
- Buchanan-Bollig IC and Smith JAC (1984a) Circadian rhythms in *Kalanchoë*: Effects of the irradiance and temperature on gas exchange and carbon metabolism. *Planta* 160: 264–271
- Buchanan-Bollig IC and Smith JAC (1984b) Circadian rhythms in Crassulacean acid metabolism: Phase relationships between gas exchange, leaf water relations and malate metabolism in *Kalanchoë daigremontiana*. *Planta* 160: 314–319
- Bush DS (1995) Calcium regulation in plant cells and its role in signaling. *Ann Rev Plant Physiol Plant Mol Biol* 46: 95–122
- Carter PJ, Nimmo HG, Fewson CA and Wilkins MB (1990) *Bryophyllum fedtschenkoi* protein phosphatase 2A can dephosphorylate phosphoenolpyruvate carboxylase. *FEBS Lett* 263: 233–236
- Carter PJ, Nimmo HG, Fewson CA and Wilkins MB (1991) Circadian rhythms in the activity of a plant protein kinase. *EMBO J* 10: 2063–2068
- Carter PJ, Wilkins MB, Nimmo HG and Fewson CA (1995a) The role of temperature in the regulation of the circadian rhythm of CO_2 fixation in *Bryophyllum fedtschenkoi*. *Planta* 196: 381–386
- Carter PJ, Wilkins MB, Nimmo HG and Fewson CA (1995b) Effects of temperature on the activity of phosphoenolpyruvate carboxylase and on the control of CO_2 fixation in *Bryophyllum fedtschenkoi*. *Planta* 196: 375–380
- Carter PJ, Fewson CA, Nimmo GA, Nimmo HG and Wilkins MB. (1996) Role of circadian rhythms, light and temperature in the regulation of phosphoenolpyruvate carboxylase in Crassulacean acid metabolism. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Vol 114, pp 46–52. Springer-Verlag, Berlin
- Caspar T, Huber SC and Somerville C (1985) Alterations in growth, photosynthesis and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. *Plant Physiol* 79: 11–7
- Cheng S-H and Edwards GE (1991) Influence of long photoperiods on plant development and expression of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *Plant Cell Environ* 14: 271–278
- Chollet R, Vidal J and O'Leary MH (1996) Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 273–298
- Chu C, Dai Z, Ku MSB and Edwards GE (1990) Induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* by abscisic acid. *Plant Physiol* 93: 1253–1260
- Cockburn W (1985) Variations in photosynthetic acid metabolism in vascular plants: CAM and related phenomena. *New Phytol* 101: 3–24
- Cockburn W, Whitelam GC, Broad A and Smith J (1996) The participation of phytochrome in the signal transduction pathway of salt stress responses in *Mesembryanthemum crystallinum* L. *J Exp Bot* 47: 647–653
- Comolli J, Taylor W, Rehman J and Hastings JW (1996) Inhibitors of serine/threonine phosphoprotein phosphatases alter circadian properties in *Gonyaulax polyedra*. *Plant Physiol* 111: 285–291
- Cook RM, Lindsay JG, Wilkins MB and Nimmo HG (1995) Decarboxylation of malate in the Crassulacean acid metabolism plant *Bryophyllum (Kalanchoë) fedtschenkoi*. *Plant Physiol* 109: 1301–1307
- Cooke R, Raynal M, Laudié M, Grellet R, Delsenay M, Morris P-C, Guerrier D, Giraudat J, Quigley F, Clabault G, Li Y-F, Mache R, Krivitzky M, Gy IJ-J, Kreis M, Lecharny A, Parmentier Y, Marbach J, Fleck J, Clément B, Philippot G, Hervé C, Bardet C, Tremousaygue D, Lescure B, Lacomme C, Roby D, Jourjon M-F, Chabrier P, Charpentier J-L, Desprez T, Amselem J, Chiapello H and Höfte H (1996) Further progress towards a catalogue of all *Arabidopsis* genes: Analysis of a set of 5000 non-redundant ESTs. *Plant J* 9: 101–124
- Cotelle V, Forestier C and Vavasseur A (1996) A reassessment of the intervention of calmodulin in the regulation of stomatal movement. *Physiol Plant* 98: 619–628
- Cushman JC (1992) Characterization and expression of a NADP-malic enzyme cDNA induced by salt stress from the facultative CAM plant, *Mesembryanthemum crystallinum*. *Eur J Biochem* 208: 259–266
- Cushman JC (1993) Molecular cloning and expression of chloroplast NADP-malate dehydrogenase during Crassulacean acid metabolism induction by salt stress. *Photosynth Res* 35: 15–27
- Cushman JC and Bohnert HJ (1992). Salt stress alters A/T-rich DNA-binding factor interactions within the phosphoenolpyruvate carboxylase promoter from *Mesembryanthemum crystallinum*. *Plant Mol Biol* 20: 411–424
- Cushman JC and Bohnert HJ (1996) Transcriptional activation of CAM genes during development and environmental stress. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Vol 114, pp 135–158. Springer-Verlag, Berlin
- Cushman JC and Bohnert HJ (1997) Molecular genetics of Crassulacean acid metabolism. *Plant Physiol* 113: 667–676
- Cushman JC, Meyer G, Michalowski CB, Schmitt JM and Bohnert HJ (1989) Salt stress leads to the differential expression of two isogenes of phosphoenolpyruvate carboxylase during

- Crassulacean acid metabolism induction in the common ice plant. *Plant Cell* 1: 715–725
- Cushman JC, Michalowski CB and Bohnert HJ (1990) Developmental control of Crassulacean acid metabolism inducibility by salt stress in the common ice plant. *Plant Physiol* 94: 1137–1142
- Cushman JC, Vernon DM and Bohnert HJ (1993) ABA and the transcriptional control of CAM induction during salt stress in the common ice plant. In: Verma DPS (ed) *Control of Plant Gene Expression*, pp 287–300. CRC Press, Boca Raton
- Dai Z, Ku MSB, Zhang Z and Edwards GE (1994) Effects of growth regulators on the induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* L. *Planta* 192: 287–294
- DeRocher EJ and Bohnert HJ (1993) Developmental and environmental stress employ different mechanisms in the expression of a plant gene family. *Plant Cell* 5: 1611–1625
- DeRocher EJ, Harkins KR, Galbraith DW and Bohnert HJ (1990) Developmentally regulated systemic endopolyploidy in succulents with small genomes. *Science* 250: 99–101
- De Santo AV and Bartoli G (1996) Crassulacean acid metabolism in leaves and stems of *Cissus quadrangularis*. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 216–229. Springer-Verlag, Berlin
- Dever LV, Blackwell RD, Fullwood NJ, Lacuesta M, Leegood RC, Onek LA, Pearson M and Lea PJ (1995) The isolation and characterization of mutants of the C₄photosynthetic pathway. *J Exp Bot* 46: 1363–1376
- Dietz KJ and Arbinger B (1996) cDNA sequence and expression of subunit E of the vacuolar H⁽⁺⁾-ATPase in the inducible Crassulacean acid metabolism plant *Mesembryanthemum crystallinum*. *Bioch Biophys Acta Gene Struct Funct* 1281: 134–138
- Earnshaw MJ, Carver KA and Charlton WA (1987) Leafy anatomy, water relations, and Crassulacean acid metabolism in the chlorenchyma and colourless internal water-storing tissue of *Carpobrotus edulis* and *Senecio mandraliscae*. *Planta* 170: 421–432
- Eastmond PJ and Ross JD (1997) Evidence that the induction of Crassulacean acid metabolism by water stress in *Mesembryanthemum crystallinum* (L.) involves root signalling. *Plant Cell Environ* 20: 1559–1565
- Edwards GE, Dai Z, Cheng SH and Ku MSB (1996) Factors affecting the induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 119–134. Springer-Verlag, Berlin
- Ehleringer JR and Monson RK (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. *Annu Rev Ecol Syst* 24: 411–439
- Feldman KA (1991) T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. *Plant J* 1:71–82
- Filzthaler B, Meyer G, Bohnert HJ and Schmitt JM (1995) Age-dependent induction of pyruvate, orthophosphate dikinase in *Mesembryanthemum crystallinum* L. *Planta* 196: 492–500
- Forsthoefel NR, Cushman MA and Cushman JC (1995a) Posttranscriptional and posttranslational control of enolase expression in the facultative Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Plant Physiol* 108: 1185–1195
- Forsthoefel NR, Vernon DM and Cushman JC (1995b) A salinity-induced gene from the halophyte *M. crystallinum* encodes a glycolytic enzymes, cofactor-independent phosphoglyceromutase. *Plant Mol Biol* 29: 213–226
- Forsthoefel NR, Cushman MAF and Cushman JC (1998) Characterization and expression of cDNA encoding a novel cysteine protease from the halophytic, facultative CAM plant, *Mesembryanthemum crystallinum* L. *Plant Science* 136: 195–206
- Foster JC, Edwards GE and Winter K (1982) Changes in the levels of phosphoenolpyruvate carboxylase with induction of CAM in *M. crystallinum* L. *Plant Cell Physiol* 23: 585–594
- Furbank RT and Taylor WC (1995) Regulation of photosynthesis in C₃ and C₄ plants: A molecular approach. *Plant Cell* 7: 797–807
- Garfinkel DJ and Nester EW (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. *J Bacteriol* 144: 732–743
- Gehrig H, Taybi T, Kluge M and Brulfert J (1995) Identification of multiple PEPC isogenes in leaves of the facultative Crassulacean acid metabolism (CAM) plant *Kalanchoë blossfeldiana* Poelln. cv. Tom Thumb. *FEBS Lett* 377: 399–402
- Giglioli-Guivarc'h N, Pierre JC, Brown S, Chollet R, Vidal J and Gadal P (1996) The light-dependent transduction pathway controlling the regulatory phosphorylation of C₄ phosphoenolpyruvate carboxylase in protoplasts from *Digitaria sanguinalis*. *Plant Cell* 8: 573–586
- Giraudat J (1995) Abscisic acid signaling. *Curr Opin Cell Biol* 7: 232–238
- Grams TEE, Beck F and Lütte U (1996) Generation of rhythmic and arrhythmic behaviour of Crassulacean acid metabolism in *Kalanchoë daigremontiana* under continuous light by varying the irradiance or temperature: Measurements *in vivo* and model simulations. *Planta* 198: 110–117
- Grams TEE, Borland AM, Roberts A, Griffiths H, Beck F and Lütte U (1997) On the mechanism of reinitiation of endogenous Crassulacean acid metabolism rhythm by temperature changes. *Plant Physiol* 113: 1309–1317
- Grasser KD (1995) Plant chromosomal high mobility group (HMG) proteins. *Plant J* 7: 185–192
- Gregory FG, Spear I and Thimann KV (1954) The interrelation between CO₂ metabolism and photoperiodism in *Kalanchoë*. *Plant Physiol* 29: 220–259
- Griffiths H (1988) Crassulacean acid metabolism: A re-appraisal of physiological plasticity in form and function. *Adv Bot Res* 15: 42–92
- Griffiths H (1989) Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. In: Lütte U (ed) *Vascular Plants as Epiphytes: Evolution and Ecophysiology*, pp 42–86. Springer-Verlag, Berlin
- Griffiths H (1992) Carbon isotope discrimination and the integration of carbon assimilation pathways in terrestrial CAM plants. *Plant Cell Environ* 15: 1051–1062
- Harding SA and Smigocki AC (1994) Cytokinins modulate stress response genes in isopentenyl transferase-transformed *Nicotiana plumbaginifolia* plants. *Physiol Plant* 90: 327–333
- Harris PJC and Wilkins MB (1978a) Evidence for phytochrome

- involvement in the entrainment of the circadian rhythm of CO_2 metabolism in *Bryophyllum*. *Planta* 138: 271–272
- Harris PJC and Wilkins MB (1978b) The circadian rhythm in *Bryophyllum* leaves: Phase control by radiant energy. *Planta* 143:323–328
- Hartwell J, Smith LH, Wilkins MB, Jenkins GI and Nimmo HG (1996) Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. *Plant J* 10: 1071–1078
- Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR and Vasil IK (1992) The viviparous-1 gene and abscisic acid activate the C1 regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes Devel* 6: 609–618.
- Herppich W, Herppich M and von Willert DJ (1992) The irreversible C_3 to CAM shift in well-watered and salt-stressed plants of *Mesembryanthemum crystallinum* is under strict ontogenetic control. *Bot Acta* 105: 34–40
- Herppich W, Herppich M and von Willert DJ (1998) Ecophysiological investigations on plants of the genus *Plectranthus* (Lamiaceae). Influence of environment and leaf age on CAM, gas exchange and leaf water relations in *Plectranthus marrubiodes* Benth. *Flora* 193: 99–109
- Heun AM, Gorham J, Lütte U and Wyn Jones RG (1981) Changes of water-relation characteristics and levels of organic solutes during salinity induced transition of *Mesembryanthemum crystallinum* from C_3 -photosynthesis to Crassulacean acid metabolism. *Oecologia* 50: 66–72
- Höfner R, Vasquez-Moreno L, Winter K, Bohnert HJ and Schmitt JM (1987) Induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum* by high salinity: Mass increase and de novo synthesis of PEP carboxylase. *Plant Physiol* 83:915–919
- Holappa LD and Walker-Simmons MK (1995) A wheat ABA-responsive protein kinase mRNA, PKABA1, is upregulated by dehydration, cold temperature, and osmotic stress. *Plant Physiol* 108:1203–1210
- Holthe PA, Sternberg LSL and Ting IP (1987) Developmental control of CAM in *Peperomia scandens*. *Plant Physiol* 84: 743–747
- Holthe PA, Patel A and Ting IP (1992) The occurrence of CAM in *Peperomia*. *Selbyana* 13: 77–87
- Holtum JAM and Winter K (1982) Activities of enzymes of carbon metabolism during the induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *Planta* 155: 8–16
- Hong SW, Jon JH, Kwak JM and Nam HG (1997) Identification of a receptor-like kinase gene rapidly induced by abscisic acid, dehydration, high salt, and cold treatments in *Arabidopsis thaliana*. *Plant Physiol* 113: 1203–1212
- Huber SC, Bachmann M and Huber JL (1996) Post-translational regulation of nitrate reductase activity: A role for Ca^{2+} and 14-3-3 proteins. *Trends Plant Sci* 1: 432–438
- Ishitani M, Majumder AL, Bornhäuser A, Michalowski CB, Jensen RG and Bohnert HJ (1996) Coordinate transcriptional induction of myo-inositol metabolism during environmental stress. *Plant J* 9: 537–548
- Jang J-C and Sheen J (1997) Sugar sensor in higher plants. *Trends Plant Sci* 2: 208–214
- Jang J-C, Leon P, Zhou L and Sheen J (1997) Hexokinase as a sugar sensor in higher plants. *Plant Cell* 9: 5–19
- Jia S-R, Yang M-Z, Ott R and Chua N-H (1989) High frequency transformation of *kalanchoe laciniata*. *Plant Cell Rep* 8: 336–340
- Jiang R and Carlson M (1996) Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. *Genes Dev* 10:3105–3115
- Jiao J-A and Chollet R (1991) Posttranslational regulation of phosphoenolpyruvate carboxylase in C_4 and Crassulacean acid metabolism plants. *Plant Physiol* 95: 981–985
- Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A and Trewavas A (1995) Circadian oscillations of cytosolic and chloroplastic free calcium in transgenic luminous plants. *Science* 269: 1863–1865
- Jonak C, Kiegerl S, Ligerink W, Barker PJ, Huskisson NS and Hirt H (1996) Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc Natl Acad Sci USA* 93: 11274–11279
- Kakimoto T (1996) CKII, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274: 982–985
- Keeley JE (1996) Aquatic CAM photosynthesis. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 281–295. Springer-Verlag, Berlin
- Keith CS, Hoang DO, Barret BM, Feigelman B, Nelson MC, Thai H and Baysdorfer C (1993) Partial sequence analysis of 130 randomly selected maize cDNA clones. *Plant Physiol* 101: 329–332
- Kloeckener-Gruisse B and Freeling M (1995) Transposon-induced promoter scrambling: A mechanism for the evolution of new alleles. *Proc Natl Acad Sci USA* 92: 1836–1840
- Kluge M and Brulfert J (1996) Crassulacean acid metabolism in the genus *Kalanchoe*: Ecological, physiological and biochemical aspects. In K. Winter and J.A.C. Smith, eds, Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Springer-Verlag, Berlin, pp 324–335
- Kluge M and Ting IP (1978) Crassulacean Acid Metabolism. Analysis of an Ecological Adaptation. Springer-Verlag, Berlin
- Kluge M, Hell R, Pfeffer A and Kramer D (1987) Structural and metabolic properties of green tissue cultures from a CAM plant, *Kalanchoe blossfeldiana* hybr. Montezuma. *Plant Cell Environ* 10: 451–462
- Knight H, Trewavas AJ and Knight MR (1996) Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* 8: 489–503
- Koch KE (1996) Carbohydrate-modulated gene expression in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 509–540
- Koreeda S, Yamashita T and Kanai R (1996) Induction of light dependent pyruvate transport into chloroplasts of *Mesembryanthemum crystallinum* by salt stress. *Plant Cell Physiol* 37: 257–262
- Kraybill AA and Martin CE (1996) Crassulacean acid metabolism in three species of the C_4 genus *Portulaca*. *Int J Plant Sci* 157: 103–109
- Ku MSB, Kano-Murakami Y and Matsuoka M (1996) Evolution and expression of C_4 photosynthesis genes. *Plant Physiol* 111: 949–957
- Kusumi K, Arata H, Iwasaki I and Nishimura M (1994) Regulation of PEP-carboxylase by biological clock in a CAM plant. *Plant Cell Physiol* 35: 233–242

- Langdale JA and Kidner CA (1994) *bundle sheath defective*, a mutation that disrupts cellular differentiation in maize leaves. *Development* 120: 673–681
- Langdale JA, Hall LN and Roth R (1995) Control of cellular differentiation in maize leaves. *Philos Trans R Soc Lond (Biol.)* 350: 53–57
- Lea PJ and Forde BG (1994) The use of mutants and transgenic plants to study amino acid metabolism. *Plant Cell Env* 17: 541–556
- Lee DM and Assmann SM (1992) Stomatal responses to light in the facultative Crassulacean acid metabolism species, *Portulacaria afra*. *Physiol Plant* 85: 35–42
- Leegood RC and Osmond CB (1990) The flux of metabolites in C_4 and CAM plants. In: Dennis DT and Turpin DH (eds) *Plant Physiology, Biochemistry and Molecular Biology*, pp 274–298. Longman, Harlow
- Li B and Chollet R (1994) Salt induction and the partial purification/characterization of phosphoenolpyruvate carboxylaseprotein-serine kinase from an inducible Crassulacean-acid-metabolism (CAM) plant, *Mesembryanthemum crystallinum* L. *Arch Bioch Biophys* 314: 247–254
- Li J, Lee Y-RJ and Assmann SM (1998) Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiol* 116: 785–795
- Lillo C, Smith LH, Nimmo HG and Wilkins MB (1996) Rhythms in magnesium ion inhibition and hysteretic properties of nitrate reductase in the CAM plant *Bryophyllum fedtschenkoi*. *Physiol Plant* 98: 140–146
- Löw R, Rockel B, Kirsch M, Ratajczak R, Hörnstensteiner, Martinio E, Lüttge U and Rausch T (1996) Early salt stress effects on the differential expression of vacuolar H^+ -ATPase genes in roots and leaves of *Mesembryanthemum crystallinum*. *Plant Physiol* 110: 259–265
- Lüttge U (1987) Carbon dioxide and water demand; Crassulacean acid metabolism (CAM), a versatile ecological adaptation exemplifying the need for integration in ecophysiological work. *New Phytol* 106: 593–629
- Lüttge U (1993) The role of Crassulacean acid metabolism (CAM) in the adaptation of plants to salinity. *New Phytol* 125: 59–71
- Lüttge U (1996) *Clusia*: Plasticity and diversity in a genus of C_3 /CAM intermediate tropical trees. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 296–311. Springer-Verlag, Berlin
- Lüttge U and Beck F (1992) Endogenous rhythms and chaos in Crassulacean acid metabolism. *Planta* 188: 28–38
- Lüttge U, Fischer-Schliebs E, Ratajczak R, Kramer D, Berndt E and Kluge M (1995) Functioning of the tonoplast in vacuolar C-storage and remobilization in Crassulacean acid metabolism. *J Exp Bot* 46: 1377–1388
- Lüttge U, Grams TEE, Hechler B, Blasius B and Beck F (1996) Frequency resonances of the circadian rhythm of CAM under external temperature rhythms of varied period lengths in continuous light. *Bot Acta* 109: 422–426
- Mawson BT and Zaugg MW (1994) Modulation of light-dependent stomatal opening in isolated epidermis following induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *J Plant Physiol* 144: 740–746
- Mazen AMA (1996) Changes in levels of phosphoenolpyruvate carboxylase with induction of Crassulacean acid metabolism (CAM)-like behaviour in the C_4 plant *Portulaca oleracea*. *Physiol Plant* 98: 111–116
- McElwain EF, Bohnert HJ and Thomas JC (1992) Light moderates the induction of phosphoenolpyruvate carboxylase by NaCl and abscisic acid in *Mesembryanthemum crystallinum*. *Plant Physiol* 99: 1261–1264
- Meiners MS, Thomas JC, Bohnert HJ and Cushman JC (1991) Regeneration of multiple shoots and plants from *Mesembryanthemum crystallinum*. *Plant Cell Rep* 9: 563–566
- Meyer G, Schmitt JM and Bohnert HJ (1990) Direct screening of a small genome: Estimation of the magnitude of plant gene expression changes during adaptation to high salt. *Mol Gen Genet* 224: 347–356
- Meyerowitz EM and Somerville CR (eds) (1994) *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Michalowski CB, Olson SW, Piepenbrock M, Schmitt JM and Bohnert HJ (1989) Time course of mRNA induction elicited by salt stress in the common ice plant (*M. crystallinum*). *Plant Physiol* 89: 811–816
- Millar AJ, McGrath RB and Chua N-H (1994) Phytochrome phototransduction pathways. *Annu Rev Genet* 28: 325–349
- Mizoguchi T, Ichimura K and Shinozaki K (1997) Environmental stress response in plants: The role of mitogen-activated protein kinases. *Trends Biotechnol* 15: 15–19
- Monson RK (1989) On the evolutionary pathways resulting in C_4 photosynthesis and Crassulacean acid metabolism (CAM). *Adv Ecol Res* 19: 57–92
- Mricha A, Brulfert J, Pierre JN and Queiroz O (1990) Phytochrome-mediated responses of cells and protoplasts of green calli obtained from leaves of a CAM plant. *Plant Cell Rep* 8: 664–666
- Nelson D, Salamini F and Bartels D (1994) Abscisic acid promotes novel DNA-binding activity to a desiccation-related promoter of *Craterostigma plantagineum*. *Plant J* 5: 451–458
- Neuffer MG, Coe EH and Wessler ST (eds) (1996) *Mutants of Maize*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Neuhaus E and Schulte N (1996) Starch degradation in chloroplasts isolated from C_3 or CAM (Crassulacean acid metabolism)-induced *Mesembryanthemum crystallinum* L. *Biochem J* 318: 945–953
- Neuhaus G, Bowler C, Kern R and Chua NH (1993) Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell* 73: 937–952
- Neuhaus G, Bowler C, Hiratsuka K, Yamagata H and Chua NH (1997) Photochrome-regulated repression of gene expression requires calcium and cGMP. *EMBO J* 16: 2554–2564
- Nimmo GA, Nimmo HG, Fewson CA and Wilkins MB (1984) Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves: A possible covalent modification. *FEBS Lett* 178: 199–203
- Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA and Wilkins MB (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi*. *Biochem J* 239: 213–220
- Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA and Wilkins MB (1987) Persistent circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase from *Bryophyllum*

- fedtschenkoi* leaves and in its sensitivity to inhibition by malate. *Planta* 170: 408–15
- Nimmo HG (1993) The regulation of phosphoenolpyruvate carboxylase by, reversible phosphorylation. In: Battey NH, Dickinson HG, and Hetherington AM (eds) Post-Translational Modifications in Plants, Society for Experimental Biology Seminar Series No 53, pp 161–170. Cambridge University Press, Cambridge
- Nishio JN and Ting IP (1987) Carbon flow and metabolic specialization in the tissue layers of the Crassulacean acid metabolism plant, *Peperomia camptotricha*. *Plant Physiol* 84: 600–604
- Nishio JN and Ting IP (1993) Photosynthetic characteristics of the palisade mesophyll and spongy mesophyll in the CAM/C₄ intermediate plant, *Peperomia camptotricha*. *Bot Acta* 106: 120–125
- Noble PS (1988) Environmental Biology of Agaves and Cacti. Cambridge University Press, New York
- Osmond CB (1978) Crassulacean acid metabolism: A curiosity in context. *Annu Rev Plant Physiol* 29: 379–414
- Osmond CB and Holtum JAM (1981) Crassulacean acid metabolism. In: Hatch MD and Boardman NK. (eds) The Biochemistry of Plants, Vol 8, Photosynthesis, pp 283–328. Academic Press, New York
- Ostrem JA, Olsen SW, Schmitt JM and Bohnert HJ (1987) Salt stress increases the level of translatable mRNA for PEPC in *Mesembryanthemum crystallinum*. *Plant Physiol* 84: 1270–1275
- Ostrem JA, Vernon DM and Bohnert HJ (1990) Increased expression of a gene coding for NAD-glyceraldehyde-3-phosphate dehydrogenase during the transition from C₃ photosynthesis to Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *J Biol Chem* 265: 3497–3502
- Park YS, Kwak JM, Kim YS, Lee DS, Cho MJ, Lee HH and Nam HG (1993) Generation of expressed sequence tags of random root cDNA clones of *Brassica napus* by single-run partial sequencing. *Plant Physiol* 103: 359–370
- Patel A and Ting IP (1987) Relationship between CAM and respiration in *Peperomia camptotricha*. *Plant Physiol* 84: 640–642
- Paul MJ, Loos K, Stitt M and Ziegler P (1993) Starch-degrading enzymes during the induction of CAM in *Mesembryanthemum crystallinum*. *Plant Cell Environ* 16: 531–538
- Peters W, Beck E, Piepenbrock M, Lenz B and Schmitt JM (1997) Cytokinin as a negative effector of phosphoenolpyruvate carboxylate induction in *Mesembryanthemum crystallinum*. *J Plant Physiol* 151: 362–367
- Piepenbrock M and Schmitt JM (1991) Environmental control of phosphoenolpyruvate carboxylase induction in mature *Mesembryanthemum crystallinum* L. *Plant Physiol* 97: 998–1003
- Piepenbrock M and Schmitt JM (1992) Environmental control of phosphoenolpyruvate carboxylase induction in mature *Mesembryanthemum crystallinum* L. *Plant Physiol* 97: 998–1003
- Piepenbrock M, von Albert C and Schmitt JM (1994) Decreasing leaf water content induces Crassulacean acid metabolism in well-irrigated *Mesembryanthemum crystallinum*. *Photosynthetica* 30: 623–628
- Poovaiah BW and Reddy ASN (1993) Calcium and signal transduction in plants. *CRC Crit Rev Plant Sci* 12: 185–211
- Quail PH, Boylan MT, Parks BM, Short TW, Xu Y and Wagner D (1995) Phytochromes: Photosensory perception and signal transduction. *Science* 268: 675–680
- Queiroz O (1968) Sur le métabolisme acide des Crassulacées. II. Variations d'activité enzymatique sous l'action du photopériodisme et du thermopériodisme. *Physiol Vég* 6: 117–136
- Queiroz O and Brulfert J (1982) Photoperiod-controlled induction and enhancement of seasonal adaptation to drought. In: Ting IP and Gibbs M (eds) Crassulacean Acid Metabolism, pp 208–230. Waverly Press, Baltimore
- Ratajczak R, Richter J and Lütge U (1994) Adaptation of the tonoplast V-type H⁺-ATPase of *Mesembryanthemum crystallinum* to salt stress, C₃-CAM transition and plant age. *Plant Cell Environ* 17: 1101–1112
- Raven JA and Spicer RA (1996) The evolution of Crassulacean acid metabolism. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Vol 114, pp 360–385. Springer-Verlag, Berlin
- Reinbothe S, Reinbothe C and Parthier B (1993a) Methyl jasmonate represses translational initiation of a specific sort of mRNAs in barley. *Plant J* 4: 459–467
- Reinbothe S, Reinbothe C and Parthier B (1993b) A methyl jasmonate-induced shift in the length of the 5' untranslated region impairs translation of the plastid *rbcL* transcript in barley. *EMBO J* 12: 1505–1512
- Reinbothe S, Reinbothe C and Parthier B (1994) JIPs and RIPS: The regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *Plant J* 6: 1197–1209
- Rippmann JF, Michalowski CB, Nelson DE and Bohnert HJ (1997) Induction of a ribosome inactivating protein following environmental stress. *Plant Mol Biol* 35: 701–709
- Robe WE and Griffiths H (1990) Photosynthesis of *Littorella uniflora* grown under two PAR regimes: C₃ and CAM gas exchange and the regulation of internal CO₂ and O₂ concentrations. *Oecologia* 85: 128–136
- Rogers JC, Lanahan MC and Rogers SW (1994) The *cis*-acting gibberellin response complex in high-*pi* alpha-amylase gene promoters. *Plant Physiol* 105: 151–158
- Roth R, Hall LN, Brutnell TB and Langdale JA (1996) *bundle sheath defective 2*, a mutation that disrupts the co-ordinate development of bundle sheath and mesophyll cells in maize. *Plant Cell* 8: 915–927
- Rygl J, Büchner K-H, Winter K and Zimmermann U (1986) Day/Night variations in turgor pressure in individual cells of *Mesembryanthemum crystallinum* L. *Oecologia* 69: 171–175
- Saitou K, Agata W, Masui Y, Asakura M, and Kubota F (1994) Isoforms of NADP-malic enzyme from *Mesembryanthemum crystallinum* L. that are involved in C₃ photosynthesis and Crassulacean acid metabolism. *Plant Cell Physiol* 35: 1165–1171
- Saitou K, Agata W, Kawarabata T, Yamamoto Y, and Kubota F (1995) Purification and properties of NAD-malate dehydrogenase from *Mesembryanthemum crystallinum* L. exhibiting Crassulacean acid metabolism. *Jpn J Crop Sci* 64: 760–766
- Sano H and Youssefian S (1994) Light and nutritional regulation of transcripts encoding a wheat protein kinase homolog is mediated by cytokinins. *Proc Natl Acad Sci USA* 91: 2582–2586

- Sasaki T, Song J, Koga-Ban Y, Matsui E, Fang F, Higo H, Nagasaki H, Hori M, Miya M, Murayama-Kayano E, Takiguchi T, Takasuga A, Niki T, Ishimaru K, Ikeda H, Yamamoto Y, Mukai Y, Ohta I, Mihadera N, Havukkala I and Minobe Y (1994) Toward cataloguing all rice genes: Large-scale sequencing of randomly chosen rice cDNAs from a callus cDNA library. *Plant J* 6: 615–624
- Schaeffer HJ, Forsthoefel NR and Cushman JC (1995) Identification of enhancer and silencer regions involved in salt-responsive expression of Crassulacean acid metabolism (CAM) genes in the facultative halophyte *Mesembryanthemum crystallinum*. *Plant Mol Biol* 28: 205–218
- Schmitt JM (1990) Rapid concentration changes of phosphoenolpyruvate carboxylase mRNA in detached leaves of *Mesembryanthemum crystallinum*. *Plant Cell Environ* 13:845–850
- Schmitt JM and Piepenbrock M (1992a) Induction of mRNA for phosphoenolpyruvate carboxylase is correlated with a decrease in shoot water content in well-irrigated *Mesembryanthemum crystallinum*. *Plant Physiol* 99: 759–761
- Schmitt JM and Piepenbrock M (1992b) Regulation of phosphoenolpyruvate carboxylase Crassulacean acid metabolism induction in *Mesembryanthemum crystallinum* L. by cytokinin: Modulation of leaf gene expression by roots? *Plant Physiol* 99: 1664–1669
- Schmitt JM, Fißlthaler B, Sheriff A, Lenz B, Bäßler M and Meyer G (1996) Environmental control of CAM induction in *Mesembryanthemum crystallinum* - a role for cytokinin, abscisic acid and jasmonate? In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Vol 114, pp 159–175. Springer-Verlag, Berlin
- Sembdner G and Parthier B (1993) Biochemistry, physiological and molecular actions of jasmonates. *Annu Rev Plant Physiol Plant Mol Biol* 44: 569–589
- Sheen J (1990) Metabolic repression of transcription in higher plants. *Plant Cell* 2: 1027–1038
- Sheen J (1994) Feedback control of gene expression. *Photosynth Res* 39: 427–438
- Sheen J (1996) Ca^{2+} -dependent protein kinases and stress signal transduction in plants. *Science* 274: 1900–1902
- Shen Q and Ho T-HD (1995) Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-activating element. *Plant Cell* 7: 295–307
- Shinozaki K and Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. *Current Opin Biotech* 7: 161–167
- Sipes D and Ting IP (1985) Crassulacean acid metabolism and Crassulacean acid metabolism modifications in *Peperomia camptotricha*. *Plant Physiol* 77: 59–63
- Smirnoff N (1996) Regulation of Crassulacean acid metabolism by water stress in the C_3/CAM intermediate *Sedum telephium*. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Vol 114, pp 176–191. Springer-Verlag, Berlin
- Smith DL and Fedoroff NV (1995) A gene expressed in lateral and adventitious root primordia of *Arabidopsis*. *Plant Cell* 7: 735–745
- Smith JAC and Bryce JH (1992) Metabolite compartmentation and transport in CAM plants. In: Tobin AK (ed) *Plant Organelles*, pp 141–167. Cambridge University Press, Cambridge
- Smith JAC and Winter K (1996) Taxonomic distribution of Crassulacean acid metabolism. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 427–436. Springer-Verlag, Berlin
- Smith RD and Walker JC (1996) Plant protein phosphatases. *Annu Rev Plant Physiol Plant Mol Biol* 47: 101–125
- Sommerville CR (1984) The analysis of photosynthetic carbon dioxide fixation and photorespiration by mutant selection. *Oxford Surveys Plant Mol Cell Biol* 1: 103–131
- Sommerville CR (1986) Analysis of photosynthesis with mutants of higher plants and algae. *Ann Rev Plant Physiol* 37:467–507
- Springer SA and Outlaw WH (1988) Histochemical compartmentation of photosynthesis in the Crassulacean acid metabolism plant *Crassula falcata*. *Plant Physiol* 88: 633–638
- Stenlid G (1982) Cytokinins as inhibitors of root growth. *Physiol Plant* 56: 500–506
- Stipe F, Barvieri L, Gorini P, Valbonesi P, Bolognesi A and Polito L (1996) Activities associated with the presence of ribosome-inactivating proteins increase in senescent and stress leaves. *FEBS Lett* 382: 309–312
- Stone JM and Walker JC (1995) Plant protein kinase families and signal transduction. *Plant Physiol* 108: 451–457
- Su WP and Howell SH (1992) A single genetic locus, *ckrl*, defines *Arabidopsis* mutants in which root growth is resistant to low concentration of cytokinin. *Plant Physiol* 99: 1569–1574
- Tallman G, Zhu J, Mawson BT, Amodeo G, Nouhi Z, Levy K and Zeiger E (1997) Induction of CAM in *Mesembryanthemum crystallinum* abolishes the stomatal response to blue light and light-dependent zeaxanthin formation in guard cell chloroplasts. *Plant Cell Physiol* 38: 236–242
- Taybi T (1995) Induction du métabolisme acide crassulacéen et synthèses enzymatiques dans les feuilles détachées du *kalanchoë blossfeldiana*: Contrôle par l'acide abscissique. Ph.D. Thesis. Pierre and Marie Curie, Université Paris 6
- Taybi T and Cushman JC (1998) Signal transduction events leading to Crassulacean acid metabolism (CAM) induction in the common ice plant, *Mesembryanthemum crystallinum*. *Plant Physiol* 117S: 47
- Taybi T, Sotta B, Gehrig H, Güclü S, Kluge M and Brulfert J (1995) Differential effects of abscisic acid on phosphoenolpyruvate carboxylase and CAM operation in *kalanchoë blossfeldiana*. *Bot Acta* 198: 2340–246
- Terzaghi WB and Cashmore AR (1995) Light-regulated transcription. *Annu Rev Plant Physiol Plant Mol Biol* 46:445–474
- Thomas JC and Bohnert HJ (1993) Salt stress perception and plant growth regulators in the halophyte, *Mesembryanthemum crystallinum*. *Plant Physiol* 103: 1299–1304
- Thomas JC, De Armond R and Bohnert HJ (1992a) Influence of NaCl on growth, proline, and phosphoenolpyruvate carboxylase levels in *Mesembryanthemum crystallinum* suspension cultures. *Plant Physiol* 98: 626–631
- Thomas JC, McElwain EF and Bohnert HJ (1992b) Convergent induction of osmotic stress-responses: Abscisic acid, cytokinin, and the effect of NaCl. *Plant Physiol* 100: 416–423
- Thomas JC, Smigocki AC and Bohnert HJ (1995) Light-induced

- expression of *ipt* from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco. *Plant Mol Biol* 27: 225–235
- Ting IP (1981) Effects of ABA on CAM in *Portulacaria afra*. *Photosynth Res* 2: 39–48
- Ting IP (1985) Crassulacean acid metabolism. *Annu Rev Plant Physiol* 36: 595–622
- Ting IP (1987) Stomata in plants with Crassulacean acid metabolism. In: Zeigler E, Farquhar GD and Cowan IR (eds) *Stomatal Function*, pp 353–366. Stanford University Press, Stanford
- Ting IP, Hann J, Sipes DL, Patel A and Walling LL (1993) Expression of P-enolpyruvate carboxylase and other aspects of CAM during the development of *Peperomia camptotricha* leaves. *Bot Acta* 106: 313–319
- Ting IP, Patel A, Sipes DL, Reid PD and Walling LL (1994) Differential expression of photosynthesis genes in leaf tissue layers of *Peperomia* as revealed by tissue printing. *Amer J Bot* 81: 414–422
- Ting IP, Patel A, Kaur S, Hann J and Walling LL (1996) Ontogenetic development of Crassulacean acid metabolism as modified by water stress in *Peperomia*. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 204–215. Springer-Verlag, Berlin
- Treichel S, Hettfleisch H, Eilhardt S, Faist K and Kluge M (1988) A possible induction of CAM by NaCl-stress in heterotrophic cell suspension cultures of *Mesembryanthemum crystallinum*. *J Plant Physiol* 133: 419–424
- Tsiantis MS (1996) Regulation of V-ATPase gene expression by ionic stress in higher plants. D. Phil thesis, University of Oxford, Oxford
- Tsiantis MS, Bartholomew DM and Smith JAC (1996) Salt regulation of transcript levels for the c subunit of a leaf vacuolar H^+ -ATPase in the halophyte *Mesembryanthemum crystallinum*. *Plant J* 9: 729–736
- Uchimiya H, Kidou S-I, Shimazaki T, Aotsuka S, Takamatsu S, Nishi R, Hashimoto H, Matsubayashi Y, Kidou N, Umeda M and Kata A (1992) Random sequencing of cDNA libraries reveals a variety of expressed genes in cultured cells of rice (*Oryza sativa* L.). *Plant J* 2: 1005–1009
- Urao T, Yamaguchi-Shinozaki K, Urao S and Shinozaki K (1993) An *Arabidopsis myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* 5: 1529–1539
- Vidal J and Chollet R (1997) Regulatory phosphorylation of C_4 PEP carboxylase. *Trends Plant Sci* 2: 230–237
- von Willert DJ, Kirst GO, Treichel S and von Willert K (1976a) The effect of leaf age and salt stress on malate accumulation and phosphoenolpyruvate carboxylase activity in *Mesembryanthemum crystallinum*. *Plant Sci Lett* 7: 341–346
- von Willert DJ, Treichel S, Kirst GO and Curdts E (1976b) Environmentally controlled changes of phosphoenolpyruvate carboxylases in *Mesembryanthemum*. *Phytochemistry* 15: 1435–1436
- Vernon DM and Bohnert HJ (1992) A novel methyl transferase induced by osmotic stress in the facultative halophyte *M. crystallinum*. *EMBO J* 11: 2077–2085
- Vernon DM, Ostrom JA and Bohnert HJ (1993) Stress perception and response in a facultative halophyte: The regulation of salinity-induced genes in *Mesembryanthemum crystallinum*. *Plant Cell Environ* 16: 437–444
- Walker RP and Leegood, RC (1996) Phosphorylation of phosphoenolpyruvate carboxykinase in plants. Studies in plants with C_4 photosynthesis and Crassulacean metabolism and in germinating seeds. *Biochem J* 317: 653–658
- Wang B and Lütte U (1994) Induction and subculture of callus and regeneration of fertile plants of *Mesembryanthemum crystallinum* L. *Polish J Environ Studies* 3: 55–57
- Webb AAR, McAinch MR, Mansfield TA and Hetherington AM (1996) Carbon dioxide induces increases in guard cell cytosolic free calcium. *Plant J* 9: 297–304
- Weigend M (1994) In vivo phosphorylation of phosphoenolpyruvate carboxylase from the facultative CAM plant *Mesembryanthemum crystallinum*. *J Plant Physiol.* 144: 654–660
- Whitehouse DG, Rogers JW and Tobin AK (1991) Photorespiratory enzyme activities in C_3 and CAM forms of the facultative CAM plant, *Mesembryanthemum crystallinum* L. *J Exp Bot* 42: 485–492
- Wilkins MB (1984) A rapid circadian rhythm of carbon dioxide metabolism in *Bryophyllum fedtschenkoi*. *Planta* 161: 381–384
- Wilkins MB (1992) Circadian rhythms: Their origin and control. *New Phytol* 121: 347–375
- Willenbrink ME and Huesemann W (1995) Photoautotrophic cell suspension cultures from *Mesembryanthemum crystallinum* and their response to salt stress. *Bot Acta* 108: 497–504
- Winter K (1973a) Zum Problem der Ausbildung des Crassulaceensäurestoffwechsels bei *Mesembryanthemum crystallinum* unter NaCl-Einfluss. *Planta* 109: 135–145
- Winter K (1973b) CO_2 -fixierungsreaktionen bei der Salzpflanze *Mesembryanthemum crystallinum* unter varierten Außenbedingungen. *Planta* 114: 75–85
- Winter K (1985) Crassulacean acid metabolism. In: Barber J and Baker NR (eds) *Photosynthetic Mechanisms and the Environment*, pp 329–387. Elsevier, Amsterdam
- Winter K (1987) Gradient in the degree of Crassulacean acid metabolism within leaves of *Kalanchoë daigremontiana*. *Planta* 172: 88–90
- Winter K and Gademann R (1991) Daily changes in CO_2 and water vapor exchange, chlorophyll fluorescence, and leafwater relations in the halophyte *Mesembryanthemum crystallinum* during the induction of Crassulacean acid metabolism in response to high NaCl salinity. *Plant Physiol* 95: 768–776
- Winter K and Smith JAC (1996) An introduction to Crassulacean acid metabolism. Biochemical principles and ecological diversity. In: Winter K and Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*, Vol 114, pp 1–13. Springer-Verlag, Berlin
- Winter K and von Willert DJ (1972) NaCl-induzierter Crassulaceensäurestoffwechsel bei *Mesembryanthemum crystallinum*. *Z Pflanzenphysiol* 67: 166–170
- Winter K, Osmond B and Pate JS (1981) Coping with salinity. In: Pate JS and McComb AF (eds) *The Biology of Australian Plants*, pp 88–113. University of Western Australia Press, Nedlands
- Winter K, Foster JG, Edwards GE and Holtum JAM (1982) Intracellular localization of enzymes of carbon metabolism in *Mesembryanthemum crystallinum* exhibiting C_3 photosynthetic

- characteristics or performing Crassulacean acid metabolism. *Plant Physiol* 69: 300–307
- Winter K, Wallace BJ, Stacker GC and Rodsandic Z (1983) Crassulacean acid metabolism in Australian vascular epiphytes and some related species. *Oecologia* 57: 129–141
- Xu R, Goldman S, Coupe S and Deikman J (1996) Ethylene control of E4 transcription during tomato fruit ripening involves two cooperative *cis* elements. *Plant Mol Biol* 31: 1117–1127
- Yen HE, Edwards GE and Grimes HD (1994) Characterization of a salt-responsive 24-kilodalton glycoprotein in *Mesembryanthemum crystallinum*. *Plant Physiol* 105: 1179–1187
- Yen HE, Grimes HD and Edwards GE (1995) The effects of high salinity, water deficit, and abscisic acid on phosphoenolpyruvate carboxylase activity and proline accumulation in *Mesembryanthemum crystallinum* cell cultures. *J Plant Physiol* 145: 557–564
- Zotz G and Winter K (1996) Seasonal changes in daytime versus nighttime CO_2 fixation of *Clusia uvitana* in situ. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, Vol 114, pp 312–323. Springer-Verlag, Berlin

Chapter 24

Ecophysiology of Plants with Crassulacean Acid Metabolism

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Summary

The taxonomic and ecological diversity of CAM plants is testament to a suite of morphological and metabolic attributes. These have evolved under contrasting selective pressures in up to 7% of all plant species many times over the past 10–100 Ma. The water and carbon conserving features of CAM impose: i) morphological constraints in terms of the diffusive limitations of succulence in cells or organs and ii) metabolic constraints in terms of maintaining two temporally separated carboxylation systems (usually within the same cell) and a reciprocating pool of carbohydrates which are unavailable for growth. Despite these limitations, the expression of CAM is characterized by a highly plastic response to environmental perturbations which not only permits survival under variable adverse conditions but can also result in high annual productivities of some species. This chapter will consider the biochemical components and physiological consequences of this photosynthetic plasticity. The implications for plant carbon balance, photosynthetic integrity and water use efficiency will be considered in both constitutive and facultative CAM species in response to daily, seasonal and possible future

changes in environmental conditions. By integrating field and laboratory-based approaches to the characterization of CAM expression, we seek to demonstrate that studies on the ecophysiology of this photosynthetic pathway can provide a key to understanding the components of metabolic plasticity in plants.

I. Introduction: Traditional Understanding and Approaches

Physiological ecologists, in defense of CAM, often invoke the phylogenetic diversity which accounts for perhaps 7% of plant species (Winter and Smith, 1996), and the biochemical convergence which maps onto associated succulent morphological characteristics. We also traditionally seek recourse to the high potential productivities of CAM encountered in some genera (Nobel, 1988). In reality, despite these broader ecophysiological implications, it is the fascination for setting the complexities of the CAM cycle in the context of molecular and biochemical compartmentation which drives forward our research (Chapter 23, Cushman et al.). Here, we hope to demonstrate that lessons from CAM, when set in an environmental context, provide a key to understanding metabolic plasticity and gene expression constrained by diffusive limitations of succulence in cells or organs.

A. CAM: Experimental Approaches and Characterization

Crassulacean acid metabolism presents a challenge to the environmental physiologist, because of the need to monitor biochemical and physiological characteristics throughout a 24 h cycle (Fig. 1). At night, when stomata open, CO_2 uptake, synthesis of organic acids and utilization of carbohydrate reserves allow low rates of transpiration. By day, CO_2 is

regenerated from the stored malic acid, usually in sufficient concentrations to close stomata and suppress Rubisco oxygenase and hence photorespiration (Cockburn et al., 1979; Spalding et al., 1979). To quantify the various components of the CAM cycle and integrate metabolic activity across any 24 h period, Osmond (1978) defined four Phases of CAM in terms of net CO_2 exchange, transpiration and stomatal conductance (Fig. 2). Such delineation of CAM can be refined through concurrent measurements of the difference in cell-sap titratable acidity at dusk and dawn (ΔH^+), since organic acid accumulation during Phase I reflects gross CO_2 fixation (external + internal respiratory CO_2). Thus, ΔH^+ provides a more direct assessment of the magnitude of night-time CAM activity, although it is often assumed that acid accumulation is not extended into the light period (see below) and that malic acid is not consumed at night to balance energetic requirements for accumulation in the vacuole. During the light period, because CO_2 regeneration is effectively internalized, PS II fluorescence offers great potential to track PS II photon utilization and sink demand (Winter et al., 1990; Roberts et al., 1998).

The day-night cycle is also accompanied by substantial changes in water status and solute concentration. Gas exchange can be used to infer daily, instantaneous patterns of water use efficiency, while the pressure chamber and osmometer remain an essential complement to many studies of CAM. One technique which has successfully been used to infer both the long-term (seasonal) and short-term (daily) changes in CAM activity is the use of carbon-isotope discrimination analysis (Chapter 17, Brugnoli and Farquhar). For constitutive CAM plants, the low discrimination towards ^{13}C produces C_4 -like organic material and clearly separates CAM material from C_3 . However, C_3 -CAM intermediates are often not distinguished by this technique (Griffiths, 1992). Measurements of instantaneous carbon-isotope discrimination during leaf gas exchange have provided unequivocal evidence for the transition from C_4 to C_3 carboxylation processes at dawn and late in the photoperiod (Fig. 2; Griffiths et al., 1990;

Abbreviations: $\delta^{13}\text{C}$ – carbon-isotope ratio (‰ relative to Pee Dee Belemnite); Δ – discrimination against ^{13}C ; ΔH^+ – overnight accumulation of titratable acids; CA1P – 2-carboxyarabinitol 1-phosphate; CAM – crassulacean acid metabolism; CCM – carbon concentrating mechanism; CHO – carbohydrate; EPI – environmental productivity index; g_i – internal (mesophyll) conductance to CO_2 ; NPQ – non-photochemical quenching; P_e – partial pressure of CO_2 at Rubisco; PCK – phosphoenolpyruvate carboxykinase; PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase; PFD – photon flux density; PGA – phosphoglycerate; P_i – partial pressure of CO_2 within substomatal cavity; PYR – pyruvate; qP – photochemical quenching; Rubisco – ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP – ribulose 1,5-bisphosphate; WSP – water storage parenchyma; WUE – water use efficiency

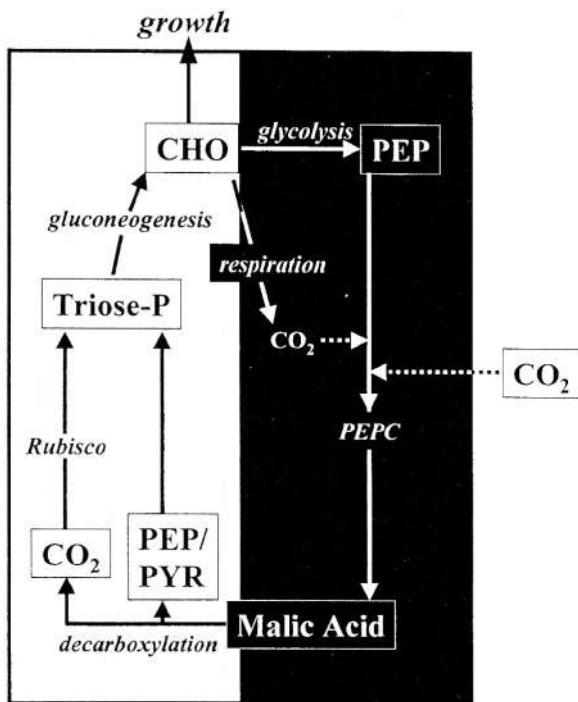


Fig. 1. Outline of the CAM pathway showing the light and dark metabolic reactions.

Borland et al., 1993; Roberts et al., 1997). As demonstrated for *Tillandsia utriculata* in Phase I of CAM, low C₄-like discrimination accompanies PEPC activity as malic acid accumulates in the vacuole overnight. Typical for bromeliads, Phase II of CAM is rather brief, with a slight increase in net CO₂ uptake as the Rubisco discrimination signal begins to dominate carboxylation as PEPC is deactivated. During Phase III, stomata can be closed by high concentrations of CO₂ generated internally. The high levels of discrimination measured at this time are associated with ¹³CO₂ preferentially leaking from the leaf, reflecting the high inherent discrimination against ¹³CO₂ which is expressed by Rubisco. Under optimal conditions of water supply and PFD, stomata often re-open to allow carboxylation mediated directly by Rubisco (Phase IV), with PEPC becoming active again before dusk associated with the shift from C₃ to C₄ discrimination signal (Fig. 2). Additionally, over a 24 h period, analysis of the ¹³C signal in metabolite pools allows the construction of detailed carbon budgets and provides an assessment of the relative contributions from C₃ and C₄ carboxylation to plant carbon balance (Borland et al., 1994; Borland, 1996).

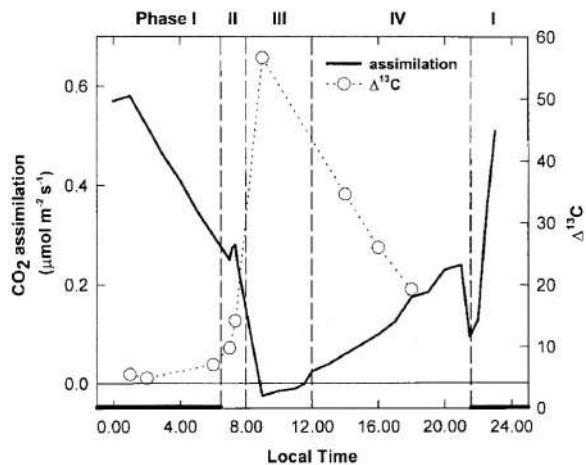


Fig. 2. Exchange of CO₂ during the four phases of CAM with concurrent measurements of carbon-isotope discrimination (Δ : ‰) for the epiphytic bromeliad *Tillandsia utriculata*. The solid bars on the x-axis indicate the periods of darkness (re-drawn from Griffiths et al., 1990).

B. CAM: Questioning Conventions and Dogmas

There are difficulties in quantifying absolute rates of gas exchange (whether as CO₂ or O₂) or carboxylation (as the interplay between PEPC and Rubisco), without recourse to more complicated stable isotope or radiolabeling methodologies (Maxwell et al., 1998). Moreover, as outlined below, a number of ecological dogmas associated with CAM still need to be re-evaluated. Are higher-energetic costs, and large pools of storage carbohydrates, really limiting for growth in a high light environment? Are CAM plants really tolerant of high light and temperatures when morphological adaptations such as self-shading and leaf/cladode orientation, suggest that adaptations to reduce PFD interception are paramount in the most exposed habitats? Why, when similar life-forms of C₃ and CAM co-specifics are compared, do C₃ plants tend to predominate in the most exposed habitats, such as the outer crown of rainforest canopy or true desert regions such as Death Valley? Thus, are CAM plants truly xerophytes, when the possession of a suite of xeromorphic adaptations prevent substantial deviations in cellular water relations? CAM has evolved in so many families in response to contrasting selective pressures, including H₂O limitation in the terrestrial habitat and CO₂ limitation in the aquatic habitat. Do the extremes of CAM distribution, from high rainfall/montane rainforests, to low temperatures

in the Rockies represent relicts (exceptions), or examples of how the metabolic plasticity central to CAM allows CO_2 uptake to be optimized over a 24 h period in succulent leaves? Ultimately, the answers to these questions lie in the metabolic and molecular regulatory mechanisms underpinning CAM. Here we have tissues which can tolerate a shift in internal conditions, whereby over a few hours, Rubisco may be operating under a range of CO_2 and O_2 concentrations equivalent to those encountered across palaeohistorical timescales. Our review in this chapter is framed by setting these conventional notions against recent developments using field and laboratory based approaches.

II. H_2O : Cellular Limitations Imposed by Deficit and Excess

A. Succulence

One of the diagnostic pre-requisites for CAM is succulence, whether at cellular or organ level: large chlorenchymatous, vacuolate cells comprise a homogenous population in many leaf succulents, typically in the Crassulaceae (Fig. 3) and Orchidaceae. Alternatively, the chlorenchyma may be heterogeneous, lying as a thin surficial layer surrounding photosynthetic stems or cladodes (e.g. the Cactaceae) or leaves (Agavaceae) above a large volume of water storage parenchyma (WSP). Many tropical epiphytes tend to show more complex arrays, with layers of WSP at the leaf surface in the Clusiaceae, (Borland et al., 1998) as well as being distributed throughout the leaf in the Bromeliaceae (Maxwell et al., 1994). Indeed, the Piperaceae may show more extreme differentiation with a multiple epidermis, palisade and spongy mesophylls showing variable carboxylation patterns (Nishio and Ting, 1988; Ting et al., 1994). Although WSP does not participate in the CAM cycle directly the contribution to water storage and recharge of chlorenchyma is important in the maintenance of overall tissue water status (Smith et al., 1987; Schulte et al., 1989; Yakir et al., 1994). However, it is interesting to note that in comparing three species of *Peperomia* and three species of *Clusia*, the cross-sectional areas of WSP are inversely correlated with the capacity for CAM (Gibeaut and Thomson, 1989; Borland et al., 1998). It is possible that the large cells of the WSP in these genera may not be capable of resisting water loss to the

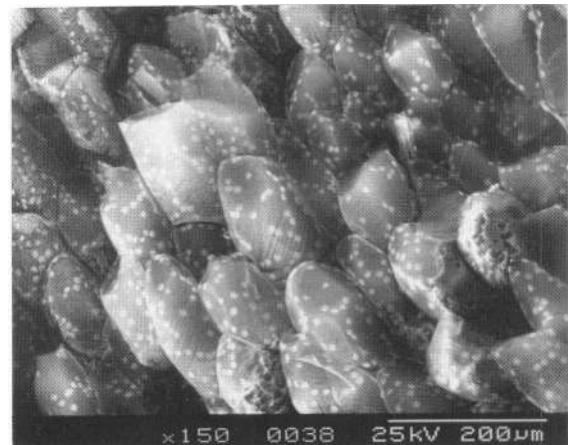


Fig. 3. Scanning electron microscope view of the closely packed mesophyll cells of *Kalanchoë daigremontiana* showing the lack of intercellular air space and scant cover of chloroplasts (from Maxwell et al., 1997).

environment under extreme conditions (Kaul, 1977). Thus, a thicker cuticle together with a lower surface area: mesophyll volume ratio may be more effective in reducing water loss under conditions of extreme exposure, as found in the constitutive CAM species of *Clusia* (Borland et al., 1998).

B. Water Relations

The succulent CAM tissues have provided model systems for evaluating cellular water relations (Smith et al., 1986, 1987; Schulte et al., 1989; Murphy and Smith, 1994, 1998). Sections of stem or cladode have required innovative solutions to allow sealing into equipment such as the pressure chamber (such as epoxy resins or cyano acrylate adhesive; Murphy and Smith, 1994), but techniques such as the pressure probe allow direct quantitation of turgor pressure and water exchange characteristics (Murphy and Smith, 1994, 1998). Cell-sap extracts are dominated by vacuolar contents and thus provide an effective proxy for the osmotic consequences of day-night changes in tissue solute concentration and can be easily measured by osmomometry on sap extracted from tissue. At the tissue level, there are inverse patterns of leaf water potential (ψ), with xylem tension becoming greater during Phase I and Phase IV when stomata are open. As malic acid accumulates, concomitant changes in leaf-sap osmotic pressure (π) are observed, which could act as a means to drive water uptake from dew fall at dawn (Smith et al.,

1986) or remobilize water from older leaves (Ruess and Eller, 1985). Concomitant changes in turgor pressure (P) can be calculated from the difference between ψ and π , but it has taken a careful study comparing indirect methods for analyzing ψ and π , with direct measurements of P (with the pressure probe) to show that the xylem osmotic component should be corrected when deriving ψ leaf using the pressure chamber (Murphy and Smith, 1994). Most recently, the pressure probe has been used to validate a model of cell-water relations parameters, including fluxes between cells via plasmodesmata (Murphy and Smith, 1998).

At the ecological level, water potentials lower than -1.0 MPa are rarely encountered in CAM plants. For *Aechmea aquilega*, normally an epiphyte but growing terrestrially at a coastal site in Trinidad, a maximum xylem tension of 0.85 MPa was measured, compared to 3.3 MPa for a nearby C_3 shrub (i.e. equivalent to ψ of -3.3 MPa). The dawn to dusk changes in ψ during the onset of the dry season in Trinidad are shown in Fig. 4 for the hemiepiphytic strangler *Clusia minor*. It is notable that greater xylem tensions (more negative ψ) developed during the rainy season, when daytime gas exchange would have predominated. High levels of CAM activity were induced at the onset of the dry season (Borland et al., 1996), when rainfall ceased (Fig. 4a) and it is notable that the dusk values of xylem tension were reduced (ψ more positive) at this time (Fig. 4b), indicating the effectiveness of CAM at conserving water on a daily basis.

These observations lead us to address two dogmas associated with CAM: one is that CAM provides higher water use efficiency than C_3 plants. When measured directly in terms of water use per unit increment of dry matter gain, or instantaneously during gas exchange, there is no doubt that C_3 mesophytes have lower WUE than CAM plants. However, when sympatric species with similar life-forms are compared under comparable environmental stress, WUE for C_3 and CAM are indeed similar (Griffiths et al., 1986; Eller and Farrari, 1997). Secondly, since CAM plants never develop severe water deficits, can we really describe them as tolerant of water deficits *sensu* true xerophytes? Succulent plants undoubtedly show remarkable adaptations to conserve water status and maintain carboxylation conductance in the face of severe diffusion limitation, as discussed below.

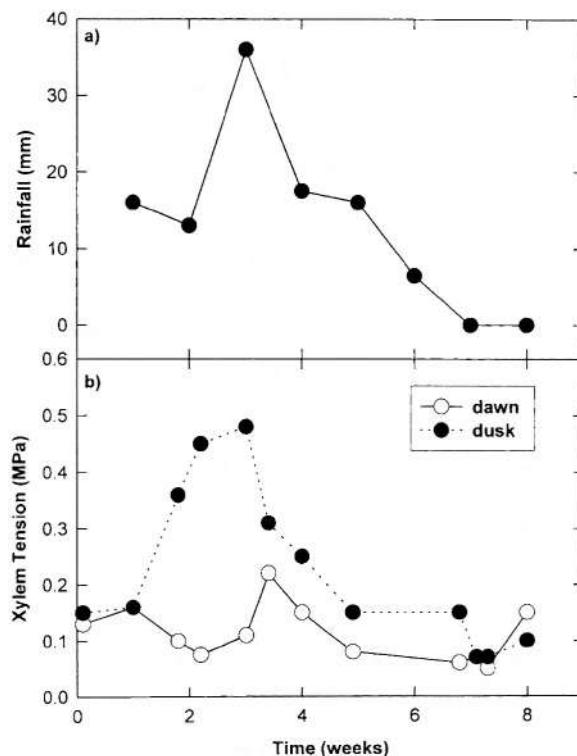


Fig. 4. Seasonal changes in a) weekly rainfall and b) leaf xylem tension measured at dawn and dusk in mature leaves of *Clusia minor* during the wet-dry season in Trinidad, West Indies (redrawn from Borland et al., 1996).

C. Root Systems and Salinity

The work of Park Nobel and colleagues has made a major contribution to our understanding of how the root/soil interface can be used to promote water uptake or prevent water loss in CAM spp, depending on soil water status (Jordan and Nobel, 1984; Nobel, 1992; North and Nobel, 1998). Mature roots, by shrinking and suberising in response to soil water deficits, create disjunctions in the soil and root hydraulic conductance pathway (Nobel, 1992), and abscission of lateral roots both reduce water uptake (or loss from the plant: Nobel, 1992; North and Nobel, 1998). Root growth is rapidly re-established within one week of re-wetting, driven by water supplied from the stem, allowing stem and leaf water storage capacity to be recharged rapidly even after extended drought periods (Nobel and North, 1998).

However, in contrast to water deficits, most CAM plants are not salt tolerant, with lateral root extension reduced by salt (Gersani et al., 1993). A comparison of stem succulent cacti and the true halophytic CAM plant *Mesembryanthemum crystallinum* (Lüttege,

1994) showed that when cacti are subjected to saline or hypersaline environments, necrosis and death in the seedling or juvenile stages of growth is common. The exception is the C₃-CAM intermediate *M. crystallinum*, in which responses to salinity and induction of CAM are definable in molecular terms (Adams et al., 1998; Cushman and Bohnert, this volume). Here, an initial suite of juvenile leaves (which can induce CAM: A. N. Dodd, personal communication) provide a critical mass of plant material using winter rains in the natural environment. As the soil dries, and salinity increases, there is a developmental shift to CAM which can be accelerated by appropriate environmental conditions (Adams et al., 1998).

D. Adaptations to Extreme Habitats: Excess Water

Given that CAM has evolved in so many families, and provides a model for analysis of the molecular and biochemical basis of responses to environmental stress, how can we account for the occurrence of CAM when water is not limiting? Aquatic macrophytes of the Isoetid lifeform, such as *Isoetes lacustris* and *Littorella uniflora*, develop CAM in the aquatic habitat and use CO₂ supplied from sediments to overcome the diffusive limitations imposed by the aquatic milieu, where CO₂ diffuses 10,000 × slower than in air. On exposure, the amphibious *L. uniflora* loses CAM activity, increasing investment in Rubisco while still relying on sedimentary CO₂ supplies (Robe and Griffiths, 1998; W. E. Robe, personal communication). This illustrates how CAM serves as an example of biochemical convergence in evolutionary terms, as a solution to diffusive limitation whether imposed externally (by water) or internally (by succulent leaf tissues: see below).

In the latter case, we should try to explain the distribution of CAM plants in extreme habitats. For instance, *Opuntia humifusa* can tolerate low temperature, high moisture habitats in Canada and eastern USA (Goldstein and Nobel, 1994; Section C below). Alternatively, endemic CAM bromeliads, such as *Aechmea arripensis* in Trinidad and *A. laesseri* in Venezuela, are often found exclusively in upper montane rainforest formations, in shaded environments where rainfall may exceed 5 meters y⁻¹! Is CAM in these habitats a relict of a previous drier environment, or could it be that CAM provides a competitive advantage over similar sympatric C₃,

lifeforms? The photosynthetic plasticity which underpins the 4 phases of CAM could be the key for optimizing CO₂ gain where films of water on the surface of wetted leaves preclude CO₂ exchange for extended periods.

III. CAM as a Carbon Concentrating Mechanism: Morphological and Biochemical Considerations

A. Mesophyll Conductance and Photorespiration

A carbon concentrating mechanism (CCM) functions to generate an elevated partial pressure of CO₂ at Rubisco active sites (P_c) and thereby increases carboxylation efficiency through a reduction in oxygenase activity. In considering CAM as a CCM, the importance of organic acid decarboxylation as a means of suppressing photorespiration has perhaps been exaggerated. Refixation of CO₂ during Phase III occurs at an internal CO₂ partial pressure (pCO₂) which may be as high as 0.8 to 2.5% (Spalding et al., 1979; Cockburn et al., 1979) and thus qualifies for CCM status. However, substantial Rubisco-mediated atmospheric CO₂ fixation occurs in many CAM and C₃-CAM plants during Phases II and IV (Borland and Griffiths, 1996). This component of day-time fixation is subject to the biochemical and biophysical limitations to carbon gain typical of C₃ plants (von Caemmerer and Farquhar, 1981; Farquhar and von Caemmerer, 1982) and does not constitute a functional CCM (Osmond et al., 1999).

CAM leaves are primarily designed for water conservation, requiring a leaf morphology which is strikingly at odds with the thin, porous blade that is optimal for atmospheric CO₂ fixation (Maxwell et al., 1997; Osmond et al., 1999). CAM plants tend towards thick, succulent leaves (Teeri et al., 1981; Winter et al., 1983; Osmond et al., 1999) or photosynthetic stems. The large, highly vacuolate cells are tightly packed creating minimal volumes of air space (Smith and Heuer, 1983). In leaves of *Kalanchoë daigremontiana* the highly succulent nature of the cells mean that air space comprises only 8% of leaf volume (Maxwell et al., 1997).

Since CO₂ and O₂ compete for Rubisco active sites, the efficiency and capacity of C₃ photosynthesis is strongly influenced by P_c . P_c is influenced by the internal (mesophyll) conductance of CO₂ (g) from

stomatal cavity to Rubisco. Recently, a positive correlation has been established between g_i and intracellular air space and a negative relationship between g_i and leaf thickness (Evans and von Caemmerer, 1996; Chapter 14, Evans and Loreto). Although a high diffusive resistance to CO_2 had previously been postulated for succulent leaves (Robinson et al., 1993b), it was calculated that in leaves of *K. daigremontiana* during Phase IV, g_i was 0.05 mol $\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$, manifested as a value of P_c of only 109 μbar at an ambient $p\text{CO}_2$ of 350 μbar (Maxwell et al., 1997). For comparison, a P_c of 209 μbar was calculated for tobacco leaves which contained a similar amount of Rubisco (Evans et al., 1994). Assuming an internal concentration of 21% O_2 and 0.01% CO_2 , a ratio of carboxylation to oxygenation will be close to parity, as compared to 2.9 for spinach (Osmond et al., 1999). Comparably low values of g_i have been demonstrated in other CAM species, most notably for three *Clusia* species which exhibit a gradation of CAM activity (C_3 , C_3 -CAM and obligate CAM) which correlated with both succulence and decreasing g_i (Borland et al., 1998; Gillon et al., 1998). Such exceptionally low values of g_i limit photosynthetic capacity of succulent species during Rubisco-mediated atmospheric CO_2 uptake. Moreover, while conventional estimates of P_i (the partial pressure of CO_2 within the sub-stomatal cavity) approximate P_c in a number of thin-leaved species, in succulent leaves P_i may be up to three-fold higher than the actual partial pressure of CO_2 which is presented to Rubisco in the chloroplast (Maxwell et al., 1997).

Light-dependent $^{18}\text{O}_2$ uptake of intact leaf discs can be used to investigate the nature of O_2 uptake during CAM (Thomas and Andre, 1987). Figure 5 shows CO_2 assimilation concomitant with O_2 uptake (corrected for dark respiration) as a function of ambient $[\text{CO}_2]$ in *Dendrobium speciosum*. During Phase IV, a low stomatal and internal conductance to CO_2 assure low rates of CO_2 assimilation and a clear negative relationship is observed between ambient CO_2 concentration and O_2 uptake (Fig. 5a). In this instance, photorespiration comprised 80% of electron transport at 590 μbar CO_2 (Maxwell et al., 1998).

During decarboxylation, being a thick leaf has mixed blessings. Firstly, high diffusive limitation coupled to stomatal closure prevents excessive CO_2 leakiness during decarboxylation. However, elevated intracellular $p\text{O}_2$ is generated from linear electron transport (approximately 42%; Spalding et al., 1979),

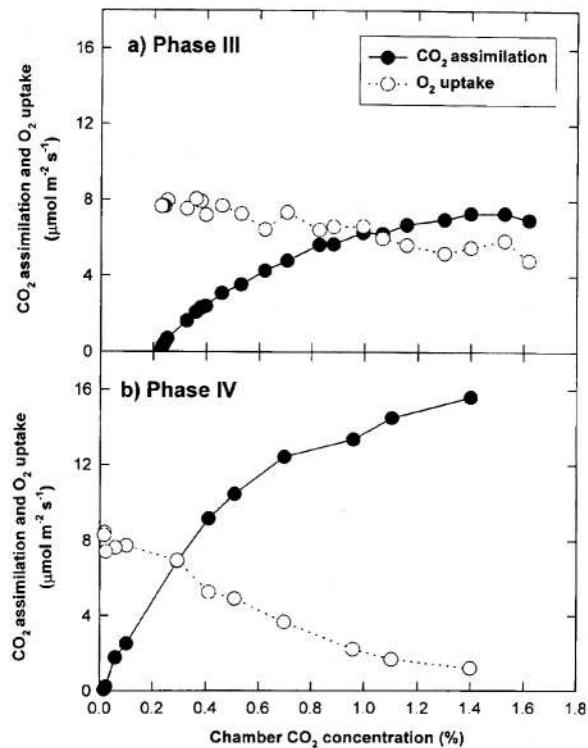


Fig. 5. Simultaneous light-dependent O_2 and CO_2 exchange in leaf discs of *Dendrobium speciosum*. Measurements of $^{18}\text{O}_2$ and $^{12}\text{CO}_2$ uptake were made during Phases III (a) and IV (b) as a function of external CO_2 concentration. (For additional technical details see Maxwell et al., 1998).

which at the very least increases the possibility of photorespiration during the CCM of Phase III (Maxwell et al., 1998), but, equally, may promote the formation of potentially damaging reactive oxygen species (Osmond et al., 1999). Indeed, increases in the transcription of genes which encode scavenging enzymes have been observed during the induction of CAM in *M. crystallinum* (Miszalski et al., 1998). The activities of ascorbate peroxidase and superoxide dismutase have also been shown to increase with the shift to CAM in *Sedum album* (Castillo, 1996). During Phase III, high rates of O_2 uptake have been observed (Thomas and André, 1987; Cote et al., 1989; Maxwell et al., 1998) which, given the biochemical and kinetic properties of Rubisco, seem only partially attributable to photorespiration (Osmond et al., 1999). In the example provided in Fig. 5b, O_2 uptake was high over the entire CO_2 range (1.2 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ at 1.4 % CO_2) and showed only a slight CO_2 sensitivity. It is possible that during Phase III, a significant proportion of electron transport is used to reduce O_2 via the Mehler ascorbate

peroxidase pathway, especially given the highly oxidizing conditions which prevail during Phase III (Osmond et al., 1999).

Accepted dogma states that CAM is a carbon concentrating mechanism and thus photorespiration should be a negligible metabolic process. However, the morphological constraints of the succulent leaf required to support CAM result in low partial pressures of CO_2 within the leaf, which increase the probability of photorespiration *throughout* the day. It is clear that photorespiration occurs in CAM plants, often at very substantial rates and must be significant in CAM-idling plants.

B. PEPC Regulation

The day/night separation of metabolism in CAM requires that PEPC is effectively down-regulated at the start of the day during Phase II. Although Phase II is usually considered as a relatively brief transition between C_4 and C_3 carboxylation processes, a literature survey of a range of obligate and facultative CAM species has indicated that up to 50% of CO_2 taken up over 24 h can occur during Phase II (Borland and Griffiths, 1996). It seems likely that an appreciable amount of this morning uptake is mediated by PEPC. Field and laboratory-based studies on hemi-epiphytic

stranglers of the genus *Clusia* have provided direct evidence that PEPC can remain active for 4–5 h after dawn as indicated by: i) a continued accumulation of organic acids over this period and ii) the low values of instantaneous carbon isotope discrimination measured during leaf gas exchange (Borland et al., 1993; Roberts et al., 1997). Flux through PEPC is regulated by reversible phosphorylation which reduces the sensitivity of the enzyme to inhibition by L-malate, with the phosphorylated, malate-insensitive (active) form of PEPC usually assumed to be present only at night (Nimmo et al., 1984; 1986; Carter et al., 1996). In *C. minor* however, PEPC can remain phosphorylated for some 3 h into the photoperiod, as indicated by measurements of the sensitivity of the enzyme to malate inhibition *in vitro* (Fig. 6b), while in *K. daigremontiana*, the enzyme is rapidly de-phosphorylated within the first hour of the photoperiod (Fig. 6a; Borland and Griffiths, 1997). Similar investigations on *Tillandsia usneoides*, an extreme atmospheric bromeliad, indicate that PEPC is only slowly de-phosphorylated some 5 h into the photoperiod (R. Haslam and A. M. Borland, unpublished). Such observations call for a reconsideration of the extent of day-time PEPC activity in diverse CAM species.

Previously, reports have suggested that the amount

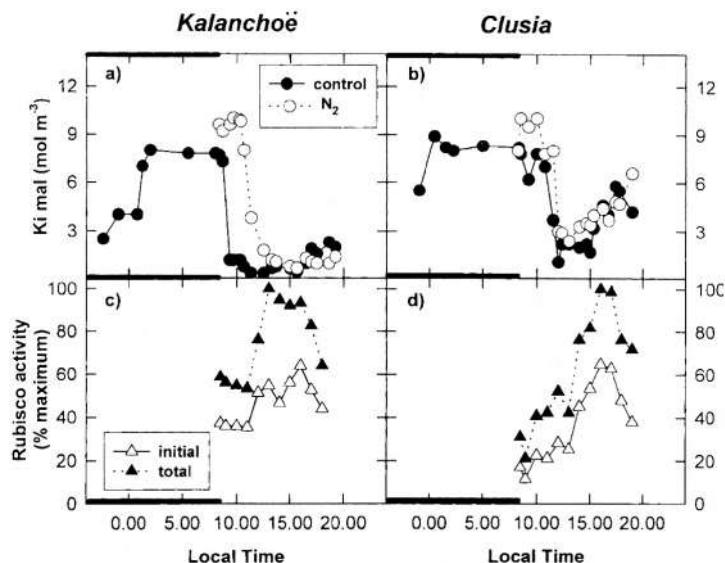


Fig. 6. Apparent activation of PEPC (a,b) and Rubisco (c,d) measured *in vitro* for extracts prepared from leaves of *Kalanchoe daigremontiana* and *Clusia minor*. Diel changes in K_i malate (a,b) indicate the apparent phosphorylation status of PEPC in control leaves and in leaves prevented from accumulating malate overnight in an atmosphere of N_2 but transferred to ambient air at the start of the photoperiod (Borland and Griffiths, 1997). The activation status of Rubisco (c,d) is indicated by changes in initial and final (pre-incubation with Mg^{2+}) activities over the photoperiod (A. Roberts and H. Griffiths, unpublished). The solid bars on the x-axes indicate the periods of darkness.

of CO_2 taken up during Phase II may be influenced by the magnitude of CO_2 fixation and malic-acid accumulation during the previous night (Medina and Delgado, 1976; Fischer and Kluge, 1984). Physiological manipulations of leaf malate content and perturbation of malate compartmentation between vacuole and cytosol have suggested that the circadian rhythm of PEPC phosphorylation (Nimmo et al., 1987) may be modulated by malate (Borland and Griffiths, 1997; Grams et al., 1997a). As illustrated in Fig. 6a,b, if leaves of *C. minor* or *K. daigremontiana* are prevented from accumulating malate overnight in an atmosphere of N_2 , subsequent transfer to ambient air at the start of the photoperiod results in an increase in apparent phosphorylation status of PEPC for the first 2–3 h of the photoperiod and de-phosphorylation now occurs some 3–4 h into the light in both species (Borland and Griffiths, 1997). The enhanced phosphorylation status of PEPC is accompanied by an increase in net CO_2 uptake during Phase II in leaves prevented from accumulating malate (Borland and Griffiths, 1997). Thus, the degree of PEPC phosphorylation can modulate carbon gain in response to short-term changes in the environment which alter the amount and/or partitioning of malate between vacuole and cytosol.

Maintenance of PEPC activity for much of the day implies that futile cycling of malate synthesis/decarboxylation could represent a substantial proportion of day-time carbon flux. A variety of experimental approaches have illustrated that futile cycling can and does occur during Phase IV in a range of CAM species (Osmond et al., 1996). However during Phase II in *C. minor* estimates of PEPC activity *in vivo* (obtained from instantaneous carbon-isotope discrimination), compared closely with the amount of malate accumulated over this period, implying that decarboxylation and thus futile cycling, were curtailed (Borland and Griffiths, 1997).

C. Rubisco Regulation

The capacity for optimizing carbon gain and WUE by variably adjusting the amount of CO_2 fixed during the 24 h CAM cycle also requires a consideration of Rubisco regulation, particularly since it is the direct uptake of CO_2 via C_3 carboxylation during Phase IV which largely determines the growth and productivity of CAM species (Nobel, 1996). Studies conducted on C_3 plants have shown that Rubisco activity may be modulated in response to changes in light intensity,

CO_2 or O_2 supply through reversible carbamylation, facilitated by the action of Rubisco activase (Portis, 1995). This aids the binding of Mg^{2+} to form a catalytically active complex. Other mechanisms of control include the binding of naturally occurring inhibitors such as 2-carboxyarabinitol 1-phosphate (CA1P) and modulation by stromal metabolites (Seemann et al., 1990; Geiger and Servaites, 1994; Parry et al., 1997). The existence of this variety of regulatory mechanisms increases the responsiveness of Rubisco to changes in the environment, and thus should serve to modulate C_3 carboxylation to the fluctuations in CO_2 supply which occur over the day-time phases of CAM. Although the diurnal regulation of Rubisco has previously received little attention in CAM plants, recent investigations have indeed indicated that in *K. daigremontiana* and *C. fluminensis*, both initial and final Rubisco activities measured *in vitro* change over the course of the day (Fig. 6c,d; A. Roberts and H. Griffiths, unpublished). In both species, the activity and the percentage activation of Rubisco, which reflects carbamylation state, is highest towards the end of the day when decarboxylation is complete, stomata have re-opened and net CO_2 uptake is evident. The up-regulation of Rubisco at this time should serve to maintain carboxylation strength (and WUE), thereby compensating in some measure for the diffusive limitations to CO_2 encountered during Phase IV (Maxwell et al., 1997; 1998). However, it is likely that CO_2 supply may also be limiting during Phase II if PEPC remains active as discussed above. Since PEPC has a high substrate affinity (i.e. is a more efficient scavenger of CO_2 than Rubisco), this will create an enormous ‘biochemical resistance’ to CO_2 in addition to that imposed by the closely packed succulent cells of the leaf. In this situation, the reduced catalytic activity of Rubisco during Phase II might reflect the inability of the chloroplast to generate RuBP.

The low activities of Rubisco measured during Phase II may also be attributed, at least in part, to the binding of endogenous inhibitors such as CA1P. The presence of such inhibitors, as indicated by the dark inactive state of Rubisco has been reported for a number of CAM species including *Ananas comosus* and *Crassula argentea* (Cu et al., 1984; Servaites et al., 1986). Moreover, studies on C_3 plants have indicated that CA1P binding is more prevalent in the morning than at an equivalent PFD later in the day (Sage et al., 1993). An additional component of Rubisco modulation may involve chloroplastic

metabolites. In C₃ plants, the concentrations of RuBP and PGA can change substantially and often reciprocally during the course of a day, producing dramatic and rapid changes in Rubisco activities (Portis, 1995). Although little is known about the intracellular distribution and diel changes of metabolites in CAM cells, it is known that levels of PGA in *K. daigremontiana* increase during deacidification and RuBP does not reach steady state for at least 20 min after onset of the photoperiod (Kenyon et al., 1981).

The extent to which Rubisco is regulated via inhibitors such as CA1P, versus changes in carbamylation state of the enzyme may well differ amongst CAM species, as has been shown to be the case in C₃ plants (Holbrook et al., 1994). Indeed, the unique conditions encountered during the day in CAM species suggest that hitherto unsuspected subtleties and complexities of Rubisco regulation may underpin the plasticity of day-time gas exchange patterns. Depending upon the CAM species, these can range from the continuous day-time net CO₂ uptake found in CAM cycling to the extreme condition of CAM-idling where stomata remain closed over 24 h.

D. Diel Carbohydrate Partitioning

A major metabolic constraint imposed by the CAM cycle is the requirement for a large reciprocating pool of carbohydrates which must be distinguished from the carbon skeletons destined for nitrogen metabolism, respiration and growth. During Phase III, 75% of the carbohydrates synthesized via gluconeogenesis and from the refixation and processing of CO₂ via C₃ photosynthesis, must be retained as a reserve to fuel carbon assimilation over the subsequent night. The remaining carbohydrates, together with any produced as a result of Phase IV photosynthesis are directed towards growth. The biochemical processes which control this partitioning of carbohydrates are poorly understood but appear to be regulated in such a manner that the demands of the dark reactions of CAM take precedence over export. Evidence for this stems largely from source-sink manipulations of *K. pinnata*, where decreasing sink demand by girdling or removing young leaves resulted in the accumulation of sucrose and abolishment of Phase IV photosynthesis, while dark CO₂ uptake was unaffected (Mayoral et al., 1991). Moreover, increasing sink strength by darkening

leaves did not affect CAM activity in the illuminated source leaf.

Understanding the metabolic processes which regulate carbohydrate partitioning in CAM plants is complicated by the diversity of strategies for cycling carbon into different carbohydrate pools. Two main groups of CAM species are commonly distinguished in terms of the most abundant storage carbohydrate; i) chloroplastic starch and glucan accumulators which include many members of the Crassulaceae such as *K. daigremontiana*, *K. pinnata* and *Sedum telephium* (Sutton, 1975; Fahrendorf et al., 1987; Borland, 1996), ii) extra-chloroplastic soluble sugar or polysaccharide accumulators which include *Ananas comosus* (vacuolar mono and di-saccharides; Carnal and Black, 1989), *Fourcroya humboldtiana* (fructans; Olivares and Medina, 1990) and *Aloe arborescens* (polymer of galactose and mannose; Verbücheln and Steup, 1984). In addition, some CAM species, including several within the genus *Clusia*, accumulate both soluble sugars and starch (Popp et al., 1987; Borland et al., 1994). Such complexity is compounded by the recent observations of Christopher and Holtum (1996; 1998) who have defused the widely accepted view that decarboxylation biochemistry (malic enzyme or PCK) determines whether carbohydrates are stored inside or outside of the chloroplast. At the last count, at least eight patterns of carbohydrate partitioning in more than twenty CAM species have been distinguished.

One approach for examining the arrangement of carbon pools in CAM plants is via quantitative measurements of the carbon-isotope composition of the various organic fractions in the leaf. Since carbohydrates synthesized directly from C₃ photosynthesis are depleted in ¹³C compared to those arising from the day-time decarboxylation of malate, studies on *K. blossfeldiana* and *K. daigremontiana* have suggested that carbohydrates derived from C₃ and C₄ metabolism are channeled into parallel pathways of glycolysis at night (Deléens and Garnier-Dardart, 1977). Thus, respiratory CO₂ was generated from soluble sugars depleted in ¹³C whereas PEP was produced from the glycolytic breakdown of ¹³C-enriched starch (Deléens et al., 1979). In species such as *C. minor* where degradation of both soluble sugars and starch is necessary to supply PEP, it is likely that the situation will be more complex at night. However, there does appear to be compartmentation between the products of C₃ and C₄ carboxylation during the day in *C. minor* (Borland et

al., 1994). Under natural field conditions, carbon lost from soluble sugars at night was considerably enriched in ^{13}C compared to CO_2 fixed during Phase IV, indicating the existence of two soluble sugar pools; i) a vacuolar pool enriched in ^{13}C from the decarboxylation of organic acids and ii) a rapidly turned over cytosolic pool of transport sugars synthesized as photosynthetic end-products during Phase IV (Borland et al., 1994). Such compartmentation provides a means by which the metabolic pathways that constitute CAM and those that fuel growth could be regulated independently of one another.

IV. Daily Integration of Environmental Conditions

A. Modulation of the CAM Phases

CAM plants tend to succeed in extreme environments which are subject to daily fluctuations in precipitation, integrated/incident photon flux density (PFD) and temperature. The expression of CAM is thus characterized by a highly plastic response to environmental perturbations which permits survival under variable adverse conditions. Over the course of 24 h, the amplitude of each of the four phases of gas exchange is determined by environmental and physiological factors and is highly species dependent (Borland and Griffiths, 1996). Under extreme conditions of drought stress, stomata may remain closed over 24 h, behind which the refixation of respiratory CO_2 at night and decarboxylation of organic acids by day permits continued flux through the pathways of carbon metabolism and electron transport. Such CAM-idling is particularly well-documented for shallow-rooted cacti (Kluge and Ting, 1978). CAM-cycling represents a less extreme modification of CAM, where continuous uptake of CO_2 over the day is accompanied by nocturnal accumulation of acids derived from refixation of respiratory CO_2 . This particular mode of photosynthetic metabolism is present in over 40 species representing at least 15 families (Martin et al., 1988), which are often found growing in habitats where water supply is unpredictable on a daily basis.

The degree of exposure is a strong determinant of C_3 and C_4 carboxylation during CAM. Figure 7 shows measurements of net CO_2 uptake made on the same day in the dry season in Trinidad on fully

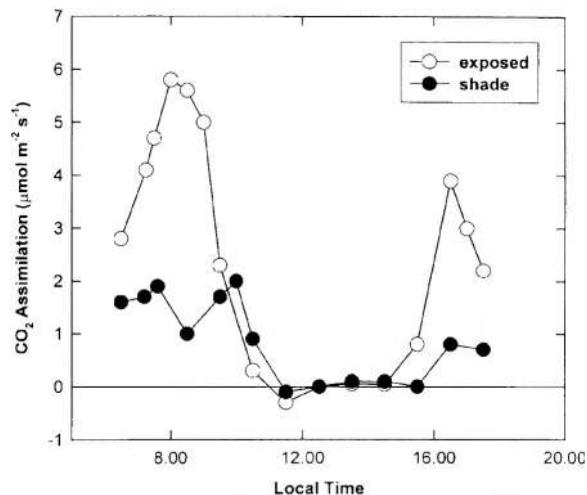


Fig. 7. Day-time net CO_2 exchange for naturally exposed and shaded leaves of *Clusia minor* measured during the dry season in Trinidad, West Indies (redrawn from Borland et al., 1993).

exposed and shaded *C. minor* plants. The shaded plants show much reduced rates of gas exchange during Phase II and particularly during Phase IV. Additionally the magnitude of CAM, measured as the dawn-dusk difference in titratable acidity (ΔH^+) was 50% lower for the shaded plants. On-line carbon-isotope discrimination has illustrated the dominance of the C_4 discrimination signal during Phase II, indicating the magnitude of prolonged PEPC activity under field conditions (Borland et al., 1993; Roberts et al., 1997).

Incident and integrated PFD also has important implications for the degree of nocturnal CO_2 uptake with a direct relationship between the amount of organic acids accumulated overnight and the photon receipt of the previous photoperiod (Nobel and Hartsock, 1983 and Fig. 8). This relationship can be attributed to the increased accumulation of glucans under high PFD which are subsequently used to generate PEP, the carbon substrate for C_4 carboxylation at night. In a study of four cactus species, Nobel (1988) demonstrated that the degree of nocturnal acidification exhibited an initial linear increase with daily photon dose and saturated at around 30 mol photon $\text{m}^{-2} \text{d}^{-1}$, presumably when vacuolar capacity was reached (Fig. 8; Nobel, 1988). A minimum of 3 mol photon $\text{m}^{-2} \text{d}^{-1}$ was required for acidification, equivalent to an incident PFD of 69 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ over a 12 h photoperiod. A similar pattern was observed for *Agaves*, although in contrast, a strikingly different pattern was obtained

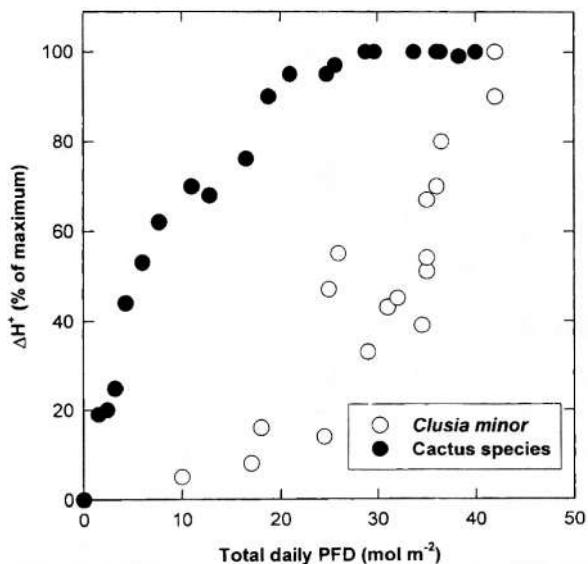


Fig. 8. Nocturnal acid accumulation as a function of total daily photon flux density for a range of desert cactus species (data taken from Nobel, 1988) and for *Clusia minor* growing in Trinidad (data taken from Borland et al., 1996).

with the facultative CAM species *C. minor* (Fig. 8). Little evidence of light saturation of ΔH^* is observed, in this species which may accumulate over 1.5 M H^* during the dry season in Trinidad (Fig. 8; Borland et al., 1992). *Clusia* species accumulate both malate and citrate with an increasing contribution from citrate at higher PFD (Borland et al., 1996). Initial observations suggested that the degree of nocturnal acidification was correlated with the level of dusk acidity (Zotz and Winter, 1993b). This view has been modified since it has now been shown that dusk citrate concentration is negatively correlated with the amplitude of nocturnal CO_2 fixation (Borland et al., 1996; Roberts et al., 1998). It has been suggested that in certain CAM species, the decarboxylation of citrate in high light generates additional carbon skeletons required for increased PEPC activity and thus modulates the expression of CAM in response to environmental conditions (Borland et al., 1996).

B. Sun and Shade Distribution

CAM plants tend to colonize extreme habitats (Lüttge, 1987) and are likely to be either fully exposed or only partially shaded. This skewed distribution towards higher light environments may be explained by the increased energetic requirements of the regeneration of PEP during Phase III photosynthesis as compared

to C_3 carboxylation (Winter and Smith, 1996) and the related minimum photon dose requirement for nocturnal acidification (Nobel, 1988). For example, the epiphytic bromeliad flora of Trinidad, W. Indies has been characterized according to habitat preference within the forest canopy viz. exposure, sun and shade tolerant (Pittendrigh, 1948). All CAM species are confined to the middle to top of the forest canopy (Griffiths and Smith, 1983). Indeed, relatively few CAM plants are distributed in deep shade, although notable exceptions exist which prove this rule. For example, *Aechmea magdalena* is a terrestrial bromeliad which successfully colonizes both deep shade and exposed habitats (Skillman and Winter, 1997). This species has overcome the problem of light limitation through an effective and rapid utilization of sunflecks which may be sufficient to support CAM within the forest understory (Skillman and Winter, 1997).

The majority of CAM plants are able to adjust the photosynthetic apparatus to reduced light regimes, yielding responses which are typical of C_3 and C_4 plants (Adams et al., 1987), albeit at the expense of reduced nocturnal acidification (Nobel and Hartsock, 1983, Nobel, 1988). Herppich and co-workers (1998a,b) have recently made a thorough examination of photosynthetic acclimation to high and low light in the CAM-cycling plant, *Delosperma tradescantoides*, which grows under exposed and semi-shaded conditions. The shaded plants exhibited a 50% reduction in diurnal CO_2 assimilation, lower dark respiration rates and light compensation point, while the quantum yield of photosynthesis was comparable with high light plants, i.e. a set of responses which are identical to those predicted for C_3 plants.

C. Photoinhibition and Photoprotective Strategies

In a consideration of the selective advantages of the evolution of CCMs, it has been postulated that CAM may prove effective in the alleviation of photo-inhibition (Gil, 1986; Griffiths, 1989), as defined by a long-term decrease in photochemical efficiency, which may incorporate damage to the photochemical apparatus. In any photosynthetic organism, photo-inhibition will arise when absorbed light energy exceeds the capacity for photosynthetic light use and thermal dissipation of the excess, parameters which are genotypically and/or phenotypically fixed. In all cases, acclimation to high light facilitates photopro-

tection and sun plants will tolerate a higher threshold PFD than shade plants, even under circumstances when shade plants exhibit CAM (Adams et al., 1987; Adams and Osmond, 1988).

The first line of defense against photoinhibition is a physical reduction in the amount of light incident on the light harvesting apparatus. CAM leaves tend towards a high reflectance of incident light, possibly as a consequence of water storage tissues and a thick epidermis. Laminar reflectance was 21% of incident light in *Hoya carnosa*, 40% in highly exposed leaves of *Dendrobium speciosum* (K. Maxwell, unpublished) and approximately 36% in young leaves of *Ananas comosus* (Adams et al., 1986). Development of wax in young leaves of *Cotyledon orbiculata* was strongly correlated with an increase in light reflectance (57% at day 35: Barker et al., 1997). Removal of wax from the leaf surface resulted in chronic and extremely prolonged photoinhibition in this species providing unequivocal evidence that wax constitutes a highly effective photoprotective barrier (Robinson et al., 1993a). Additionally, it has been suggested that anthocyanins may mitigate photoinhibition in young leaves of *C. orbiculata* prior to wax deposition (Barker et al., 1997).

In CAM species, the predicted phases of the pathway impose a contrasting pattern of photosynthetic light use over the diurnal course which may mediate photoprotection and photoinhibition. It has long been postulated that since decarboxylation events are energetically costly and are coincident with the periods of maximum incident PFD, photoinhibition will be minimized during Phase III. Indeed, a very large body of evidence has emerged in support of this hypothesis and it seems clear that decarboxylation will alleviate photoinhibition (Osmond, 1982; Adams et al., 1988; Griffiths et al., 1989) provided that the plants are pre-acclimated to high light (Adams et al., 1987; Adams and Osmond, 1988; Maxwell et al., 1994; Grams et al., 1997b; Roberts et al., 1997; Barker et al., 1998; Herppich et al., 1998a,b). The overnight accumulation of citrate has been observed to increase in certain plants exposed to high light and it has been suggested that decarboxylation of this organic acid may generate a higher $p\text{CO}_2$ compared to malate, and thereby prevent photoinhibition (Haag-Kerwer et al., 1992; Grams et al., 1997b), although a precise photoprotective role has yet to be substantiated. Phase II occurs at the beginning of the photoperiod when light levels are relatively low and therefore the likelihood of photoinhibition is reduced,

although this will in part depend upon leaf orientation (Barker and Adams, 1997; Barker et al., 1998). Extended PEPC activity may delay decarboxylation until PFD is maximal and thereby maximize photosynthetic light use when the risk of photodamage is greatest (Roberts et al., 1998).

The duration of Phase III decreases with increasing PFD and subsequent Phase IV activity represents Rubisco mediated atmospheric CO_2 uptake. Despite very low internal $p\text{CO}_2$, this phase is coincident with high Rubisco activity (Fig. 6c,d). Since photorespiration rates are high at this time (Maxwell et al., 1997, 1998), it is feasible that Rubisco oxygenase maintains photon use and alleviates photoinhibition in CAM plants in a mechanism identical to that found in C_3 plants (Heber et al., 1996; Osmond et al., 1997).

At light levels which exceed photosynthetic saturation, a proportion of absorbed light energy may be harmlessly dissipated from the light harvesting complex, in a photoprotective process which is manifested as an increase in non-photochemical quenching (NPQ) of chlorophyll fluorescence (Horton et al., 1996). Almost without exception, dissipation is linked with the de-epoxidation of the carotenoids violaxanthin to antheraxanthin and zeaxanthin within the light harvesting complex, in reactions which comprise the xanthophyll cycle (Demmig-Adams and Adams, 1996; Horton et al., 1996). Daily variations in both the expression of NPQ and the xanthophyll cycle have been observed in CAM species.

On a daily basis, the highest values of NPQ (Winter and Demmig, 1987) and zeaxanthin (Winter et al., 1990) have been observed during the transient phases of CAM. These observations, made at constant light intensity, reinforce the concept of an increased requirement for thermal dissipation when light use is lowest. However, despite high levels of titratable acidity and delayed decarboxylation, significant rates of thermal dissipation are also required to prevent chronic photoinhibition during Phase III in the genus *Clusia* (de Mattos et al., 1997; Grams et al., 1997b; Roberts et al., 1998). The operation of the xanthophyll cycle during CAM has most elegantly been described in recent work on two *Opuntia* species (Barker and Adams, 1997; Barker et al., 1998). Both exhibit a positive relationship between photon dose and the xanthophyll pool size in cladodes with different orientations. Equally, both studies provide convincing evidence for a dependency of thermal dissipation on the formation of antheraxanthin and zeaxanthin.

Cladodes of *Opuntia stricta* are subject to sub-zero winter temperatures during the early morning and are thought to decrease photon use by an inhibition of Calvin cycle enzymes and a reduction in membrane fluidity which prevents malic acid efflux and decarboxylation (Barker et al., 1998). Therefore, when low temperatures coincide with high incident PFD, the increased probability of photoinhibition is clear. Under these conditions, the cladodes exhibited significant reductions in photochemical efficiency, which was linked to xanthophyll cycle activity and whereby approximately 80% absorbed energy was diverted through energy dissipation (Barker et al., 1998). North-facing cladodes retained a high proportion of de-epoxidized xanthophylls overnight, thereby effectively creating a system which is poised for thermal dissipation at the onset of adverse conditions the following morning (Maxwell et al., 1995). With increasing temperature, a greater proportion of light energy was used photochemically, coincident with decarboxylation events (Barker et al., 1998). Very similar responses have been observed with cladodes of *O. macrorhiza* growing in North America (Barker and Adams, 1997) and are likely to be typical for a number of CAM species subject to combined light and chill stresses.

Epiphytes are subject to large diurnal and seasonal variations in the light environment which necessitate key, rapid adjustments in the photosynthetic apparatus, despite extreme resource limitation. Mature leaves may experience a 60% increase in integrated PFD within 5–6 days and an extreme response is predicted if species are to succeed within the epiphytic niche (Maxwell et al., 1992, 1994, 1995). Figure 9 provides a summary of the photoprotective strategies employed by the C₃-CAM epiphytic bromeliad *Guzmania monostachia* over a diurnal course during the dry season in Trinidad, 1995. Net CO₂ assimilation rates are characteristically low and photon use during the middle of the day is supplemented by the reversible induction of CAM (Fig. 9b). Despite high photorespiratory activity, photon use still only accounted for 10% absorbed light energy. With such low photosynthetic activity, photoinhibition is predicted, although PS II was maintained in an oxidized state, manifested as a high level of photochemical quenching ($qP = 0.596$ at 12.30). Photoprotection was mediated by exceptionally high levels of thermal dissipation coupled to zeaxanthin formation (Fig. 9d).

In summary, effective photoprotection requires a

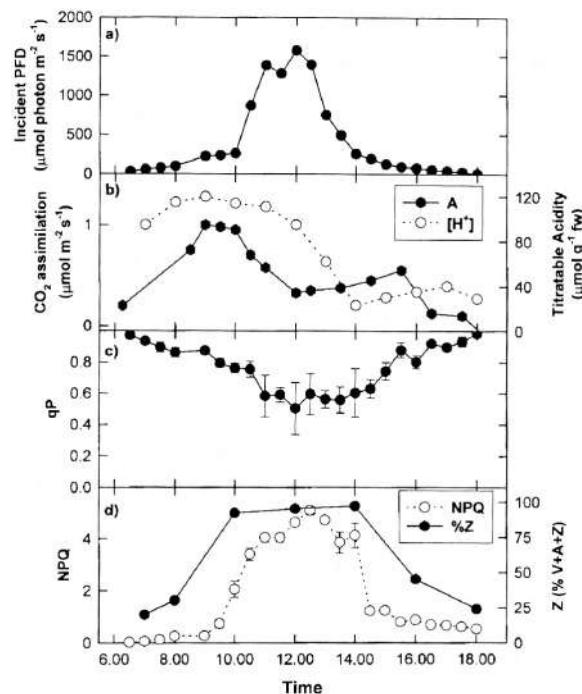


Fig. 9. Measurements of incident light (a), net CO₂ assimilation and organic acid decarboxylation (b) in leaves of the epiphytic bromeliad, *Guzmania monostachia*. Changes in photochemical (c) and non-photochemical quenching of chlorophyll fluorescence together with changes in zeaxanthin content (d) are also shown (K. Maxwell, unpublished).

regulated interplay between absorbed light energy use and dissipation, particularly in CAM plants, whereby CO₂ supply, pCO₂ and Rubisco activity varies considerably both over the diurnal course, imposed by the photosynthetic pathway and in response to environmental variables.

V. Seasonal Integration of CAM Performance and Productivity

A. Seasonal Changes in Constitutive CAM Expression

The dynamic shifts in carboxylation processes described above serve to illustrate the range of biochemical and molecular mechanisms which fine-tune carbon gain to fluctuations in the environment. However, it is important that laboratory-based manipulations are viewed in the ecophysiological context by considering seasonal and longer-term changes in the expression of CAM. Water is the key environmental variable limiting CO₂ uptake by desert

succulents and long-term water use efficiencies integrated over an entire year can range from 0.016 $\text{CO}_2/\text{H}_2\text{O}$ in *Agave deserti* to 0.005 $\text{CO}_2/\text{H}_2\text{O}$ in *Opuntia basilaris* (Szarek and Ting, 1974; Nobel, 1976). Despite the seasonal and annual variations in precipitation in many of the worlds deserts, under natural conditions values of carbon isotope discrimination (Δ) for stem succulents such as agaves and cacti are remarkably conserved, ranging from only 2–10 ‰ (Griffiths, 1992). Such observations may be related to low rates of CO_2 uptake during Phases II and IV (in comparison to leaf succulents), which may be abolished under severe drought. Under more mesic conditions, the proportion of day-time CO_2 uptake may increase in stem succulents, but any effect on Δ has been shown to be masked by a concomitant increase in dark CO_2 fixation (Griffiths, 1992). Some desert succulents can experience severe drought when successive years without rainfall lead to CAM-idling. Such long-term responses are possible because of the remarkable tissue tolerance to water loss in cacti and agaves. Some species can lose 80–90% of their water content and still survive, sometimes up to six years without water in the case of the cactus *Copiapoa cinerea* (Nobel, 1988). In such situations, the cycling of carbon between the C_3 and C_4 pathways behind closed stomata serves to maintain photosynthetic integrity and a positive carbon balance at the expense of growth.

Aside from deserts, the other major biome for CAM species is the tropical rainforest, which tends to conjure an image of relatively stable environmental conditions. However, many seasonal forest formations are subject to increasing drought and light intensity as the dry season progresses, with concomitant changes in temperature and vapor pressure deficit. This combination of ‘stresses’ can enhance the expression of CAM in constitutive CAM species; the extreme atmospheric bromeliad *T. usneoides* increases investment of leaf proteins in PEPC and PCK in response to long-term drought/high light (R. Haslam, H. Griffiths and A. M. Borland, unpublished). In many leaf succulents of the genus *Kalanchoë*, rather than switching to CAM-idling in conditions of severe drought, dark CO_2 uptake is maintained since leaf water status can be preserved through the mobilization of water reserves from the abscission of older leaves (Kluge and Brulfert, 1996).

B. Seasonal Carbon Gain in Facultative CAM Plants

Facultative CAM comprises both annual and perennial species which tend to use the C_3 pathway to establish high rates of growth at times of sufficient water supply but switch to CAM as a means of reducing water loss while maintaining photosynthetic integrity during dry periods. Measurements of carbon-isotope discrimination (Δ) in leaf organic material are commonly used to identify and quantify the contribution of CAM to seasonal carbon gain within plant populations (Griffiths, 1992). The unprecedented range of CAM expression which can be provoked by environmental manipulations for different *Clusia* species for example is generally reflected in leaf Δ taken from plants growing in their natural habitats (Popp et al., 1987; Franco et al., 1994). Thus Δ can range from 6‰ in *C. rosea* growing in dry seasonal forest where CO_2 uptake by PEPC dominates photosynthetic carbon metabolism to 20‰ in *C. aripoensis* growing in moist montane rainforest where most CO_2 uptake is taken up by Rubisco over the annual cycle of leaf growth (Popp et al., 1987; Borland et al., 1992). However, organic Δ is dominated by structural material which comprises at least 50% of leaf dry weight (Borland et al., 1994). Thus, if leaves flush during periods of adequate rainfall, the deposition of structural material will be dominated by a C_3 signal which may obscure the later contributions from C_4 carboxylation, which in the case of species such as *C. minor* ($\Delta = 20\text{\textperthousand}$) can be substantial (Borland et al., 1993).

A more direct assessment of the relative contributions of C_3 photosynthesis and CAM in a facultative CAM species is provided by the elegant studies of Zottz and Winter (1993b; 1996). Direct measurements of leaf gas exchange at intervals over an entire growing season demonstrated that CAM dominated carbon gain in epiphytic individuals of *C. uvitana*. In contrast, while dark CO_2 uptake contributed significantly to diel carbon gain in hemi-epiphytic plants on most days throughout the year, day-time photosynthesis usually exceeded night-time uptake. From this, the authors concluded that light was the main climatic factor determining carbon gain and expression of CAM in hemi-epiphytic plants while water availability was the critical factor in determining CAM expression in epiphytic plants (Zottz and Winter, 1996). These studies also revealed a close correlation

between maximum instantaneous rates of net CO_2 uptake and total 24 h carbon gain in *C. uvitana*, as has been found for C_3 plants (Zotz and Winter, 1993a). Extending this approach to a range of sympatric C_3 and CAM epiphytes growing in a moist semi-evergreen rainforest indicated that annual carbon gain and long term nitrogen use efficiency were similar for all the species although long-term water use efficiency in the CAM species was more than twice as high as in the two C_3 epiphytes investigated (Zotz and Winter, 1994).

While the studies of Zotz and Winter would tend to reinforce theoretical considerations that the energetic costs associated with CAM are at most only 10% higher than those attributed to C_3 photosynthesis (Winter and Smith, 1996), there can be occasions when under extreme and unpredictable environmental conditions in nature, CAM might represent a liability in terms of plant carbon balance. Field investigations of a sympatric weak CAM and constitutive CAM species of *Clusia* growing on sand dune formations in Rio de Janeiro revealed that after a particularly prolonged dry season with record high temperatures, the CAM species became deciduous while the weak CAM species remained green (Roberts et al., 1996). This surprising observation might reflect the energetic costs and requirements for large pools of carbohydrates committed to the CAM pathway. More recently, the generation of constitutive CAM mutants of *M. crystallinum* in which the juvenile C_3 growth phase is abolished, suggest that the extremely slow growth rate of these mutants reflects the costs associated with the CAM pathway (Adams et al., 1998).

A variation of C_3 -CAM intermediacy is exhibited by plants showing CAM-cycling where diel fluctuations in malate are found but all uptake of external CO_2 occurs during the day. The rapid induction or enhancement of CAM-cycling serves to recapture increasing amounts of respiratory CO_2 at night under conditions of increasing drought (Borland and Griffiths, 1990, 1992; Martin, 1996). Indeed an increase in the extent of respiratory CO_2 fixation appears to be a common first step during CAM induction in a number of diverse facultative species (Borland and Griffiths, 1996). Given that the leaf organic Δ of CAM-cycling species are typically in the C_3 range, it would appear that C_4 carboxylation is of minor importance to long-term carbon balance. A model for the partitioning of photosynthetically fixed carbon in source leaves was employed to consider the ecophysiological significance of CAM-cycling

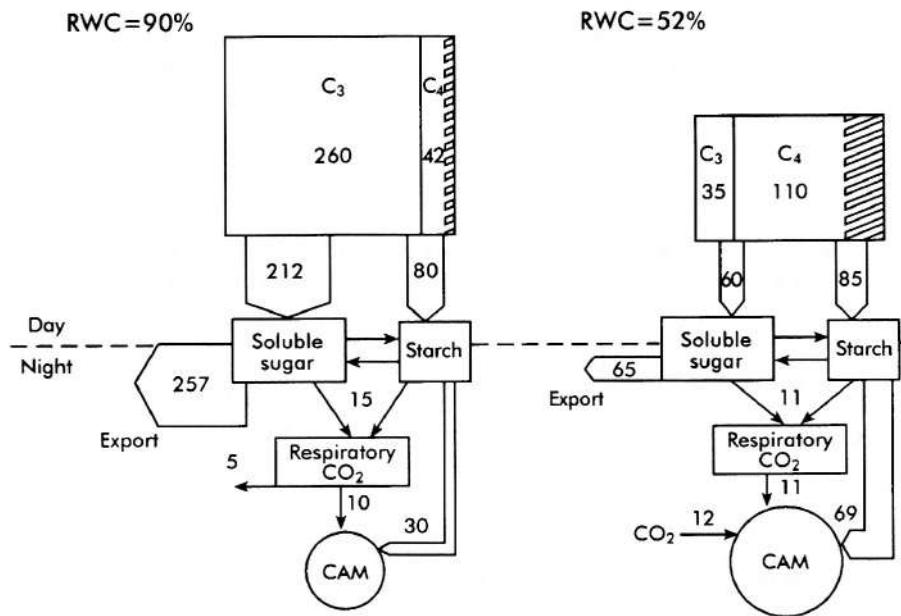
in *S. telephium* by assessing the extent to which CAM is induced or enhanced at the expense of growth (Fig. 10; Borland, 1996). The resulting carbon budgets demonstrate the key role for carbohydrate partitioning in the C_3 -CAM transition and subsequent magnitude of CAM expression. Starch degradation increases at least twofold with severe drought stress, with the flux of carbon into starch maintained at the expense of the soluble sugar pool. Moreover, under high PFD, the enhancement of CAM-cycling in *S. telephium* conserves water while permitting continued export of carbon and growth, albeit at a reduced rate under conditions of severe drought (Fig. 9). Under low PFD however, the induction of CAM-cycling serves mainly as a maintenance mechanism whereby water is conserved and a positive carbon balance is preserved by recycling carbon skeletons at the expense of export and growth during periods of severe water stress.

C. CAM and Reproductive Output

For constitutive species, CAM is undoubtedly crucial for growth, survival and reproductive success although relatively few studies have assessed the direct contribution from carbon fixed via PEPC to the construction of reproductive tissues. However, for the prickly pear cactus, *O. ficus-indica*, fruits can exhibit CAM activity, in contrast to the predominantly C_3 -like flower buds and young cladodes (Ingleses et al., 1994). For this species, nocturnal CO_2 uptake per unit area of fruit averaged 20% of that of the cladodes for 15 to 45 days after flowering before the activities of PEPC and Rubisco declined in line with fruit chlorophyll content. Thereafter fruit growth was supported by both the bearing and surrounding cladodes.

It might be supposed that in constitutive CAM species and C_3 -CAM shifts which are developmentally programmed, such as that exemplified by the well studied *M. crystallinum*, CAM has evolved as a means of partitioning available resources to maximize growth and reproductive output in habitats which are usually seasonally predictable with regard to water supply (Sayed and Hegazy, 1994). Evidence to support this hypothesis was provided by Winter and Zeigler (1992) who showed that, under controlled conditions, salt-stressed plants of *M. crystallinum* which were prevented from fixing CO_2 at night, produced only 10% of the number of seeds produced by plants performing CAM. It was also demonstrated

a) High PFD



b) Low PFD

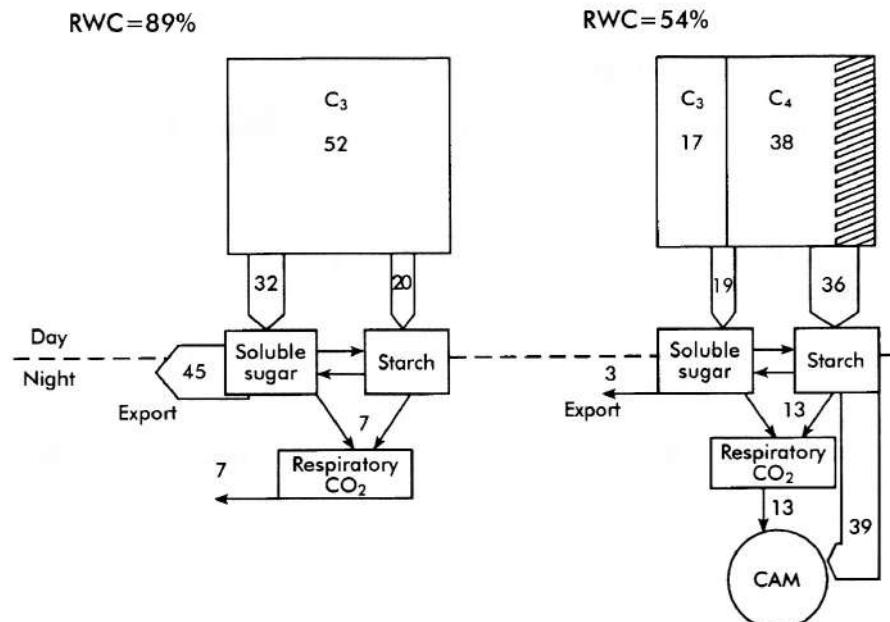


Fig. 10. Diel carbon budgets for shoots of the C_3 -CAM intermediate *Sedum telephium* grown under a) high PFD- $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ or b) low photon flux density— $75 \mu\text{mol m}^{-2} \text{s}^{-1}$ and subjected to extended drought stress, as reflected by leaf relative water contents (RWC). Units are $\text{mmol C m}^{-2} \text{d}^{-1}$. (See Borland, 1996 for full description of model parameters).

that for *M. crystallinum*, C₄ carboxylation makes a direct and substantial contribution to carbon gain in leaves and reproductive structures as determined from carbon isotope discrimination in organic material (Winter and Ziegler, 1992).

A comparison of organic Δ of mature fruits and leaves of the C₃-CAM intermediate *C. minor* and the constitutive CAM *C. rosea* taken from the field indicate distinct partitioning of C₃ and C₄-derived assimilates between vegetative and reproductive growth (A. M. Borland, unpublished). Thus, while the Δ of leaf structural material reflected the relatively greater contribution from carbon fixed by PEPC to seasonal carbon gain in *C. rosea* (12%) compared to *C. minor* (17%), structural material of the fruits from both species showed similar and C₃-like discrimination (18%). Such observations are in line with suggestions that the existence of a transport pool of sugars, synthesized predominantly from Rubisco-mediated carboxylation during Phase IV, will ensure a predominantly C₃ isotopic signature for sugars imported to sinks, regardless of the degree of CAM (Borland et al., 1994). Thus, the unpredictability of resource availability which accompanies the hemi-epiphytic life style of *Clusia* species suggests that while a capacity for the modification of CAM activity undoubtedly maximizes growth and survival, the complete reversibility of such processes and the maintenance of C₃ carboxylation potential throughout these metabolic shifts ultimately ensures reproductive success.

D. Productivity Indices and a Changing Environment

Despite the requirement for maintaining two carboxylation systems and a reciprocating pool of carbohydrates, annual productivities of some CAM species are amongst the highest recorded for any C₃ plants. Pineapple (*Ananas comosus*) is the only CAM plant that is intensively cultivated as a food crop, being grown over some 60 degrees of latitude and a wide range of environmental conditions. Through the use of trickle irrigation which maximizes the contribution from day-time CO₂ uptake during Phase IV, the growth rate and productivity of these plants can approach 35 Mg ha⁻¹ y⁻¹. The optimal spacing and periodic pruning of *O. ficus-indica* to maintain optimal light interception can produce productivities up to 50 Mg ha⁻¹ y⁻¹, exceeding that of the highest producing C₃ crops and trees (Nobel, 1988).

Productivity represents the cumulative, integrative and interactive effects of water supply, PFD and temperature as they relate to the acquisition and allocation of carbon, as detailed in the preceding sections. Nobel and colleagues have modeled the influences of these environmental factors on the productivity of various constitutive agaves and cacti by the calculation of environmental productivity indices. EPI is the product of water, temperature and PFD indices, each with a maximum value of one when that factor does not limit net CO₂ uptake over 24 h (Nobel, 1988). Thus, EPI ranges from zero when one or more factors abolish net CO₂ uptake to one when net CO₂ uptake is maximal. Commonly, EPI is calculated by monitoring 24 h gas exchange. However, the observations by Zotz and Winter (1993a,b; 1994) that the maximum instantaneous rate of net CO₂ uptake can be equated to total 24 h carbon gain could aid the calculation of EPIs for a wider range of species. More recently, a nutrient index has been incorporated into EPI to quantify the effects of soil elements on net CO₂ uptake (Nobel, 1996). This should provide some much needed information on the influence of particular nutrients on the expression of CAM.

By quantifying how changes in various environmental conditions affect productivity, EPI can have a number of practical applications. With a view to optimizing agronomic practice, Nobel (1985) calculated that by irrigating in winter and early spring to induce a water index of one for each month, annual EPI would increase from 0.42 to 0.52 for *Agave fourcroydes* growing in Yucatan, Mexico. In ecological studies, EPI has been used to estimate the growth of particular species along an elevational transect, to investigate plant competition and to estimate plant age in natural populations by calculating the relative growth expected each year (Nobel, 1988).

In the light of climate change, EPI's have been used to predict both the productivity and geographical regions which CAM plants might successfully exploit (Nobel, 1991). It is generally accepted that over the next century, atmospheric [CO₂] will double alongside changes in temperature and rainfall patterns. In long-term studies with *O. ficus-indica*, doubling [CO₂] resulted in an increased rate of annual dry-matter accumulation of up to 40%, reaching 65 Mg ha⁻¹ y⁻¹, in plots where optimal light interception was maintained by frequent pruning (Nobel and Israel, 1994). Moreover, simulations based on EPI have

indicated that *O. ficus-indica* may presently be effectively cultivated over a substantial fraction of the earth's surface (Nobel, 1991). It is likely that the continuing development of arid and semi-arid regions of the world will result in an even greater area which CAM crops might successfully exploit.

For CAM plants, in which primary CO_2 fixation via PEPC is commonly thought to be CO_2 -saturated at present-day ambient $[\text{CO}_2]$, it might be predicted that nocturnal CO_2 fixation will be little affected by elevated $[\text{CO}_2]$. Thus, any influence of elevated $[\text{CO}_2]$ on dry-matter accumulation might largely be expected as a result of increased day-time fixation via Rubisco (Winter and Englebrecht, 1994). The substantially increased productivity of *O. ficus indica* in elevated CO_2 described above is therefore particularly unexpected for a plant in which over 80% of net diel CO_2 uptake occurs at night. Moreover, given the high rates of photorespiration measured during Phase IV in some CAM species, which may be related to the low internal conductance to CO_2 (Maxwell et al., 1997, 1998), it will be of considerable interest to determine how constitutive and facultative CAM species respond to elevated CO_2 in their natural habitats.

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References

- Adams P, Nelson DE, Yamada S, Chmara W, Jensen RG, Bohnert HJ and Griffiths H (1998) Tansley Review No. 97: Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). *New Phytol* 138: 171–190
- Adams III WW and Osmond CB (1988) Internal CO_2 supply during photosynthesis of sun and shade grown CAM plants in relation to photoinhibition. *Plant Physiol* 86: 117–123
- Adams III WW, Nishida K and Osmond B (1986) Quantum yields of CAM plants measured by photosynthetic O_2 exchange. *Plant Physiol* 81: 297–300
- Adams III WW, Osmond CB and Sharkey TD (1987) Responses of two CAM species to different irradiances during growth and susceptibility to photoinhibition by high light. *Plant Physiol* 83: 213–218
- Adams III WW, Terashima I, Brugnoli E and Demmig B (1988) Comparisons of photosynthesis and photoinhibition in the CAM vine *Hoya australis* and several C_3 vines growing on the coast of eastern Australia. *Plant Cell Environ* 11: 173–181
- Barker DH and Adams III WW (1997) The xanthophyll cycle and energy dissipation in differently oriented faces of the cactus *Opuntia macrorhiza*. *Oecologia* 109: 353–361
- Barker DH, Seaton GR and Robinson SA (1997) Internal and external photoprotection in developing leaves of the CAM plant *Cotyledon orbiculata*. *Plant Cell Environ* 20: 617–624
- Barker DH, Logan BA, Adams III WW and Demmig-Adams B (1998) Photochemistry and xanthophyll cycle-dependent energy dissipation in differently oriented cladodes of *Opuntia stricta* during the winter. *Aust J Plant Physiol* 25: 95–104
- Borland AM (1996) A model for the partitioning of photosynthetically fixed carbon during the $\text{C}_3\text{-CAM}$ transition in *Sedum telephium*. *New Phytol* 134: 433–444
- Borland AM and Griffiths H (1990) The regulation of CAM and respiratory recycling by water supply and light regime in the $\text{C}_3\text{-CAM}$ intermediate *Sedum telephium*. *Funct Ecol* 4: 33–39
- Borland AM and Griffiths H (1992) Properties of phosphoenolpyruvate carboxylase and carbohydrate accumulation in the $\text{C}_3\text{-CAM}$ intermediate *Sedum telephium* L. grown under different light and watering regimes. *J Exp Bot* 43: 353–361
- Borland AM and Griffiths H (1996) Variations in the phases of crassulacean acid metabolism and regulation of carboxylation patterns determined by carbon-isotope discrimination techniques. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 230–249. Springer-Verlag, Berlin
- Borland AM and Griffiths H (1997) A comparative study on the regulation of C_3 and C_4 carboxylation processes in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* and the $\text{C}_3\text{-CAM}$ intermediate *Clusia minor*. *Planta* 201: 368–378
- Borland AM, Griffiths H, Maxwell C, Broadmeadow MSJ, Griffiths NM and Barnes JD (1992) On the ecophysiology of the Clusiaceae in Trinidad: Expression of CAM in *Clusia minor* L. during the transition from wet to dry season and characterization of three endemic species. *New Phytol* 122: 349–357
- Borland AM, Griffiths H, Broadmeadow MSJ, Fordham MC and Maxwell C (1993) Short-term changes in carbon-isotope discrimination in the $\text{C}_3\text{-CAM}$ intermediate *Clusia minor* L. growing in Trinidad. *Oecologia* 95: 444–453
- Borland AM, Griffiths H, Broadmeadow MSJ, Fordham MC and Maxwell C (1994) Carbon-isotope composition of biochemical fractions and the regulation of carbon balance in leaves of the $\text{C}_3\text{-Crassulacean}$ acid metabolism intermediate *Clusia minor* L. growing in Trinidad. *Plant Physiol* 106: 493–501
- Borland AM, Griffiths H, Maxwell C, Fordham MC and Broadmeadow MSJ (1996) CAM induction in *Clusia minor* L. during the transition from wet to dry season in Trinidad: The role of organic acid speciation and decarboxylation. *Plant Cell Environ* 19: 655–664

- Borland AM, Técsi LI, Leegood RC and Walker RP (1998) Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterization and intercellular localization of carboxylation and decarboxylation processes in three species which exhibit different degrees of CAM. *Planta* 205: 342–351
- Carnal NW and Black CC (1989) Soluble sugars as the carbohydrate reserve for CAM in pineapple leaves. Implications for the role of pyrophosphate:6-phosphofructokinase in glycolysis. *Plant Physiol* 90: 91–100
- Carter PJ, Fewson CA, Nimmo GA, Nimmo HG and Wilkins MB (1996) Roles of circadian rhythms, light and temperature in the regulation of phosphoenolpyruvate carboxylase in crassulacean acid metabolism. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 46–52. Springer-Verlag, Berlin
- Castillo FJ (1996) Antioxidative protection in the inducible CAM plant *Sedum album* L following the imposition of severe water stress and recovery. *Oecologia* 107: 469–477
- Christopher JT and Holtum JAM (1996) Patterns of carbon partitioning in leaves of Crassulacean acid metabolism species during deacidification. *Plant Physiol* 112: 393–399
- Christopher JT and Holtum JAM (1998) Carbohydrate partitioning in the leaves of Bromeliaceae performing C3 photosynthesis or Crassulacean acid metabolism. *Aust J Plant Physiol* 25: 371–376
- Cockburn W, Ting IP and Sternberg LO (1979) Relationships between stomatal behaviour and internal carbon dioxide concentration in Crassulacean acid metabolism plants. *Plant Physiol* 63: 1029–1032.
- Cote FX, Andre M, Folliot M, Massimino D and Daguenet A (1989) CO_2 and O_2 exchanges in the CAM plant *Ananas comosus* (L.) Merr. *Plant Physiol* 89: 61–68
- de Mattos EA, Grams TEE, Ball E, Franco AC, Haag-Kerwer A, Herzog B, Scarano FR and Lüttge U (1997) Diurnal patterns of chlorophyll a fluorescence and stomatal conductance in species of two types of coastal vegetation in southeastern Brazil. *Trees* 11: 363–369
- Deléens E and Garnier-Dardart J (1977) Carbon isotope composition of biochemical fractions isolated from leaves of *Bryophyllum daigremontianum* Berger, a plant with crassulacean acid metabolism: Some physiological aspects related to CO_2 dark fixation. *Planta* 135: 241–248
- Deléens E, Garnier-Dardart J and Querioz O (1979) Carbon-isotope composition of intermediates of the starch-malate sequence and level of the crassulacean acid metabolism in leaves of *Kalanchoë blossfeldiana* Tom Thumb. *Planta* 146: 441–449
- Demmig-Adams B and Adams III WW (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends Plant Sci* 1: 21–26
- Eller BM and Ferrari S (1997) Water use efficiency of two succulents with contrasting CO_2 fixation pathways. *Plant Cell Environ* 20: 93–100
- Evans JR and von Caemmerer S (1996) Carbon dioxide fixation in leaves. *Plant Physiol* 110: 339–346
- Evans JR, von Caemmerer S, Setchell BA and Hudson GS (1994) The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. *Aust J Plant Physiol* 21: 475–495
- Fahrendorf T, Holtum JAM, Mukherjee U and Latzko E (1987) Fructose 1,6-bisphosphate, carbohydrate partitioning and crassulacean acid metabolism. *Plant Physiol* 84: 182–187
- Farquhar GD and von Caemmerer S (1982) Modelling of photosynthetic responses to environmental conditions. In: Lange OL, Nobel PS, Osmond CB and Ziegler H (eds) *Plant Physiological Ecology II*, Vol 12B, pp 549–587. Springer-Verlag, Heidelberg
- Fetene M, Lüttge U and Zeigler H (1991) Flexibility in CO_2 fixation pathway of a highland *Kalanchoë*, *Kalanchoë petitiana* A. Rich. *Botanica Acta* 104: 374–378
- Fischer A and Kluge M (1984) Studies on carbon flow in crassulacean acid metabolism during the initial light period. *Planta* 160: 121–128
- Franco AC, Olivares E, Ball E, Lüttge U and Haag-Kerwer A (1994) In situ studies of Crassulacean acid metabolism in several sympatric species of tropical trees of the genus *Clusia*. *New Phytol* 126: 203–211
- Geiger DR and Servaites JC (1994) Diurnal regulation of photosynthetic carbon metabolism in C_3 plants. *Annu Rev Plant Physiol Plant Mol Biol* 45: 235–56
- Gersani M, Graham EA and Nobel PS (1993) Growth responses of individual roots of *Opuntia ficus indica* to salinity. *Plant Cell Environ* 16: 827–834
- Gibeaut DM and Thomson WW (1989) Leaf ultrastructure of *Peperomia obtusifolia*, *P. camptotricha* and *P. scandens*. *Bot Gaz* 150: 108–114
- Gil F (1986) Origin of CAM as an alternative photosynthetic carbon fixation pathway. *Photosynthetica* 20: 494–507
- Gillon JS, Borland AM, Harwood KG, Roberts A, Broadmeadow MSJ and Griffiths H (1998) Carbon dioxide discrimination in terrestrial plants: carboxylations and decarboxylations. In: Griffiths H (ed) Stable isotopes. Integration of Biological, Ecological and Geochemical Processes, pp 111–131. Bios, Oxford
- Goldstein G and Nobel PS (1994) Water relations and low-temperature acclimation for cactus species varying in freezing tolerance. *Plant Physiol* 104: 675–681
- Grams TEE, Borland AM, Roberts A, Griffiths H, Beck F and Lüttge U (1997a) On the mechanism of reinitiation of endogenous crassulacean acid metabolism rhythm by temperature changes. *Plant Physiol* 113: 1309–1317
- Grams TEE, Haag-Kerwer EO, Ball E, Arndt S, Popp M, Medina E and Lüttge U (1997b) Comparative measurements of chlorophyll a fluorescence, acid accumulation and gas exchange in exposed and shaded plants of *Clusia minor* L. and *Clusia multiflora* H.B.K. in the field. *Trees* 11: 240–247
- Griffiths H (1989) Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. In: Lüttge U (ed) *Ecological Studies 76: Vascular Plants as Epiphytes*, pp 42–85. Springer-Verlag, Berlin
- Griffiths H (1992) Carbon isotope discrimination and the integration of carbon assimilation pathways in terrestrial CAM plants. *Plant Cell Environ* 15: 1051–1062
- Griffiths H and Smith JAC (1983) Photosynthetic pathways in the Bromeliaceae of Trinidad: Relations between life-forms, habitat preference and the occurrence of CAM. *Oecologia* 60: 176–184
- Griffiths H, Lüttge U, Stimmel K-H, Crook CE, Griffiths NM and Smith JAC (1986) Comparative ecophysiology of CAM and C_3 bromeliads. III. Environmental influences on CO_2 assimilation and transpiration. *Plant Cell Environ* 9: 385–393

- Griffiths H, Ong BL, Avadhani PN and Goh CJ (1989) Recycling of respiratory CO_2 during crassulacean acid metabolism: Alleviation of photoinhibition in *Pyrrosia piloselloides*. *Planta* 179: 115–122
- Griffiths H, Broadmeadow MSJ, Borland AM and Hetherington CS (1990) Short-term changes in carbon-isotope discrimination identify transitions between C_3 and C_4 carboxylation during crassulacean acid metabolism. *Planta* 181: 604–610
- Haag-Kerwer A, Franco AC and Lütte U (1992) The effect of temperature and light on gas exchange and acid accumulation in the $\text{C}_3\text{-CAM}$ plant *Clusia minor* L. *J Exp Bot* 248: 345–352
- Heber U, Bligny R, Streb P and Douce R (1996) Photorespiration is essential for the protection of the photosynthetic apparatus of C_3 plants against photoinactivation under sunlight. *Bot Acta* 109: 307–315
- Herppich WB, Herppich M, Tüffers A, von Willert DJ, Midgley GF and Veste M (1998a) Photosynthetic responses to CO_2 concentration and photon fluence rates in the CAM-cycling plant *Delosperma tradescantiooides* (Mesembryanthemaceae). *New Phytol* 138: 433–440
- Herppich WB, Midgley GF, Herppich M, Tüffers A, Veste M and von Willert DJ (1998b) Interactive effects of photon fluence rates and drought on CAM-cycling in *Delosperma tradescantiooides* (Mesembryanthemaceae). *Physiol Plant* 102: 148–154
- Holbrook GP, Campbell WJ, Rowland-Bamford A and Bowes G (1994) Intraspecific variation in the light/dark modulation of ribulose 1,5-bisphosphate carboxylase-oxygenase activity in soybean. *J Exp Bot* 45: 1119–1126
- Horton P, Ruban AV and Walters RG (1996) Regulation of light harvesting in green plants. *Annu Rev Plant Physiol Plant Mol Biol* 47: 655–684
- Inglese P, Israel AA and Nobel PS (1994) Growth and CO_2 uptake for cladodes and fruit of the Crassulacean acid metabolism species *Opuntia ficus-indica* during fruit development. *Physiol Plant* 91: 708–714
- Kaul RB (1977) The role of the multiple epidermis in foliar succulence of *Peperomia* (Piperaceae). *Bot Gaz* 138: 213–218
- Kenyon WH, Holaday AS and Black CC (1981) Diurnal changes in metabolite levels and crassulacean acid metabolism in *Kalanchoë daigremontiana* leaves. *Plant Physiol* 68: 1002–1007
- Kluge M and Brulfert J (1996) Crassulacean acid metabolism in the genus *Kalanchoë*: ecological, physiological and biochemical aspects. In: Winter K, Smith JAC (eds) *Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution*. Springer, Berlin pp 324–335
- Kluge M and Ting IP (1978) Crassulacean acid metabolism: Analysis of an ecological adaptation. Springer-Verlag, Berlin
- Lütte U (1987) Carbon dioxide and water demand: Crassulacean acid metabolism (CAM) a versatile ecological adaptation exemplifying the need for integration in ecophysiological work. *New Phytol* 106: 593–629
- Martin CE (1996) Putative causes and consequences of recycling CO_2 via crassulacean acid metabolism. In: Winter K and Smith JAC (eds) *Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution*, pp 192–203. Springer-Verlag, Berlin
- Martin CE, Higley MH and Wang WZ (1988) Recycling of CO_2 via crassulacean acid metabolism in the rock outcrop succulent *Sedum pulchellum* Michx. (Crassulaceae). *Photosynth Res* 18: 337–343
- Maxwell C, Griffiths H, Borland AM, Broadmeadow MSJ and McDavid CR (1992) Photoinhibitory responses of the epiphytic bromeliad *Guzmania monostachia* during the dry season in Trinidad maintain photochemical integrity under adverse conditions. *Plant Cell Environ* 15: 37–47
- Maxwell C, Griffiths H and Young AJ (1994) Photosynthetic acclimation to light regime and water stress by the $\text{C}_3\text{-CAM}$ epiphyte *Guzmania monostachia*; gas-exchange characteristics, photochemical efficiency and the xanthophyll cycle. *Funct Ecol* 8: 746–754
- Maxwell C, Griffiths H, Borland AM, Young AJ, Broadmeadow MSJ and Fordham MC (1995) Short-term photosynthetic responses of the $\text{C}_3\text{-CAM}$ epiphyte *Guzmania monostachia* var. *monostachia* to tropical seasonal transitions under field conditions. *Aust J Plant Physiol* 22: 771–781
- Maxwell K, von Caemmerer S and Evans JR (1997) Is a low internal conductance to CO_2 diffusion a consequence of succulence in plants with crassulacean acid metabolism? *Aust J Plant Physiol* 24: 777–786
- Maxwell K, Badger MR and Osmond CB (1998) A comparison of CO_2 and O_2 exchange patterns and the relationship with chlorophyll fluorescence during photosynthesis in C_3 and CAM plants. *Aust J Plant Physiol* 25: 45–52
- Mayoral ML, Medina E and Garcia V (1991) Effect of source-sink manipulations on the crassulacean acid metabolism of *Kalanchoë pinnata*. *J Exp Bot* 42: 1123–1129
- Medina E and Delgado M (1976) Photosynthesis and night CO_2 fixation in *Echeveria columbiana* v. Poell nitz. *Photosynthetica* 10: 155–163
- Miszalski Z, Slesak I, Niewiadomska E, Baczk-Kwinta R, Lütte U and Ratajczak R (1998) Subcellular localization and stress response of superoxide dismutase isoforms from leaves in the $\text{C}_3\text{-CAM}$ intermediate halophyte *Mesembryanthemum crystallinum* L. *Plant Cell Environ* 21: 169–179
- Murphy R and Smith JAC (1994) A critical comparison of the pressure-probe and pressure-chamber techniques for estimating leaf-cell turgor pressure in *Kalanchoë daigremontiana*. *Plant Cell Environ* 17: 15–29
- Murphy R and Smith JAC (1998) Determination of cell water-relation parameters using the pressure probe: Extended theory and practice of the pressure-clamp technique. *Plant Cell Environ* 21: 637–657
- Nimmo GA, Nimmo HG, Fewson CA, Wilkins MB (1984) Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves: A possible covalent modification. *FEBS Lett* 178: 199–203
- Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA, Wilkins MB (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi*. *Biochem J* 239: 213–220
- Nimmo, GA, Wilkins MB, Fewson CA, Nimmo HG (1987) Persistent circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi* leaves and its sensitivity to inhibition by malate. *Planta* 170: 408–415
- Nishio J and Ting IW (1987) Carbon flow and metabolic specialisation in the tissue layers of the CAM plant *Peperomia camptotricha*. *Plant Physiol* 84: 600–604
- Nobel PS (1976) Water relations and photosynthesis of a desert

- CAM plant, *Agave deserti*. *Plant Physiol* 58: 576–582
- Nobel PS (1985) PAR, water and temperature limitations on the productivity of cultivated *Agave fourcroydes* (Henequen). *J Appl Ecol* 22: 157–173
- Nobel PS (1988). Environmental Biology of Agaves and Cacti. Cambridge University Press, Cambridge
- Nobel PS (1991) Environmental productivity indices and productivity for *Opuntia ficus-indica* under current and elevated atmospheric CO₂ levels. *Plant Cell Environ* 14: 637–646
- Nobel PS (1996) High productivity of certain agronomic CAM species. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 255–265. Springer-Verlag, Berlin
- Nobel PS and Hartsock TL (1983) Relationships between photosynthetically active radiation, nocturnal acid accumulation and CO₂ uptake for a crassulacean acid metabolism plant, *Opuntia ficus-indica*. *Plant Physiol* 71: 71–75
- Nobel PS and Israel AA (1994) Cladode development, environmental responses of CO₂ uptake and productivity for *Opuntia ficus-indica* under elevated CO₂. *J Exp Bot* 45: 295–303
- North GB and Nobel PS (1998) Water uptake and structural plasticity along roots of a desert succulent during prolonged drought. *Plant Cell Environ* 21: 705–713
- Olivares E and Medina E (1990) Carbon dioxide exchange, soluble carbohydrates and acid accumulation in a fructan accumulating plant: *Fourcroya humboldtiana* Treal. *J Exp Bot* 41: 579–585
- Osmond B, Maxwell K, Popp M and Robinson S (1999) On being thick: Fathoming apparently futile pathways of photosynthesis and carbohydrate metabolism in succulent CAM plants. In: Burrel M, Bryant J and Kruger N (eds) Carbohydrate Metabolism in Plants, pp 183–200. BIOS, Oxford
- Osmond CB (1978) Crassulacean acid metabolism: A curiosity in context. *Annu Rev Plant Physiol* 29: 379–414
- Osmond CB (1982) Carbon cycling and the stability of the photosynthetic apparatus in CAM. In: Ting IP and Gibbs M (eds) Crassulacean Acid Metabolism, pp 128–153. American Society of Plant Physiologists, Rockville
- Osmond CB, Popp M and Robinson SA (1996) Stoichiometric nightmares: Studies in photosynthetic O₂ and CO₂ exchanges in CAM plants. In: Winter K and Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution, pp 19–30. Springer-Verlag, Berlin
- Osmond CB, Badger MR, Maxwell K, Björkman O and Leegood RC (1997) Too many photons: Photorespiration, photo-inhibition and photooxidation. *Trends Plant Sci* 2: 119–121
- Parry MAJ, Andraloic PJ, Parmar S, Keys AJ, Habash D, Paul MJ, Aired R, Quick WP and Servaites JC (1997) Regulation of Rubisco by inhibitors in the light. *Plant Cell Environ* 20: 528–534
- Pittendrigh CS (1948) The bromeliad-Anopheles-malaria complex in Trinidad. I. The bromeliad flora. *Evolution* 2: 58–89
- Popp M, Kramer D, Lee H, Diaz M, Zeigler H and Luttge U (1987) Crassulacean acid metabolism in tropical trees of the genus *Clusia*. *Trees* 1: 238–247
- Portis AR (1995) The regulation of Rubisco by Rubisco activase. *J Exp Bot* 46: 1285–1291
- Roberts A, Griffiths H, Borland AM and Reinert F (1996) Is crassulacean acid metabolism activity in sympatric species of hemi-epiphytic stranglers such as *Clusia* related to carbon cycling as a photoprotective process? *Oecologia* 106: 28–38
- Roberts A, Borland AM and Griffiths H (1997) Discrimination processes and shifts in carboxylation during the phases of crassulacean acid metabolism. *Plant Physiol* 113: 1283–1291
- Roberts A, Borland AM, Maxwell K and Griffiths H (1998) Ecophysiology of the C₃-CAM intermediate *Clusia minor* L. in Trinidad: Seasonal and short-term photosynthetic characteristics of sun and shade leaves. *J Exp Bot* 49: 1563–1573
- Robinson SA, Lovelock CE and Osmond CB (1993a) Wax as a mechanism for protection against photoinhibition—a study of *Cotyledon orbiculata*. *Botanica Acta* 106: 307–312
- Robinson SA, Osmond CB and Giles L (1993b) Interpretations of gradients in δ¹³C value in thick photosynthetic tissues of plants with Crassulacean acid metabolism. *Planta* 190: 271–276
- Sage RF, Reid CD, Moore BD and Seemann JR (1993) Long-term kinetics of the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in plants with and without 2-carboxyarabinitol 1-phosphate. *Planta* 191: 222–230
- Sayed OH and Hegazy AK (1994) Growth-specific phytomass allocation in *Mesembryanthemum nodiflorum* as influenced by CAM induction in the field. *J Arid Environ* 27: 325–339
- Schulte PJ, Smith JAC and Nobel PS (1989) Water storage and osmotic pressure influences on the water relations of a dicotyledonous desert succulent. *Plant Cell Environ* 12: 831–842
- Seemann JR, Kobza J and Moore BD (1990) Metabolism of 2-carboxyarabinitol 1-phosphate and regulation of ribulose-1,5-bisphosphate carboxylase activity. *Photosynth Res* 23: 119–130
- Servaites JC, Parry MAJ, Gutteridge S and Keys AJ (1986) Species variation in the predawn inhibition of Ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol* 82: 1161–1163
- Skillman JB and Winter K (1997) High photosynthetic capacity in a shade-tolerant Crassulacean acid metabolism plant. *Plant Physiol* 113: 441–450
- Smith JAC and Heuer S (1981) Determination of the volume of intercellular spaces in leaves and some values for CAM plants. *Ann Bot* 48: 915–917
- Smith JAC and Nobel PS (1986) Water movement and storage in a desert succulent: Anatomy and rehydration kinetics for leaves of *Agave deserti*. *J Exp Bot* 37: 1044–1053
- Smith JAC, Griffiths H, Lütte U, Crooks CE, Griffiths NM and Stimmel K-H (1986) Comparative ecophysiology of CAM and C₃ bromeliads. IV. Plant water relations. *Plant Cell Environ* 9: 395–410
- Smith JAC, Schulte PJ and Nobel PS (1987) Water-flow and water storage in *Agave deserti*—osmotic implications of crassulacean acid metabolism. *Plant Cell Environ* 10: 639–648
- Spalding MH, Stumpf DK, Ku MSB, Burris RH and Edwards GE (1979) Crassulacean acid metabolism and diurnal variations of CO₂ and O₂ concentrations in *Sedum praealtum* DC. *Aust J Plant Physiol* 6: 557–567
- Sutton BG (1975) The path of carbon in CAM plants at night. *Aust J Plant Physiol* 2: 377–387
- Szarek SR and Ting IP (1974) Seasonal patterns of acid metabolism and gas exchange in *Opuntia basilaris*. *Plant Physiol* 54: 76–81

- Teeri JA, Tonsor SJ and Turner M (1981) Leaf thickness and carbon isotope discrimination in the Crassulaceae. *Oecologia* 50: 367–369
- Thomas DA and André M (1987) Oxygen and carbon dioxide exchanges in crassulacean acid metabolism plants: I. Effect of water stress on hourly and daily patterns. *Plant Physiol Biochem* 25: 85–93
- Ting IP, Patel A, Sipes DL, Reid PD and Walling LL (1994) Differential expression of photosynthesis genes in leaf tissue layers of *Peperomia* as revealed by tissue printing. *Am J Bot* 8: 414–422
- Verbücheln O and Steup M (1984) Carbon metabolism and malate formation in the CAM plant *Aloe arborescens*. *Adv Photosynth Res* 3: 421–123
- von Caemmerer S and Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153: 376–387
- Vu V, Allen LH and Bowes G (1984) Dark/light modulation of ribulose bisphosphate carboxylase activity in plants from different photosynthetic categories. *Plant Physiol* 76: 843–845
- Winter K and Demmig B (1987) Reduction state of Q and nonradiative energy dissipation during photosynthesis in the leaves of crassulacean acid metabolism plant, *Kalanchoë daigremontiana* Hamet et Perr. *Plant Physiol* 85: 1000–1007
- Winter K and Engelbrecht B (1994) Short-term CO_2 responses of light and dark CO_2 fixation in the Crassulacean acid metabolism plant *Kalanchoë pinnata*. *J Plant Physiol* 144: 462–467
- Winter K and Smith JAC (1996) Crassulacean acid metabolism: Current status and perspectives. In: Winter K, Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution. Springer, Berlin pp 389–426
- Winter K and Ziegler H (1992) Induction of crassulacean acid metabolism in *Mesembryanthemum crystallinum* increases reproductive success under conditions of drought and salinity stress. *Oecologia* 97: 475–479
- Winter K, Wallace BJ, Stocker GC and Roksandic Z (1983) Crassulacean acid metabolism in Australian vascular epiphytes and some related species. *Oecologia* 57: 129–141
- Winter K, Lesch M and Diaz M (1990) Changes in xanthophyll-cycle components and in fluorescence yield in leaves of a crassulacean-acid-metabolism plant, *Clusia rosea* Jacq., throughout a 12-hour photoperiod of constant irradiance. *Planta* 182: 181–185
- Yakir D, Ting I and DeNiro M (1994) Natural abundance $^{2}\text{H}/^{1}\text{H}$ ratios of water storage in leaves of *Peperomia congesta* HBK during water stress. *J Plant Physiol* 144: 607–612
- Zotz G and Winter K. (1993a) Short-term photosynthesis measurements predict leaf carbon balance in tropical rainforest canopy plants. *Planta* 191: 409–412
- Zotz G and Winter K (1993b) Short-term regulation of Crassulacean acid metabolism activity in a tropical hemiepiphyte, *Clusia uvitana*. *Plant Physiol* 102: 835–841
- Zotz G and Winter K (1994) Annual carbon balance and nitrogen-use efficiency in tropical C₃ and CAM epiphytes. *New Phytol* 126: 481–192
- Zotz G and Winter K (1996) Seasonal changes in daytime versus nighttime CO_2 fixation of *Clusia uvitana* in situ. In: Winter K, Smith JAC (eds) Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution. Springer, Berlin, pp 312–323

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