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Plasticity in light reactions of photosynthesis for energy production and photoprotection

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Abstract

Plant photosynthesis channels some of the most highly reactive intermediates in biology, in a way that captures a large fraction of their energy to power the plant. A viable photosynthetic apparatus must not only be efficient and robust machinery, but also well integrated into the plant's biochemical and physiological networks. This requires flexibility in its responses to the dramatically changing environmental conditions and biochemical demands. First, the output of the energy-storing light reactions must match the demands of plant metabolism. Second, regulation of the antenna must be flexible to allow responses to diverse challenges that could result in excess light capture and subsequent photoinhibition. Evidence is presented for the interplay of two types of mechanistic flexibility, one that modulates the relative sensitivity of antenna down-regulation to electron flow, and the other, which primarily modulates the output ratio of ATP/NADPH, but also contributes to down-regulation.

Key words: ATP synthase, cyclic electron transfer, proton motive force, q_E quenching, water–water cycle.

Photosynthesis and its down-regulation

Light is captured by a set of light-harvesting complexes (LHCs) that funnel light energy into photochemical reaction centres, photosystem (PS) I and PSII (Fig. 1) (see review by Ort and Yocum, 1996). Special subsets of chlorophyll molecules in these photosystems are excited by light energy, allowing electrons on them to be transferred through a series of redox carriers called the electron transfer chain (ETC), beginning from the oxygen evolving complex (OEC) of PSII (which oxidizes H_2O and releases O_2 and protons) (Diner and Babcock, 1996), through the plastoquinone (PQ) pool, the cytochrome (cyt) b_6f complex (Sacksteder *et al.*, 2000) and plastocyanin (PC), and finally through PSI (Malkin, 1996). Electrons from PSI are transferred to ferredoxin (Fd), which, in turn, reduces $NADP^+$ to NADPH via ferredoxin:$NADP^+$ oxidoreductase (FNR) (Knaff, 1996). This linear electron flux (LEF) to $NADP^+$ is coupled to proton release at the OEC, and 'shuttling' of protons across the thylakoid membrane by the PQ pool and the Q-cycle at the cyt b_6f complex, which establishes an electrochemical potential of protons, or proton motive force (*pmf*) that drives the synthesis of ATP by chemiosmotic coupling through the chloroplast ATP synthase (McCarty, 1996; Mitchell, 1966).

Following the absorption of photons by chlorophyll, the transfer of excitons to reaction centre chlorophyll and the initiation of electron transfer must be well regulated to prevent 'over-excitation' of the photosystems (i.e. more excitation than can be processed by the reaction centres), which favours the formation of highly reactive species and photoinhibition of the photosynthetic machinery (Anderson and Barber, 1996; Kramer and Crofts, 1996). In general, overexcitation of PSII is prevented largely by antenna down-regulation, which dissipates excess excitation energy

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Abbreviations: CEF1, cyclic electron flow around PSI; cyt, cytochrome; ΔpH, transthylakoid pH gradient; $\Delta\psi$, transthylakoid electric field; DIRK, Dark Interval Relaxation Kinetics; ECS, electrochromic shift; ECS_{inv}, inverted ECS; ECS_{ss}, steady-state ECS; ECS_t, full extent of the ECS decay; ETC, electron transfer chain; Fd, ferredoxin; FNR, ferredoxin:$NADP^+$ oxidoreductase; g_{H^+}, proton conductivity; H^+/e^-, proton to electron stoichiometry; LEF, linear electron flux; LHCs, light-harvesting complexes; NPQ, non-photochemical exciton quenching; OEC, oxygen evolving complex; PC, plastocyanin; *pmf*, proton motive force; PQ, plastoquinone; PS, photosystem; q_E, energy-dependent quenching; SOD, superoxide dismutase; τ_{ECS}, ECS decay time; VDE, violaxanthin deepoxidase; WWC, water–water cycle.

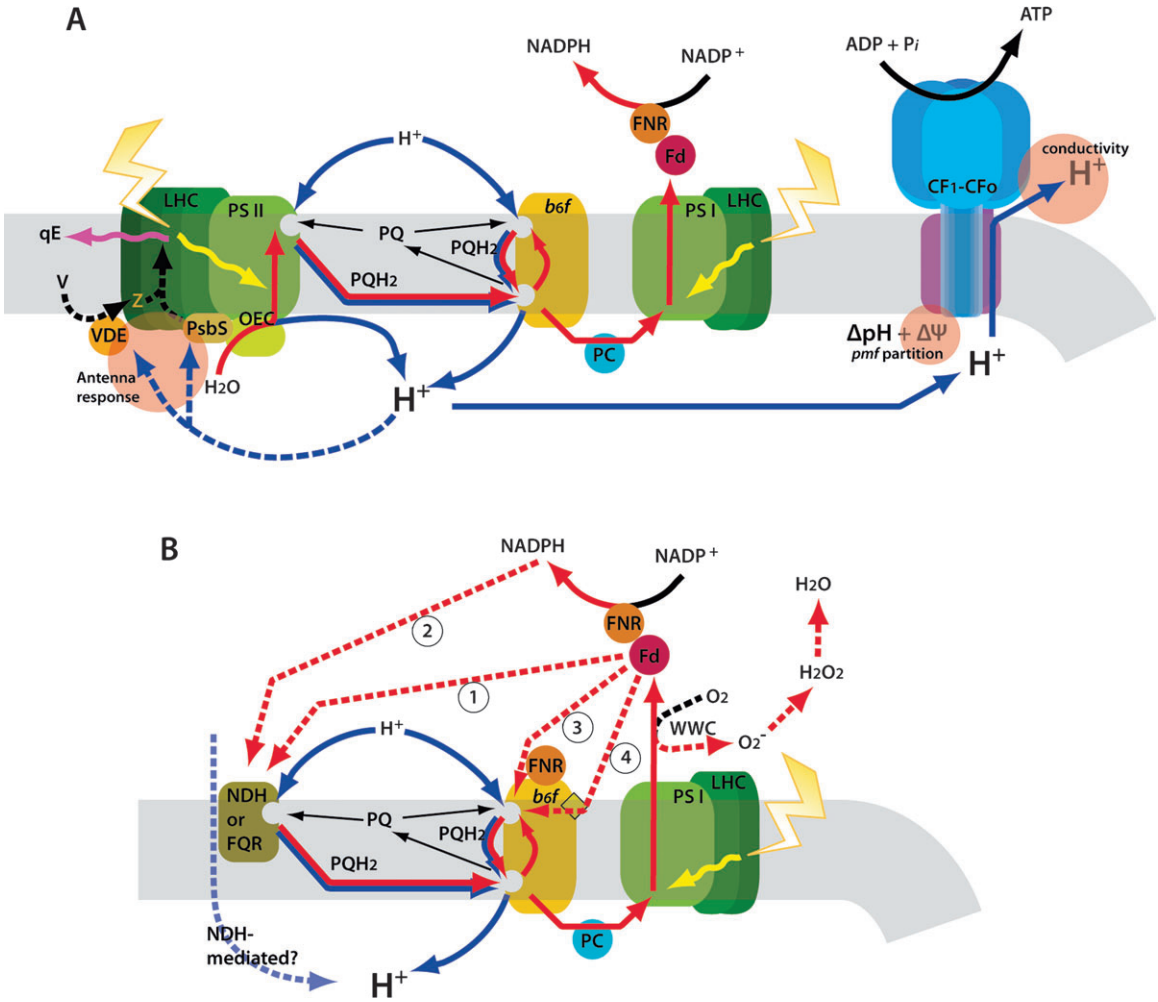


Fig. 1. Primary routes of proton/electron flux and mechanisms of Type I and II flexibility. (A) Energy storage begins with the absorption of light energy (lightning bolts) by light-harvesting complexes (LHC) associated with photosystem (PS) II and I, respectively. Depicted is the linear electron flux (LEF, red arrows) of electrons derived from the oxidation of H_2O at the oxygen evolving complex (OEC) through PSII reducing sequentially plastoquinone (PQ) to a quinol (PQH_2). Bifurcated oxidation of PQH_2 occurs at the cytochrome b_6f complex (b_6f) where half of the electrons are linearly transferred to the $NADP^+$/NADPH couple via plastocyanin (PC), PSI, ferredoxin (Fd), and ferredoxin-$NADP^+$ oxidoreductase (FNR), and the other half of the electrons will return to the PQH_2 pool. Proton flux (blue arrows) originates from H_2O splitting at the OEC and the cyclic reduction and oxidation of PQ/PQH_2, establishing an electrochemical gradient of protons across the thylakoid membrane (*pmf*), comprised of pH (ΔpH) and electric field (Δψ) components. Total *pmf* drives ATP synthesis from ADP and P_i as protons move down their electrochemical gradient through the CF_1-CF_O ATP synthase. Energy dissipation by q_E (purple arrow) is pH-dependent due to the pH-dependent activity of violaxanthin de-epoxidase (VDE), which sequentially reduces violaxanthin (V) to zeaxanthin (Z), and protonation of PsbS. Type II mechanisms (highlighted in red) involve variability in: (i) the response of the antenna to lumen pH, (ii) the conductive properties of the ATP synthase, and (iii) the relative partitioning of *pmf* into Δψ and ΔpH. Type I mechanisms (B) involve alternate routes of electron transfer at the reducing side of PSI, including the water–water cycle (WWC) and cyclic electron flow around PSI (CEF1). The WWC uses the same electron transfer pathways as normal LEF except at the reducing side of PSI it reduces O_2 to O_2^- which is subsequently detoxified to H_2O. As depicted, four carrier pathways have been proposed for the cycling of electrons from PSI back to the PQ pool (CEF1): (1) a ferredoxin-PQ oxidoreductase (FQR), (2) a NADPH-PQ oxidoreductase (NDH), (3) oxidation of Fd by a FNR/b_6f super complex, and (4) oxidation of, for example, Fd by a newly discovered haem associated with the stromal side of the b_6f complex.

as heat. This involves a series of processes, which are collectively termed non-photochemical exciton quenching (NPQ) and typically measured by the quenching of chlorophyll *a* fluorescence (reviewed by Maxwell and Johnson, 2000). Under most physiological conditions, the major form of NPQ is termed q_E, for the 'quenching' of light energy in the antenna that is dependent on the 'energization' of the thylakoid membrane (reviewed by Horton *et al.*, 1996; Müller *et al.*, 2001; Owens, 1996; Yamamoto and Bassi, 1996). Activation of q_E involves at least two processes (Fig. 1): (i) the conversion of the xanthophyll carotenoid violaxanthin to antheraxanthin and zeaxanthin, catalysed by violaxanthin deepoxidase (VDE) (Eskling *et al.*, 2001); and (ii) protonation of amino acid side-chains on an antenna-associated, chlorophyll binding protein, PsbS (Li *et al.*, 2004). Both of these processes are activated by acidification of the lumen by the ΔpH component of *pmf*. In this analysis, the proton gradient is considered to equilibrate across the entire continuous lumenal space, i.e. it is not necessary to invoke proton domains to explain

these data. Thus, *pmf* not only drives the synthesis of ATP, but is also a key signal for feedback regulation of the light reactions.

The need for modulation of down-regulatory sensitivity (q_E-modulation)

q_E sensitivity is defined as the responsiveness of q_E to LEF, because both parameters are readily and frequently measured using chlorophyll fluorescence measurements. Alternatively, under most conditions NPQ may be substituted for q_E, since q_E makes up a significant fraction of NPQ. If the light reactions behaved in a static fashion, q_E sensitivity would be constant, i.e. q_E would be a continuous function of LEF. However, such rigidity in down-regulation of the photosynthetic apparatus would leave it prone to catastrophic failure (Asada, 1996; Heber and Walker, 1992; Kanazawa and Kramer, 2002). For example, if photosynthesis became limited by the lack of PSI electron acceptors, as might be expected under conditions of metabolic stress, LEF and its proton pumping will be attenuated. A static model would predict a decrease in q_E, precisely under the conditions where photoprotection is needed most to prevent the build-up of reduced intermediates, which could lead to 'acceptor side' photoinhibition (Anderson *et al.*, 1997). Clearly, a more flexible model must be invoked to account for the response of antenna regulation to the fluctuating physiological status of the plant (Horton, 1989; Horton *et al.*, 1999).

Indeed, such flexibility has been demonstrated in C_3 plants (Avenson *et al.*, 2004; Kanazawa *et al.*, 2001; Kanazawa and Kramer, 2002). Rather than a continuous relationship, as the static model would predict, a series of distinct curves was observed, with q_E becoming increasingly more sensitive to LEF as $[CO_2]$ was lowered (Kanazawa and Kramer, 2002). Physiologically, this is desirable because the availability of PSI electron acceptors, and thus overall LEF, is expected to decrease with decreasing CO_2; to maintain reasonable levels of photoprotection, q_E should become more sensitive to LEF.

The need to balance ATP/NADPH ratios

With LEF to $NADP^+$, ATP synthesis and NADPH production are coupled, and within a static model the output ratio of ATP to NADPH would be fixed. However, this would work only in a system where consumption of ATP and NADPH occurs at the same fixed ratio; that is, their relative consumption by chloroplast metabolism (including fixation of carbon, nitrogen, phosphorus, and sulphur) and other plastid maintenance processes continuously matches output by energized thylakoids. Yet each individual process imposes a different demand for ATP/NADPH. Again, this leaves a static model susceptible to failure in cases where differential flux is required to respond to the changing demands on the chloroplast. If shortage of a single metabolite decreases relative metabolic flux through the pathway that fixes it, then the demand for ATP versus NADPH may change. Also, the resulting mismatch between production and consumption ratios would create 'back pressure' on the light reactions from excess product (ATP or NADPH) or lack of substrate ($ADP+P_i$, $NADP^+$), sensitizing the photosynthetic apparatus to photoinhibition. Therefore, contrary to a static model, a certain measure of flexibility in the LEF output ratio is expected in order to compensate for changes in demand.

The need for balancing mechanisms is further exemplified by potential mismatch between the LEF-dependent ouput and the demand of CO_2 fixation. If one considers only LEF, the ATP/NADPH ratio is defined by the proton coupling stoichiometries for the ETC (H^+/e^-) and that for ATP synthesis (H^+/ATP, termed *n*) (Allen, 2002; Kramer *et al.*, 2003). There is strong evidence that H^+/e^- for LEF remains at 3 under physiological conditions (Sacksteder *et al.*, 2000). New information about the structure and mechanism of the ATP synthase implies that *n* is likely to be 4.67 (reviewed by Allen, 2002; Kramer *et al.*, 2003). With these stoichiometries, ATP/NADPH should be 1.3, which as discussed later, would provide insufficient ATP to support CO_2 fixation in C_3 plants. Without flexible responses, even larger supply–demand mismatch would occur in species using modified CO_2 fixation strategies, for example, in plants with some types of C_4 photosynthesis.

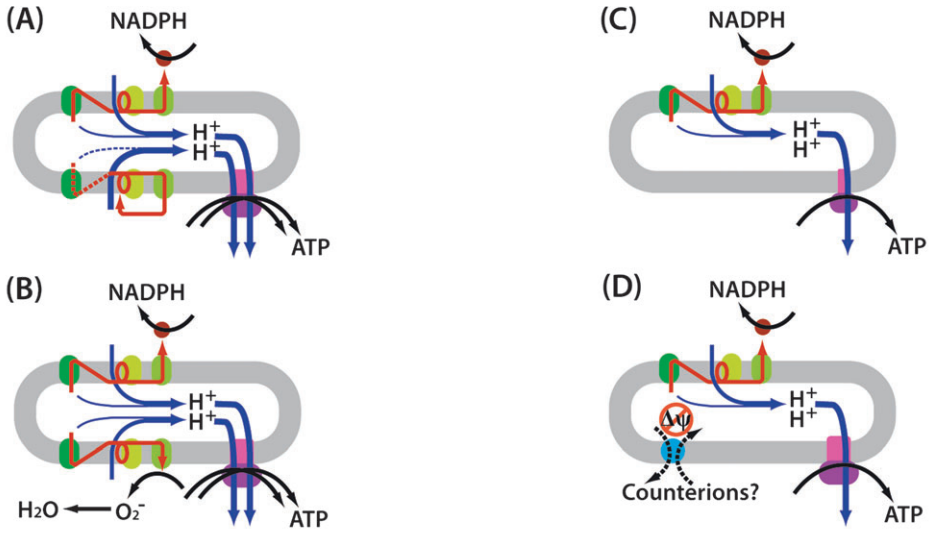
As discussed in (Kanazawa and Kramer, 2002) and extended here, there are several models that could, together or separately, account for q_E modulation (Fig. 1), some of which will also affect the output ratio of ATP/NADPH, and these were termed Type I flexibility mechanisms. Other mechanisms will have no effect on the ATP/NADPH output ratio, and these were termed Type II flexibility mechanisms. This distinction is critical for understanding the relative roles of these processes.

Type I: Flexibility mechanisms affecting the ATP/NADPH ratio

In accordance with the general model for electron and proton transfer, any process increasing the rate of proton translocation into the lumen will tend to activate q_E by increasing *pmf*. If such processes supplement proton flux supplied by LEF, they will increase q_E sensitivity as it has been defined here. They will also tend to increase the ATP/NADPH ratio, because the resulting increase in proton flux will drive more ATP synthesis (Fig. 2), without a net increase in the reduction of $NADP^+$. There have been several proposals for this type of mechanism.

Changes in the H^+/e^- ratio for LEF

The Q-cycle is a catalytic mechanism which couples electron transfer through the cyt b_6f complex to the



	NADPH	*ATP*	*pmf*	*ΔpH*	*qE / LEF*
(A) ↑ CEF1	*NC*	↑	↑	↑	↑
(B) ↑ WWC	*NC*	↑	↑	↑	↑
(C) ↓ gH^+	*NC*	*NC*	↑	↑	↑
(D) ↓ Δψ/pmf	*NC*	*NC*	*NC*	↑	↑

Fig. 2. Relationships between energy-transduction and q_E sensitivity. As determined by its sensitive components, PsbS and VDE, q_E (and thus NPQ) will be a function of lumenal pH. As pH drops from ~6.5 to ~5.8, q_E will continuously increase to saturation. If the steady-state pH of the stroma is constant, then q_E will be a function of ΔpH. Therefore, factors affecting the extent to which ΔpH forms will influence q_E induction. Depicted are simplified schematics of chloroplastic energy transduction with proton and electron fluxes indicated in blue and red, respectively. The table indicates relative changes in ATP output, NADPH output, *pmf*, and ΔpH (NC indicates no change). The *pmf* (and by extension ΔpH) will depend, in part, on the steady-state rate of proton accumulation. Supplementing the rate of proton accumulation through CEF1 (A) or WWC (B) will increase *pmf*, the rate of proton efflux and, consequentially, the rate of ATP synthesis. However, since electrons on the reducing side of PSI return to the PQ/PQH_2 pool via CEF1 or to water via WWC, NADPH output does not change. Since at steady-state, the rate of efflux will equal the rate of accumulation, *pmf* will also depend on how conductive the membranes are to proton flux. Thus, decreasing conductivity (C) will require an increase in *pmf* to balance proton accumulation with efflux. Since the steady-state rate of proton flux does not change in proportion to electron flux to NADPH, the relative outputs of ATP and NADPH remain constant. Finally, if, under most conditions the ΔpH partition is approximately 50% of *pmf*, collapsing the electric field component through counterion movements (D) would require an increase in ΔpH to sustain steady-state proton flux. In all cases, the sensitivity of q_E to LEF (q_E/LEF) increases.

translocation of protons from the stroma to the lumen (reviewed by Kurisu *et al.*, 2003; Sacksteder *et al.*, 2000). For each electron transferred through LEF, one proton is released into the lumen from water oxidation, and one proton is taken up during PQ reduction at the Q_B site of PSII and released when PQH_2 is oxidized at the Q_o site of the cyt b_6f complex. An additional proton is translocated by the Q-cycle, making the overall H^+/e^- stoichiometry for LEF 3. Although several authors have proposed that the Q-cycle is facultative (reviewed by Berry and Rumberg, 1999; Cornic *et al.*, 2000; Ivanov, 1993; Kramer and Crofts, 1993; Sacksteder *et al.*, 2000), disengaging it (see review by Sacksteder *et al.*, 2000) would lower the H^+/e^- ratio to 2, thereby lowering the *pmf* generated by LEF and, consequently, the ATP/NADPH output ratio and q_E sensitivity.

On the other hand, *in vitro* mechanistic studies of the cyt b_6f complex indicated that the Q-cycle was very likely obligatory (Kramer and Crofts, 1993; Rich, 1988). Furthermore, comparisons of estimated fluxes of protons with LEF and with electron flux through the cyt b_6f complex *in vivo* suggested a constant H^+/e^- ratio from low to saturating light intensities (Sacksteder *et al.*, 2000). It was concluded that the Q-cycle is ptobably continuously engaged under normal, non-stressed photosynthetic conditions.

These arguments are bolstered by recent structural studies of the mitochondrial cyt bc_1 complexes (Zhang *et al.*, 1998) (which are homologous to the chloroplast cyt b_6f complex) and cyt b_6f complexes (Kurisu *et al.*, 2003; Stroebel *et al.*, 2003) which have led to proposals that the 'Rieske' iron-sulphur protein 'gates' electron transfer by undergoing large-scale conformational changes during catalysis, essentially forcing the complex to shuttle protons via the Q-cycle (reviewed by Roberts *et al.*, 2001; Zhang *et al.*, 1998). It was concluded that there is a strong

mechanistic basis for a constant H^+/e^- ratio at the cyt b_6f complex and that differential engagement of the Q-cycle probably does not account for ATP/NADPH balancing or for variable sensitivities of down-regulatory processes.

Alternate electron transfer cycles

Various light-driven cyclic electron transfer pathways have been proposed to translocate protons across the thylakoid and thus drive ATP production or initiate q_E in the absence of $NADP^+$ reduction. Two of the pathways, cyclic electron flow around PSI (CEF1) and the water-water cycle (WWC), have gained support in recent years and are discussed here.

Cyclic electron flux around PSI

CEF1 bypasses the photosynthetic Z-scheme by involving only one of the two photosystems, PSI. Light excites PSI, resulting in reduction of its FeS centres and oxidation of its primary chlorophyll donor, P_{700}. Just as in LEF, the oxidized P_{700}^+ is reduced by electrons from the PQ pool, via the cyt b_6f complex and PC (Bendall and Manasse, 1995). Electrons on the reducing side of PSI eventually reduce PQ, completing the cycle. There is no net reduction of Fd or $NADP^+$ but flux of electrons through the cycle will translocate protons to the lumen, resulting in *pmf*, which can drive ATP synthesis and activate q_E.

At least four pathways have been proposed to link the reducing side of PSI with the PQ pool (Fig. 1B, paths 1–4). First, a linkage may occur via a ferredoxin-PQ oxidoreductase (FQR) (path 1), a pathway that has been shown to be sensitive to antimycin A (Bendall and Manasse, 1995). Recently, Shikanai and coworkers (Munekage *et al.*, 2002) identified an *Arabidopsis* mutant, *pgr5*, lacking antimycin A-sensitive Fd reduction of the PQ pool, preliminary evidence that the PGR5 gene product may be involved in FQR-mediated CEF1. Second, an enzyme homologous to complex I of mitochondria and bacteria (Edwards and Walker, 1983; Kubicki *et al.*, 1996), NAD(P)H-PQ oxidoreductase (NDH) (path 2), has been suggested to be involved in CEF1, but its role *in vivo* remains ambiguous, as suggested by deletion studies under normal (Endo *et al.*, 1999; Horvath *et al.*, 2000) and stress conditions (Barth and Krause, 2002; Sazanov *et al.*, 1998). However, evidence presented by Shikanai and coworkers (Munekage *et al.*, 2004) suggests that paths 1 and 2 act in parallel. Third, Cramer and co-workers (Zhang *et al.*, 2001) suggested that, based on the copurification of FNR with the b_6f complex, an FNR/b_6f super complex (path 3) may operate in a third type of CEF1, a pathway that has been verified *in vitro* (Zhang *et al.*, 2001) but the details of which remain unresolved (Kramer, 1990). Lastly, based on recent structures of the b_6f complex (Kurisu *et al.*, 2003; Stroebel *et al.*, 2003), an unexpected haem group in between b_H and the stromal phase has been identified, hinting at a role for this extra haem in mediating electron transfer from the reducing side of PSI (path 4), although a physiological pathway has yet to be identified (Stroebel *et al.*, 2003). While several potential PQ reduction pathways have been identified, none can be established as dominant, nor can specific roles for the individual pathways be identified. Indeed, it is possible that the PQ reductase activities serve functions other than photosynthetic (Sazanov *et al.*, 1998).

In vivo *estimates of CEF1 rates*

Care must be taken before accepting *in vitro* rates as reflecting those that can occur *in vivo*, especially since CEF1 is known to be well-regulated and a measurable change in its relative rate may appear only under special conditions. There is strong evidence for participation of CEF1 in ATP synthesis in green algae (e.g. *Chlamydomonas*) and cyanobacteria (Depege *et al.*, 2003; Finazzi *et al.*, 2002), as well as in C_4 plant bundle sheath chloroplasts (Kubicki *et al.*, 1996). However, the situation in C_3 vascular plants is clearly unresolved, with the bulk of the evidence pointing to only minor contributions of CEF1 under steady-state conditions.

Several groups have estimated CEF1 rates in C_3 vascular plants under steady-state conditions. These measurements are difficult because the electrons flow in a cycle, and no readily measurable, stable products are formed. One approach to indicate the activation of CEF1 is to estimate steady-state transthylakoid ΔpH using pH-indicator dyes, or the onset of q_E with LEF. The argument is that at a given LEF, CEF1 will increase *pmf*, thereby decreasing lumen pH, and thus increasing q_E (Cornic and Briantais, 1991; Heber, 2002). However, it is argued below that such effects can equally result from the engagement of Type II mechanisms, which have been shown to alter the relationship between LEF and steady-state *pmf*, as well as between *pmf* and q_E.

Most commonly, the relative fluxes of electrons through different parts of the electron transfer chain are compared to estimate the relative engagements of LEF and CEF1. In steady-state LEF, the rates of electron transfer through PSII should equal that through PSI (Genty *et al.*, 1990; Klughammer and Schreiber, 1994; Kramer and Crofts, 1996; Ort and Baker, 2002) or the cyt b_6f complex (Klughammer and Schreiber, 1994; Sacksteder and Kramer, 2000). The engagement of CEF1 should increase electron flux through PSI over that through PSII. Likewise, the ratio of proton translocation to LEF should increase with the engagement of CEF1 (Sacksteder *et al.*, 2000). The fraction of overall photosynthetic energy storage attributable to PSII will change with the engagement of CEF1 (Herbert *et al.*, 1990). Unfortunately, each of these techniques measures CEF1 only as a fraction of LEF and is only sensitive to changes in the ratio of CEF1:LEF (Bendall and Manasse, 1995; Kramer and Crofts, 1996), and low rates are not readily detected. A number of studies using such assays have found little evidence for changes in the fractional turnover of

CEF1 in steady states as conditions were altered, and thus the general consensus appears to be that, in C_3 vascular plants, CEF1 is either negligible or a fairly constant fraction of steady-state LEF (Genty *et al.*, 1990; Herbert *et al.*, 1990; Klughammer and Schreiber, 1994; Kramer and Crofts, 1996; Ort and Baker, 2002; Sacksteder and Kramer, 2000). On the other hand, in more recent papers other groups have reported substantial rates of CEF1 (15–100% of LEF) during photosynthetic induction (Joliot and Joliot, 2002) or anaerobiosis (Joet *et al.*, 2001) or under high light, low temperature conditions (Clarke and Johnson, 2001) or drought stress (Golding and Johnson, 2003).

The water-water cycle (WWC)

In the WWC, electrons extracted from H_2O by PSII are transferred through the ETC to PSI, where O_2 acts as the terminal acceptor forming superoxide (O_2^-, Fig. 1B, WWC). O_2^- is dismutated to hydrogen peroxide and dioxygen, a reaction that is catalysed by superoxide dismutase (SOD), and the hydrogen peroxide is reduced to H_2O by ascorbate peroxidase, thereby completing the cycle. Since O_2 is reduced more slowly by Fd than FNR, the WWC has been proposed to operate to a higher extent when concentrations of $NADP^+$ are low. Although the WWC produces no net reductant, it does generate *pmf*, which may serve to drive ATP synthesis or to initiate down-regulation (Asada, 1996).

Because it shares nearly all reactions with LEF, the WWC is very difficult to distinguish from LEF (Heber, 2002) and thus it is not surprising that issues concerning the relative contribution of the WWC to overall electron transfer have not yet been resolved. Much of the literature (Foyer and Noctor, 2000; Heber, 2002) suggests that, *in vivo*, the WWC is a relatively minor contributor to LEF. An estimate based on a survey of more recent work (Badger *et al.*, 2000) suggests that, at most, WWC operates at 10% of LEF of C_3 photosynthesis, even under conditions of extreme stress. Moreover, others have observed little to no WWC under conditions that should favour NADPH accumulation, such as lowered RUBISCO levels (Ruuska *et al.*, 2000) or low temperatures (Clarke and Johnson, 2001).

By contrast, higher flux capacities for WWC have been observed in isolated chloroplasts of C_3 plants (Backhausen *et al.*, 2000; Badger *et al.*, 2000), suggesting that conditions which favour WWC may not be simple to produce *in vivo*. However, there is evidence for the active engagement of the WWC in conjunction with CEF1 in rice leaves, during photosynthetic induction (Makino *et al.*, 2002). It was suggested that the supplemental proton flux was required to generate additional ATP for the initiation of the Calvin–Benson cycle from a dark-adapted state. Furthermore, suppressed expression of thylakoid-associated Cu/Zn-SOD in *Arabidopsis* suppressed photosynthetic activity and growth, which is consistent with the need for detoxification of O_2^- generated by photosynthesis (Rizhsky *et al.*, 2003). While this observation supports the presence of the WWC *in vivo*, it does not necessarily support a role for the WWC supplementing *pmf* during steady-state photosynthesis.

Type II: Flexibility without altering ATP/NADPH output ratio

While Type I mechanisms could be modulators of q_E, effective engagement would require them to comprise a large fraction of total electron flux, leading to mismatch in supply and demand for ATP and NADPH. By contrast, Type II mechanisms, as depicted in Fig. 1, allow the regulation of q_E sensitivity without perturbing the ATP/NADPH ratio.

Alteration of q_E response to lumen pH

One way to alter q_E sensitivity would be to change the response capacity of q_E to lumen pH. Over developmental time-scales, the differential accumulation of antenna and xanthophyll components has been shown to alter q_E sensitivity (Demmig-Adams and Adams III, 1996). Hypothetically, more dynamic changes in q_E sensitivity could occur though alterations in the pH response of the molecular components of q_E. For example, covalent modification of VDE or PsbS could shift either pH dependence of VDE or pK_as of protonatable groups on PsbS, respectively. Alternatively, components in the membrane could be modified, affecting the propensity of LHCs to aggregate or associate with the xanthophyll components, processes which have been linked to exciton dissipation by q_E (reviewed by Horton *et al.*, 1996). The predicted outcome, in all cases, would be a range of sensitivities of q_E to ΔpH. However, in tobacco, a constant relationship was observed between q_E and estimates of light-driven *pmf* changes, over conditions where q_E sensitivity was substantially altered by changing CO_2 levels (Kanazawa and Kramer, 2002), while under extreme acceptor limiting conditions q_E was a continuous function of ΔpH (Avenson *et al.*, 2004). These observations suggest that a constant relationship exists between lumen pH and q_E and that modifications in antenna response do not account for short-term changes in q_E sensitivity, under these conditions.

The importance of pmf *composition for modulating q_E response*

Since q_E is triggered by the ΔpH, but not the $\Delta\psi$ (electric field) component of thylakoid *pmf*, one way to change q_E sensitivity would be to alter the manner in which *pmf* is stored. The chemisomotic mechanism, first described by Peter Mitchell, states that *pmf* is thermodynamically composed of the sum of the ΔpH and $\Delta\psi$ potentials (Mitchell, 1966). Many of the earlier characterizations of *pmf* were performed by monitoring ATP synthesis in intact thylakoids

as a function of ΔpH produced by pH jump and/or by measuring ΔpH-dependent uptake of radiolabelled or fluorescent amines (Davenport and McCarty, 1986; Junesch and Gräber, 1985; Schuldiner *et al.*, 1972). While useful for defining the thresholds of activation and other energetic parameters, these studies ignored and actively suppressed the Δψ component of *pmf* through the use of uncouplers and/or relatively high concentrations of counterions. Direct measurements of Δψ, made using salt-filled microelectrodes (Vredenberg and Tonk, 1975), helped to popularize the notion that it contributed little or negligibly to steady-state *pmf*, despite changes observed *in vivo* in the electrochromic shift (ECS) (Finazzi and Rappaport, 1998; Joliot and Joliot, 1989; Sacksteder *et al.*, 2000) or measurement of Δψ-dependent ATP synthesis (Hangarter and Good, 1982; Junesch and Gräber, 1991), which suggested the contrary. Lately, it has been argued that under permissive conditions, it is unlikely that ΔpH solely comprises *pmf* (reviewed in Cruz *et al.*, 2001; Kramer *et al.*, 1999). In essence, a ΔpH requirement of 2–3 to activate ATP synthesis (Kramer and Crofts, 1989) yields a lumen pH that is inconsistent with the pH sensitivities of PSII and PC and with the pH-dependent rates of VDE and cyt b_6f, observed *in vivo*.

In much of the authors' recent work, the ECS has been exploited as an endogenous probe for changes in transthylakoid Δψ during light-to-dark transitions (Avenson *et al.*, 2004; Cruz *et al.*, 2001; Kanazawa and Kramer, 2002; Sacksteder *et al.*, 2000). The relevance of the ECS to *pmf* was first reported by Junge and Witt (1968). ECS refers to a Δψ-induced 'shift' in the absorption spectrum of pigments (i.e. chlorophyll and carotenoids) embedded in the thylakoid membrane. The peak of the difference spectrum occurs at 515–520 nm and has been shown to be a linear indicator of the strength of the transthylakoid Δψ (Witt and Zickler, 1973). One particular advantage of using the ECS is that it is non-invasive, allowing *in vivo* measurements on intact leaves. Generally, two techniques were employed when using ECS to probe *pmf*, both of which are variations of Dark Interval Relaxation Kinetic (DIRK) analysis (Sacksteder and Kramer, 2000). The DIRK technique uses brief (<500 ms) dark intervals to create reproducible perturbations in steady-state electron and proton fluxes. The initial rate of the ECS decay has been attributed to proton flux through the ATP synthase (Kramer and Crofts, 1989), and initial rates have been argued to reflect steady-state LEF or proton flux linearly (Sacksteder *et al.*, 2000). From steady-state conditions, these rapid ECS decay kinetic traces are fit to monoexponential decays (Fig. 3A), giving decay times (τ_{ECS}) inversely proportional to proton conductivity (g_{H^+}) across the membrane (i.e. predominantly through the ATP synthase). The full extent of the decay (ECS_t) should be proportional to the light-induced, steady-state *pmf* (Avenson *et al.*, 2004; Kanazawa and Kramer, 2002; Sacksteder and Kramer, 2000).

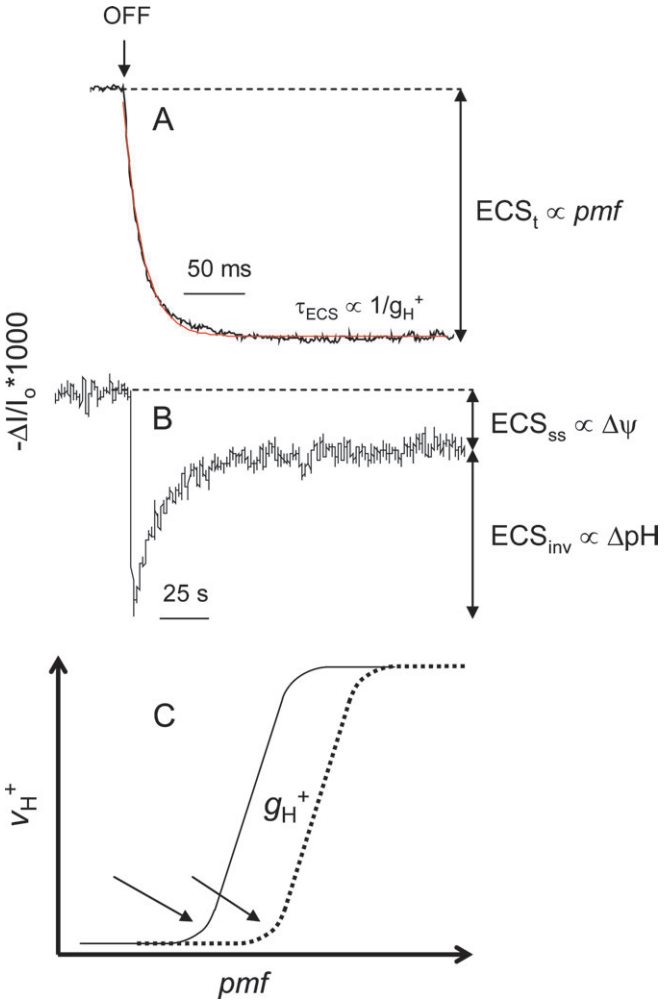


Fig. 3. DIRK analysis of the ECS estimates *pmf* and its relative partitioning into Δψ and ΔpH. Tobacco leaves from intact plants were clamped into the measuring chamber of the spectrophotometer, which was purged with a stream of water-saturated air. Following 15 min of actinic illumination with 520 μmol of photons m^{-2} s^{-1} red light, ECS decay kinetic traces were collected for 300 ms (A) and 120 s (B) dark intervals. Over the 300 ms time-scale the ECS signal predominates and the extent of the decay estimates light-induced *pmf*, or ECS_t. The time constant for decay of the signal (τ_{ECS}), estimated from a first order fit (red line) of the data (r^2=0.99) is inversely proportional to the proton conductivity of the ATP synthase (g_{H^+}). Over longer periods of analysis (i.e. minutes), light scattering contributes more significantly to apparent absorbance changes at 520 nm, necessitating deconvolution of the ECS kinetic trace (as described in Kramer and Sacksteder, 1998). From the deconvoluted trace, arbitrary amplitudes for Δψ (ECS_{ss}) and ΔpH (ECS_{inv}) may be derived from the extents of the decay from steady-state to the dark baseline (ECS_{ss}) and from the dark baseline to full extent of the ECS inversion (ECS_{inv}), respectively. (C) depicts the 'ohmic' relationship between force (*pmf*) and flux (v_{H^+}). Flux increases linearly with force, with a slope equal to the conductivity, g_{H^+}, when the force is above the threshold of activation (arrows). With tight coupling of flux and ATP synthesis, v_{H^+} is equivalent to the H^+/ATP ratio, *n*, multiplied by the ATP synthesis rate. Increasing the threshold of activation (as happens with oxidation of the ATP synthase γ disulphide (Junesch and Gräber, 1985) or with an increase in ΔG_{ATP} (Hangarter and Good, 1982)) does not change the conductivity above activation (dotted line).

With longer dark intervals (i.e. minutes), following the rapid, initial decay the ECS relaxes to a dark level (Fig. 3B). Determination of the dark baseline allows tentative separation of $\Delta\psi$- and ΔpH-driven decays of *pmf* (Cruz *et al.*, 2001). Since the ECS is a linear indicator of $\Delta\psi$, the extent of its decay from steady-state to baseline (ECS_{ss}) should be proportional to the light-induced $\Delta\psi$. However, the ECS continues to decay below baseline, indicating 'inversion' of the electric field with respect to steady-state levels. This effect arises from the continued efflux of protons from the lumen, driven by ΔpH (and beyond that driven by $\Delta\psi$ alone). Since the ECS decay should continue until ΔpH essentially reaches equilibrium with the inverted $\Delta\psi$, the extent to which ECS drops below baseline (ECS_{inv}) should be proportional to the light-induced ΔpH, at least under appropriate conditions (Cruz *et al.*, 2001). The sum of the amplitudes for ECS_{ss} and ECS_{inv} (i.e. ECS_t) should be proportional to the light-induced *pmf*. Using this 'partition analysis', the fraction of *pmf* stored as $\Delta\psi$ (or ΔpH) may be expressed as the ECS_{ss} (or ECS_{inv}) divided by ECS_t.

Modulation of pmf by proton conductivity

Above the threshold of activation for ATP synthesis, a linear relationship exists between *pmf* and proton efflux until the ATP synthase pool reaches the maximum rate of turnover (Hangarter and Good, 1982; Junesch and Gräber, 1985; Kramer and Crofts, 1989). The slope of this relationship, g_{H^+}, is a measure of the response of transthylakoid efflux to changes in driving force (Fig. 3C). It is important to note that changing g_{H^+} will not change the steady-state rate of H^+ efflux at a given LEF, but it will change the *pmf* required to sustain this rate (Fig. 2). Since q_E should be a continuous function of *pmf*, provided that ΔpH is a constant fraction of *pmf*, q_E sensitivity could be modulated by changes in g_{H^+}. Indeed, correlative changes in q_E sensitivity and g_{H^+} have been observed in tobacco. Through DIRK analysis of the ECS, large decreases in g_{H^+} were observed as CO_2 levels were lowered from 2000 to nearly 0 ppm with coincident increases in q_E sensitivity (Kanazawa *et al.*, 2001; Kanazawa and Kramer, 2002). Further analysis suggested that essentially all changes in q_E sensitivity could be explained by modulation of g_{H^+} alone.

Variable partitioning of pmf

Variable *pmf* partitioning allows a more flexible relationship to exist between steady-state ATP synthesis and q_E. For example, increasing the fraction ΔpH at a given *pmf* will increase the q_E response without affecting the ATP synthetic rate (Fig. 2). Such effects would be important in cases where the physiological status of the leaf requires q_E to be large, but LEF is too low to support a large enough *pmf*. Variable *pmf* partitioning will lead to discontinuities in relationships between *pmf* and q_E as well as LEF and q_E, and recently, this effect was observed in tobacco leaves under low O_2 and low CO_2 (Avenson *et al.*, 2004). Partition analysis of the ECS kinetics indicated a relative increase in the fraction of *pmf* stored as ΔpH, suggesting that variable partitioning of *pmf* was responsible for enhancing the sensitivity of q_E. Furthermore, decreases in g_{H^+} were observed as well, suggesting that variable *pmf* partitioning and g_{H^+} modulation may act in concert to increase q_E sensitivity.

Previous work (Cruz *et al.*, 2001) suggested that, if the thylakoid membrane contains only passive channels to allow counterions to dissipate $\Delta\psi$, the extent to which *pmf* will be stored as $\Delta\psi$ and ΔpH will depend largely on the proton buffering capacity of the lumen and the concentration of counterions. The collective work of several groups suggest that buffering capacity will depend on the concentration of fixed buffering groups (Ewy and Dilley, 2000; Junge *et al.*, 1979; van Kooten *et al.*, 1986) and that *in vivo* this is unlikely to change to a large extent (Junge *et al.*, 1979; van Kooten *et al.*, 1986). Thus, postulating the existence of active ion transport mechanisms in the thylakoid (e.g. coporters, antiporters, ion-transporting pumps), the most likely mechanism to alter *pmf* partitioning is the concentration of counterions, and it is not difficult to imagine that these could be regulated in some way *in vivo* to adjust q_E sensitivity.

An integrated model for photosynthetic flexibility

As discussed earlier, without Type I flexibility, LEF would yield a ratio of 1.3 ATP/NADPH (Allen, 2002). As estimated from the combined energy requirements for CO_2 fixation under photorespiring conditions and nitrate assimilation to glutamate, a ratio of ~1.43 ATP/NADPH (Edwards and Walker, 1983) would be needed through photochemistry in the chloroplast, yielding a deficit of about 0.13 ATP per NADPH. The net contributions of Type I mechanisms to increasing relative ATP output will depend on their H^+/e^- coupling stoichiometries. The WWC, which uses essentially the same reactions as LEF, probably produces an H^+/e^- of 3. In the case of CEF1, the H^+/e^- will be partly determined by the pathway for PQ reduction, which is not well understood, especially in C_3 vascular plants. Conservative estimates, based on various models, place the lower-upper bounds for H^+/e^- between 2 and 4. Using these values, the WWC would need to run at a rate of about 12%, or CEF1 between 18% and 9%, that of LEF to fill the ATP deficit between the light reactions and downstream metabolism. While current estimates of WWC and CEF1 capacity are close to and in some cases exceed this requirement, as noted by Makino *et al.* (2002), to balance output it is probable that they run in concert, possibly with other electron sinks such as the malate valve (Fridlyand *et al.*, 1998) or chlororespiration (Cardol *et al.*, 2003) or with mitochondrial respiration (Noctor and Foyer, 1998). Although this contribution may seem small, the impact of Type

I mechanisms on C_3 photosynthesis might be quite significant. Indeed, *Arabidopsis* double mutants for PGR5 and NDH show substantial decreases in capacity for NPQ, probably due to the observed decreases in LEF (and subsequent *pmf* formation), created by an uncorrected imbalance in the supply and demand ratios for ATP and NADPH, leading to metabolic conjestion (Munekage *et al.*, 2004). Thus, these data are consistent with the view presented here, in that the relatively small contributions indirectly affect proton translocation.

Assignment of this specific role to Type I mechanisms is supported by the observed evolutionary adaptations of the photosynthetic apparatus. For example, if carbon fixation is looked at specifically, there is considerable evidence that steady-state CEF1 is small in C_3 vascular plants, where the expected output balance of LEF is close to, but does not precisely match, biochemical demand. The exception is induction of photosynthesis when priming of the carbon cycle might also require additional ATP (Poolman *et al.*, 2003), as inferred from high CEF1 rates reported by Joliot and Joliot (2002) and Cardol *et al*. (Cardol *et al.*, 2003). However, some C_4 plants require a ratio of ATP/NADPH of 5:2. In species like maize and sorghum, mesophyll chloroplasts generate most, if not all, of the reductive power while bundle sheath chloroplasts function to produce ATP, likely via a CEF1 pathway (Edwards and Walker, 1983; Ivanov *et al.*, 2001). Similarly, cyanobacteria and green algae, for which robust CEF1 is well-documented, need a higher PSI/PSII ratio to fix CO_2 than do C_3 vascular plants, in part because they possess ATP-driven CO_2 concentrating mechanisms (Ogawa and Kaplan, 2003; Turpin and Bruce, 1990).

However, extremes in acceptor limitation (namely O_2 and CO_2) can induce rather dramatic (up to ~6-fold) changes in the sensitivity of q_E to LEF (Avenson *et al.*, 2004; Kanazawa and Kramer, 2002). To account for such a robust response by themselves, CEF1 or WWC would have to occur at rates about 5-fold larger than that of LEF. Even the largest estimates of CEF1 and WWC capacity fall far short of this. Thus, it is argued that CEF1 cannot by itself account for the observed large changes in q_E sensitivity to LEF, and that contributions from Type II mechanisms are therefore necessary.

It is important to keep in mind that all protons translocated into the lumen, either via LEF or a cyclic process, pass back across the thylakoid membrane, mainly through the ATP synthase. This implies that any increase in *pmf* generation by CEF1 or WWC (Type I mechanisms) will result in additional ATP synthesis, as long as uncoupling or 'slip' in the ATP synthase reaction is negligible, as shown by Junge and coworkers (Groth and Junge, 1993). Moreover, the augmented *pmf* should also increase regulatory sensitivity via q_E, as long as static *pmf* partitioning etc., accompany changes in Type I mechanisms. However, as discussed previously, large increases in Type I flux could result in an excessive increase in the supply ratio of ATP/NADPH. While, in principle, the problem could be solved by dissipating ATP non-productively in a futile cycle, no such futile ATPase activity has yet been identified. By contrast, Type II mechanisms increase regulatory sensitivity without altering ATP/NAPDH output ratios, and rather strong evidence has been presented that when an increase in q_E sensitivity is *all* that is needed, for example, under LEF-limited conditions, Type II mechanisms are activated.

These observations form the basis of an integrated model, where Type I mechanisms (CEF1 and WWC) provide plasticity at the level of ATP/NADPH, no doubt also impacting q_E sensitivity, while Type II mechanisms play a more significant role in adjusting q_E sensitivity without altering the ATP/NADPH output ratio. Furthermore, these mechanisms are not mutually exclusive and may act separately or in parallel to modulate q_E sensitivity.

One implication of the integrated model is that, for both types of mechanisms, regulation will be mediated through metabolic pools of the reactants or products of the light reactions. For example, for Type I mechanisms to operate effectively, the levels of their induction would need to be dictated by fluctuations in ATP consumption relative to NADPH. One possible model is that induction will be sensitive to the redox poise of the $NADP^+$/NADPH couple or the intermediate carriers of the ETC. Indeed, there is strong evidence that CEF1 must be properly redox poised to operate in vascular plants (Bendall and Manasse, 1995; Joet *et al.*, 2001; Joliot and Joliot, 2002). Moreover, in *Chlamydomonas*, state transitions, which have a large effect on excitation energy distribution between PSI and PSII (Delosme *et al.*, 1996), appear to trigger CEF1 (Depege *et al.*, 2003; Finazzi *et al.*, 2002), and this process is regulated by the phosphorylation of antenna complexes and is initiated by changes in the redox state of the PQ pool (reviewed in Allen and Forsberg, 2001; Haldrup *et al.*, 2001).

Similarly, engagement of Type II mechanisms would be expected to be linked to proportionate changes in overall flux of NADPH and ATP. In fact, these mechanisms appear to be induced under conditions where LEF and ATP synthesis are limited by the availability of electron acceptors and P_i acceptors (e.g. low CO_2). This fits adequately with a proposed model where g_{H^+} is modulated by stromal P_i levels (Kanazawa and Kramer, 2002). P_i levels in chloroplasts are typically 20 mM and may drop to 10 mM during photosynthesis (Furbank *et al.*, 1987; Usuda, 1988). Under stress conditions P_i concentrations may dip as low as 2 mM (Sharkey and Vanderveer, 1989). Since 1–2 mM of stromal P_i is inactive (Furbank *et al.*, 1987; Robinson and Giersch, 1987), and not available for photophosphorylation, active P_i concentration could be close to the reported K_m, 0.6 mM (Selman and Selman-Reimer, 1981). Indeed, decreases in the g_{H^+} of spinach and *Arabidopsis* leaf discs have been observed when pretreated with mannose, which has been

shown to act *in vivo* as a phosphate sink. Commensurate increases in *pmf* and q_E were also observed, and all effects were reversed with phosphate replenishment (K Takizawa, unpublished data). An alternative possibility is that ATP synthase activity is modulated allosterically. Interestingly, evidence has been presented that a 14-3-3 protein can bind a phosphorylated chloroplast ATP synthase and that binding partially inhibits turnover (Bunney *et al.*, 2001).

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