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Trends in Plant Science

Review

Physiological functions of malate shuttles in plants and algae

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Subcellular compartmentalization confers evolutionary advantage to eukaryotic cells but entails the need for efficient interorganelle communication. Malate functions as redox carrier and metabolic intermediate. It can be shuttled across membranes through translocators. The interconversion of malate and oxaloacetate mediated by malate dehydrogenases requires oxidation/reduction of NAD(P)H/NAD(P)$^+$; therefore, malate trafficking serves to transport reducing equivalents and this is termed the 'malate shuttle'. Although the term 'malate shuttle' was coined more than 50 years ago, novel functions are still emerging. This review highlights recent findings on the functions of malate shuttles in photorespiration, fatty acid β-oxidation, interorganelle signaling and its putative role in CO_2-concentrating mechanisms. We compare and contrast knowledge in plants and algae, thereby providing an evolutionary perspective on redox trafficking in photosynthetic eukaryotes.

Highlights

The malate shuttle, as a valve for photosynthetic electron dissipation, has been proposed for >50 years, but only recently has this function been clearly demonstrated.

The plastidial NAD-MDH is essential for embryogenesis and chloroplast development. This role is not due to its enzymatic activity but rather to its ability to stabilize a large AAA-ATPase complex at the inner envelope. The plNAD-MDH is therefore a moonlighting protein.

The malate shuttle connects fatty acid biosynthesis in the chloroplast to mitochondrial reactive oxygen species (ROS) production and to programmed cell death in plants.

The malate shuttle connects fatty acid catabolism in the peroxisome to photosynthesis and chloroplast metabolism in algae.

Expression of malate shuttle components is responsive to CO_2 levels. The latest results indicate its critical role in plant photorespiration and points to a possible role in the algal CO_2-concentrating mechanism.

Malate as a major cellular redox carrier

Eukaryotic cells are compartmentalized, and distinct subcellular organelles house specific subsets of metabolic reactions that are physically separated from each other by biological membranes. Exchange of information and energy between compartments, mostly mediated by metabolites, is indispensable to achieve whole-cell homeostasis and ensure optimal growth [1,2]. While some of these exchanges occur through passive diffusion, most of them are mediated by transporters [3]. Understanding the complex interplay of energy and information between subcellular compartments is key towards the domestication of photosynthetic organisms for tailor-made production of valuable compounds.

Malate is a C4 dicarboxylic acid and is involved in a number of metabolic pathways, including the tricarboxylic acid (TCA), C4-dicarboxylic acid, and **glyoxylate cycles** (see Glossary) in plants and algae [4–6]. Malate can be produced by malate synthase (MAS) and metabolized malic enzyme (ME) or malate dehydrogenase (MDH) (Box 1). Amongst these, only MDH catalyzes a reversible reaction that is coupled to the reduction/oxidation of NAD(P)$^+$/NAD(P)H, while interconverting malate and oxaloacetate (OAA). In contrast to the main cellular redox couple, that is, the pyridine nucleotides NAD(P)(H), malate can be efficiently transported across membranes through di- or tricarboxylate translocators. Although specific transporters of NAD$^+$ are present in the membrane of the chloroplast, mitochondrion, or peroxisome [7,8], their activity is restricted in importing NAD$^+$ into organelles and is probably important in *de novo* loading of organelles with the NAD$^+$ pool. But these transporters show a very low affinity for NAD(P)H [9] and are therefore probably not sufficient for redox exchange between compartments. Malate trafficking thus serves as an indirect but efficient way of transporting reducing equivalents.

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Box 1. Malate metabolism in photosynthetic cells

Malate is a versatile compound and its cellular metabolism involves malate synthase (MAS), malic enzyme (ME), or malate dehydrogenase (MDH) (Figure I). MAS catalyzes the formation of malate from acetyl-CoA and glyoxylate, which is a key step in the glyoxylate cycle [4,88]. The glyoxylate cycle allows cells to utilize two-carbon compounds (C2), such as acetate, and it is essential for the heterotrophic growth of some algae such as chlamydomonas [89]. The concerted action of MAS and isocitrate lyase (ICL) in the glyoxylate cycle forms succinate that enters the mitochondrial TCA cycle, bypassing the steps that result in carbon loss [4,64].

ME catalyzes the decarboxylation of malate to pyruvate, resulting in the production of NAD(P)H and CO_2. This reaction has been shown to be an important source of reducing equivalents for *de novo* fatty acid synthesis as well as desaturation in fungi [90], plants [91], and more lately in algae [92]. It is also a crucial reaction in C4 and CAM (Crassulacean acid metabolism) photosynthetic metabolism [93].

MDHs are the only enzymes catalyzing a reversible reaction, important for its function in modulating redox status. In arabidopsis, nine genes encoding MDHs are present in the genome, and the activities of MDH could be detected in most subcellular compartments, including the mitochondrion (mNAD-MDH1, 2), peroxisome (pNAD-MDH1, 2), cytosol (cyNAD-MDH1, 2, 3), and plastid (plNAD-MDH and plNADP-MDH) [10]. In contrast to land plants, five isoforms of MDH (MDH1-5) are encoded in the genome of chlamydomonas [94]. CrMDH5 (the only $NADP^+$ requiring MDH) is predicted to be chloroplastic, CrMDH2 peroxisomal [4,63], CrMDH3 is considered cytosolic, and CrMDH4 mitochondrial [95]. The situation is more complex with CrMDH1 which is predicted to be chloroplast-based on PredAlgo [95], but is found peroxisomal when the 25 aa of its N terminus is fused to yellow fluorescent protein (YFP) [4]. Therefore, subcellular localization(s) of CrMDH1 using full-length protein fusion or immunogold labeling would be needed to clarify its location.

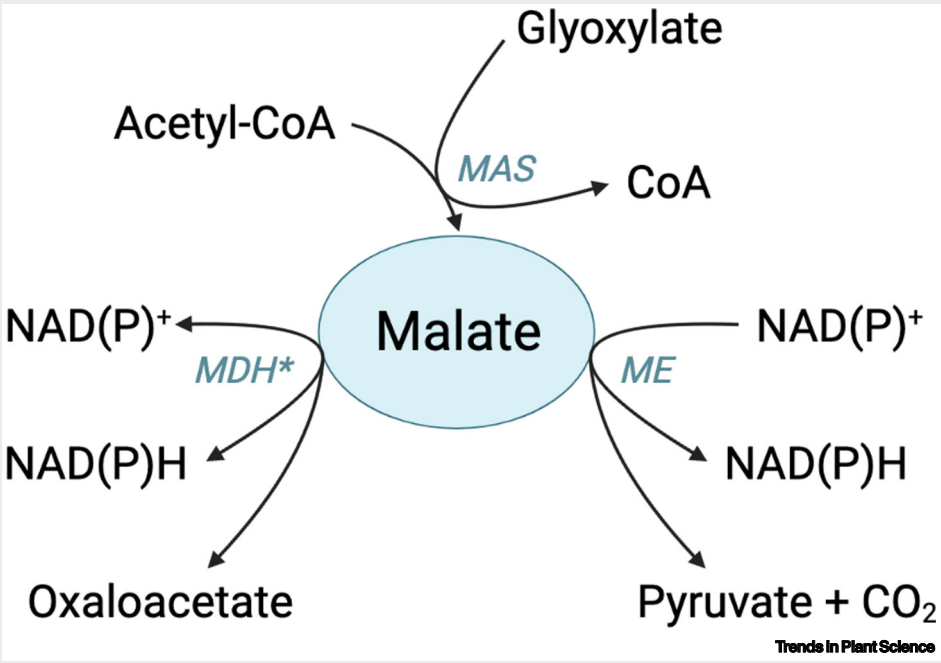

Figure I. Metabolic reactions that involve malate. Malate dehydrogenase (MDH), malic enzyme (ME), and malate synthase (MAS) are found in higher plants and algae. MAS catalyzes the biosynthesis of malate by condensing glyoxylate and acetyl-CoA. This reaction is part of the glyoxylate cycle. ME uses either $NADP^+$ or NAD^+ to decarboxylate malate into pyruvate, releasing CO_2; it is present in several subcellular compartments. MDH catalyzes the reversible interconversion between malate and oxaloacetate using either $NAD(P)^+$ or NAD(P)H as cofactor. This reaction occurs in the plastid, cytosol, peroxisome, and mitochondrion. CoA, Co-enzyme A; $NAD(P)^+$, nicotinamide adenine dinucleotide (phosphate). *Denotes that the MDH reaction is reversible.

Trafficking of malate between different subcellular compartments has been extensively studied in plants (Box 2) [10]. The inner membrane of the chloroplast and mitochondrion in plant and algal cells is equipped with several types of di- and tricarboxylate translocators catalyzing malate

Glossary

CO_2-concentrating mechanism (CCM): CCM, as the name implies, is a process of concentrating CO_2 to the active site of RuBisCo, the major protein of the carbon photoreduction cycle (i.e., often called Calvin–Benson–Bassham cycle). It is therefore considered a mechanism to reduce the rate of photorespiration. Depending on species, CCM could refer to the dicarboxylic acid cycle in C4 plants, CAM-based CCM in CAM plants, carboxysome based-CCM in cyanobacteria, and the biophysical CCM occurring in algae (the conversion of CO_2 to bicarbonate and its transport to the pyrenoid).

Cyclic electron flow (CEF): the recycling of electrons from the acceptor side of photosystem I through the cyt b_6/f complex, increasing the proton motive force (*pmf*). The *pmf* is then used to drive ATP synthesis. The CEF promotes ATP synthesis without additional NADPH production and is therefore considered a way to increase the ATP/NADPH ratio in response to metabolic or environmental situations. Several pathways of CEF have been characterized: (i) the plant type I NADPH dehydrogenase complex (NDH); (ii) the type II NDH (NDA2) unique to algae; and (iii) the proton gradient regulator 5 (PGR5)/PGR-like 1 (PGRL1)-mediated CEF pathway, which is common to plants, algae, and cyanobacteria.

Fatty acid β-oxidation: one of the major mechanisms of fatty acid degradation. Oxidation of fatty acids can occur either at the α-, β- or ω-carbon position of a given fatty acid; β-oxidation is found as a major pathway in plants and algae. This pathway ultimately breaks down fatty acids into acetyl-CoAs, which are further metabolized either for energy production, through the mitochondrial electron transport chain, or for the synthesis of sugars when coupled to glyoxylate and gluconeogenesis pathways.

Glyoxylate cycle: an anabolic pathway occurring widely in plants, bacteria, fungi, and algae. It allows the conversion of acetyl-CoA to succinate for the synthesis of carbohydrates, therefore allowing microorganisms to utilize two-carbon compounds as a carbon source. For example, it plays an essential role in the heterotrophic growth of green algae on acetate. It shares several steps

Box 2. Dicarboxylate and tricarboxylate translocators

In addition to MDHs, the malate shuttle requires translocators of malate/or other dicarboxylates or tricarboxylates from one compartment to another. Different translocators have been predicted and experimentally verified in arabidopsis chloroplasts and mitochondria [12,96]. The transfer of malate through the chloroplast envelope membrane can be mediated by at least three different translocators. The 2-oxoglutarate (OG)/malate-translocator 1 (OMT1 or DiT1) catalyzes the transport of malate in exchange for 2-OG [31]. AtOMT1 was also shown to have high affinity for OAA in reconstituted liposomes [22]. The dicarboxylate translocators 1 and 2 (DCT1 and 2, also sometimes called DiT2.1 and 2.2) are glutamate/malate-translocators importing malate in counterexchange with glutamate [31]. Very likely also, malate uniporters exist in the chloroplast envelope membrane. However, the corresponding genes have not been identified.

The transfer of malate across the mitochondrial inner membrane is mediated by at least three dicarboxylate carriers (DIC1, DIC2, DIC3) and one dicarboxylate-tricarboxylate carrier (DTC) belonging to the mitochondrial carrier family (MCF) [85,86]. The recombinant DICs, when introduced into liposomes, catalyze the transport of a wide range of dicarboxylates such as malate, OAA, succinate, but with low affinity for 2-OG. These DICs were proposed to be involved in the mitochondrial malate-OAA shuttle [86]. Very recently, the physiological function of DIC2 has been demonstrated in arabidopsis as being a high-affinity malate–citrate antiporter in the mitochondria, through evidence from the reverse genetic approach in combination with comprehensive *in vitro*, *in organello*, and *in vivo* analyses [87]. Conversely, in addition to dicarboxylates (malate, OAA, 2-OG, and succinate), DTC was shown to also transport tricarboxylates (such as citrate or isocitrate) [85]. Therefore, DTC can serve as a malate/2-OG translocator. A malate/aspartate shuttle has been proposed by [67] connecting mitochondria to the peroxisome, which requires coordinated functions of two types of translocators, that is, malate/2-OG carrier (likely DTC) and a glutamate/aspartate carrier.

Genes encoding putative proteins homologous to the plant di- or tricarboxylate translocators are present in the chlamydomonas genome (see Table 1 in main text). Three putative plastidial 2-OG/malate translocators (annotated as OMT1, OMT2, and low-carbon-inducible20 – LCI20), and one mitochondrial translocator known as MiTC14 and belonging to the MCF family, have been identified [94]. The protein sequence of LCI20 shares 55% and 60% similarity with DiT2.1 and DiT2.2, respectively, whereas AtDiT1 shares 63% of similarity with both CrOMT1 and CrOMT2, suggesting that they are evolutionarily related dicarboxylate translocators.

transport in counter-exchange with, for example, OAA, glutamate, or aspartate. It can function in either direction depending on the concentration gradient resulting from a metabolic demand [11,12]. Collectively, the trafficking of malate through specialized translocators from one compartment to another, combined with the malate processing enzymes (MDHs), is termed the **malate shuttle** [10].

Although the concept of the 'malate shuttle' has been around for over 50 years ago, components of this pathway remain hypothetical in microalgae, despite considerable progress has been made in land plants [10]. Depending on the type of carboxylates being transported, several variations of the 'malate shuttle' occur, which are, malate/OAA shuttle, malate/aspartate shuttle (Box 3), and malate/2-oxoglutarate shuttle. These various types of malate shuttle, their subcellular locations, and their involvement in different metabolic pathways are illustrated in Figure 1.

Box 3. Other players in the malate shuttle

Apart from MDH and the malate translocators listed in Table 1, other enzymes could also be involved in functioning of variants of the malate shuttle. One notable example is the malate–aspartate shuttle, which requires aspartate aminotransferases (AspATs), sometimes called glutamate-oxaloacetate transaminases (GOTs). AspATs are ubiquitous enzymes encoded by a multigenic family catalyzing the reversible interconversion between aspartate and 2-oxoglutarate with glutamate and OAA. In arabidopsis, five isoforms of eukaryotic type AspAT are present, that is, AspAT1 (mitochondrial), AspAT2 and AspAT4 (cytosolic), AspAT3 (peroxisomal), AspAT5 (chloroplastic), and one prokaryotic-type AspAT (PTA) localized in the chloroplast [97,98]. Activity of AspAT was also detected in chlamydomonas [99], and five genes *AST1–AST5* (phytozome v13) encode AspAT proteins [94], respectively: AST1, AST3 and AST4 (likely mitochondrial or peroxisomal), AST2 (chloroplastic), and AST5 (cytosolic). The plant peroxisomal and mitochondrial isoforms of AspAT have been proposed, in conjunction with MDHs, to play a role in shuttling reducing equivalents between both organelles through the malate–aspartate shuttle during photorespiration [100], but none of the algal proteins has been studied.

with the tricarboxylic acid (TCA) cycle but bypasses the steps that release CO_2.

Malate shuttle: this refers to the means of redox trafficking from one subcellular compartment to another through malate transport. It comprises two major protein components, that is, malate dehydrogenases and di- or tricarboxylate translocators.

Photorespiration: a metabolic pathway found in organisms that carry out oxygenic photosynthesis in which the major enzyme RuBisCo can fix either CO_2 or O_2 depending on their stoichiometric ratio. The oxygenation reaction produces 2-phosphoglycolate, a toxic metabolite, which is detoxified by conversion to the Calvin–Benson–Bassham Cycle intermediate 3-phosphoglycerate. This conversion involves a set of reactions occurring in four subcellular compartments, that is, chloroplast, cytosol, peroxisome, and mitochondria, and it entails the loss of CO_2, NH_4^+ and energy in the process.

Pseudo-cyclic electron flow (pseudo-CEF): a pathway in which electrons, generated from the splitting of water at PSII, are used to reduce O_2 to H_2O, thereby driving photosynthetic electron flow and generating a proton gradient, without NADPH production. In green algae, O_2-photoreduction is mostly mediated by the flavodiiron (FLV) proteins.

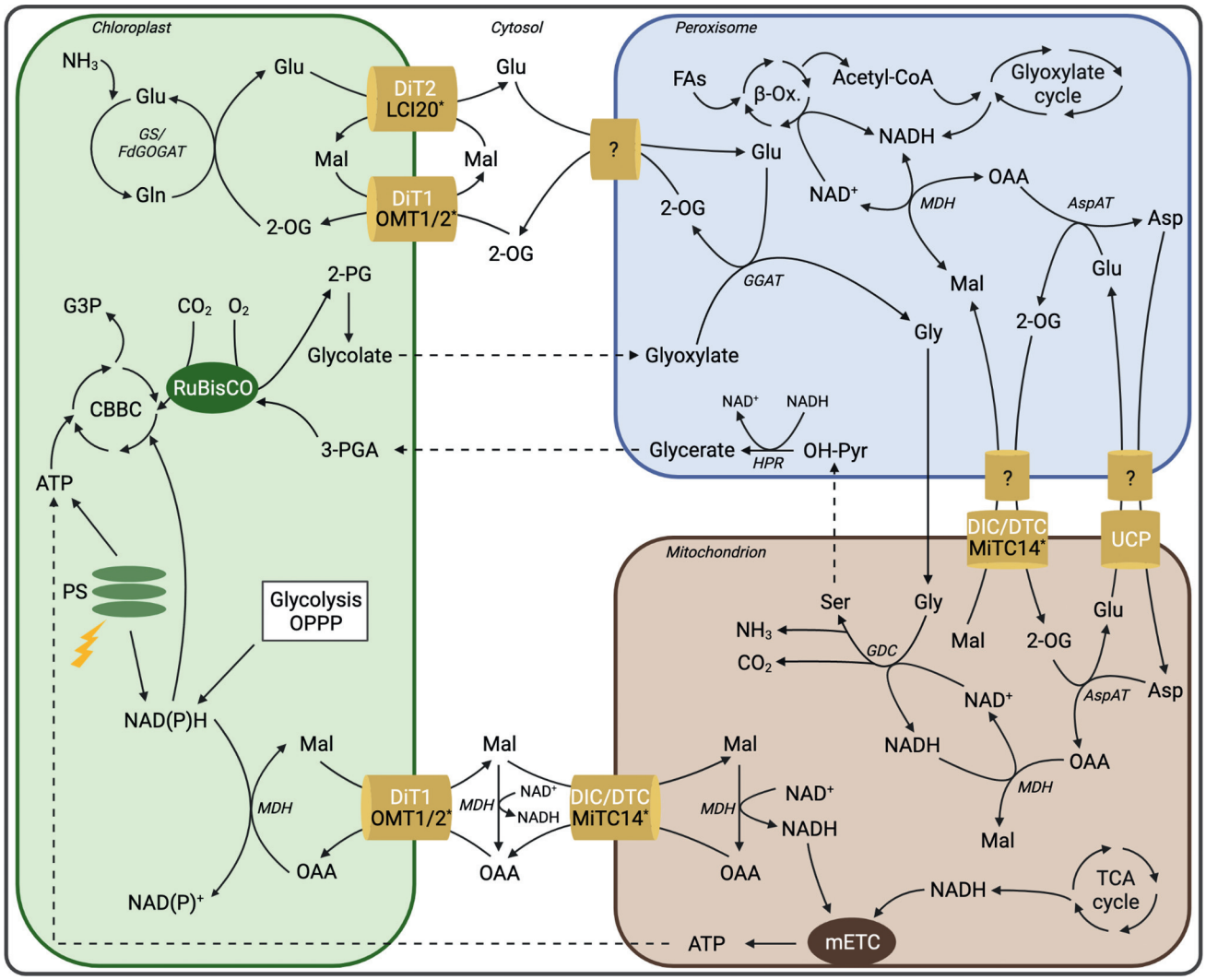

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Figure 1. Known and putative steps of malate trafficking in a subcellular context. The known dicarboxylate/tricarboxylate translocators based on either *in vivo* or *in vitro* characterization are reported in Table 1. Transporter name in white refers to arabidopsis proteins and black refers to the chlamydomonas putative transporters (*) based on homology without any functional data. The question mark (?) represents the putative translocators involved in the malate shuttle. Dashed lines indicate reactions with several intermediate steps. Created with BioRender.com. ADP, adenosine diphosphate; ATP, adenosine triphosphate; Asp, aspartate; AspAT, aspartate aminotransferase (Box 3); β-Ox., β-oxidation; CBBC, Calvin–Benson–Bassham cycle; DIC/DTC, mitochondrial dicarboxylate/dicarboxylate-tricarboxylate carrier; DiT1/2, dicarboxylate translocator 1/2; FA, fatty acid; FdGOGAT, ferredoxin-dependent glutamine 2-oxoglutarate aminotransferase; G3P, glyceraldehyde-3-phosphate; GDC, glycine decarboxylase; GGAT, glutamate-glyoxylate aminotransferase; Gln, glutamine; Glu, glutamate; GS, glutamine synthase; HPR, hydroxypyruvate reductase; Mal, malate; MiTC14, mitochondrial substrate carrier protein 14; MDH, malate dehydrogenase; mETC, mitochondrial electron transport chain; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); OAA, oxaloacetate; 2-OG, 2-oxoglutarate; OH-Pyr, hydroxypyruvate; OMT1/2, 2-oxoglutarate/malate translocator; OPPP, oxidative pentose phosphate pathway; 2-PG, 2-phosphoglycolate; 3-PGA, 3-phosphoglycerate; PS, photosystems; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TCA cycle, tricarboxylic acid cycle; UCP, uncoupling protein.

Here, we review some of the most recent discoveries on the roles of malate shuttles in connecting carbon, nitrogen, and energy metabolism in plants and algae. While isoforms of MDHs and di-/tricarboxylate translocators have been well characterized in land plants, evidence is just emerging regarding their functions in algae. We provide a comparative analysis of the corresponding proteins between the model plant arabidopsis (*Arabidopsis thaliana*) and the model green alga chlamydomonas (*Chlamydomonas reinhardtii*) (Table 1). We discuss the malate shuttle's physiological

Table 1. Main components of the malate shuttle in arabidopsis and chlamydomonas[a,b]

Protein family	Chlamydomonas	Phytozome ID	Arabidopsis	TAIR ID	Localization	Refs
Malate dehydrogenases	CrMDH1	Cre03.g194850	plNAD-MDH	At3g47520	Chloroplast (arabidopsis) Peroxisome[c] (chlamydomonas)	[4,50,78–80]
	CrMDH2	Cre10.g423250	pMDH1	At2g22780	Peroxisome	[25,37,68]
			pMDH2	At5g09660		
	CrMDH3	Cre02.g145800	cyNAD-MDH1	At1g04410	Cytosol	[81,82]
			cyNAD-MDH2	At5g43330		
			cyNAD-MDH3	At5g56720		
	CrMDH4	Cre12.g483950	mMDH1	At1g53240	Mitochondria	[33,83,84]
			mMDH2	At3g15020		
	CrMDH5	Cre09.g410700	cpNADP-MDH	At5g58330	Chloroplast	[19,20,23]
Malate translocators	CrOMT1	Cre17.g713350	DiT1 (OMT1)	At5g12860	Chloroplast	[22,32,50]
	CrOMT2	Cre17.g713200				
	CrLCI20	Cre06.g260450	DiT2.1 (DCT1)	At5g64290		[30–32]
			DiT2.2 (DCT2)	At5g64280		
	CrMiTC14	Cre16.g672650	DIC1	At2g22500	Mitochondria	[11,85,86]
			DIC2	At4g24570		
			DIC3	At5g09470		
			DTC	At5g19760		

[a]Abbreviations: At, *Arabidopsis thaliana*; Cr, *Chlamydomonas reinhardtii*; 'pl', plastidial; 'p', peroxisome; 'cy', cytosol and 'm', mitochondria.
[b]Homology between chlamydomonas and arabidopsis proteins was identified through reversible BlastP search of TAIR (for arabidopsis) and Phytozome v13 (for chlamydomonas) proteins. Arabidopsis protein sequences were used first as baits. Note that the function assigned to mitochondrial malate translocators in arabidopsis is only based on *in vitro* experiments except for DIC2, which has recently been characterized *in vivo* [87]. Other players in the malate shuttle can be found in Boxes 2 and 3.
[c]Refers to a putative localization of CrMDH1 in the peroxisome based on fusion of N-terminus transit peptide with fluorescent tag.

importance in photosynthesis, **photorespiration**, and **fatty acid β-oxidation**, and we point to its possible implication in algal **CO_2-concentrating mechanisms** (**CCMs**). Finally, we highlight the role of malate shuttle in interorganelle communication through interaction with reactive oxygen species (ROS)-mediated signal transduction.

Malate shuttle as a valve for photosynthetic electron dissipation

During the linear electron flow (LEF) of photosynthesis, light energy is converted by two photosystems (PSII and PSI) into chemical energy in the form of NADPH and ATP, which are subsequently used to drive metabolic reactions, particularly CO_2 fixation by RuBisCo and its conversion into triose phosphates through the Calvin–Benson–Bassham (CBB) cycle [13]. However, the LEF is known to produce an insufficient amount of ATP (as compared to NADPH) than that required for optimal CO_2 photoreduction, leading to an imbalance [14]. The imbalance in ATP and NADPH could be further accentuated by changes in environmental conditions. For example, under CO_2 limiting conditions, photorespiration in plants, or a CCM in algae, requires additional ATP [14,15]. Excess reducing equivalents could potentially lead to ROS overproduction [16]. Coordination between production and usage of ATP and NAD(P)H is therefore essential for cell fitness and survival, particularly in a changing environment.

ATP and NADPH balance can be achieved by increasing ATP production in the chloroplast or through import, or by lowering the NADPH level through enhancing sink strength or export to other compartments (Figure 2). Several mechanisms are involved in the supply of extra-ATP, which include **cyclic electron flow** (**CEF**) around PSI, or the O_2-photoreduction by flavodiiron

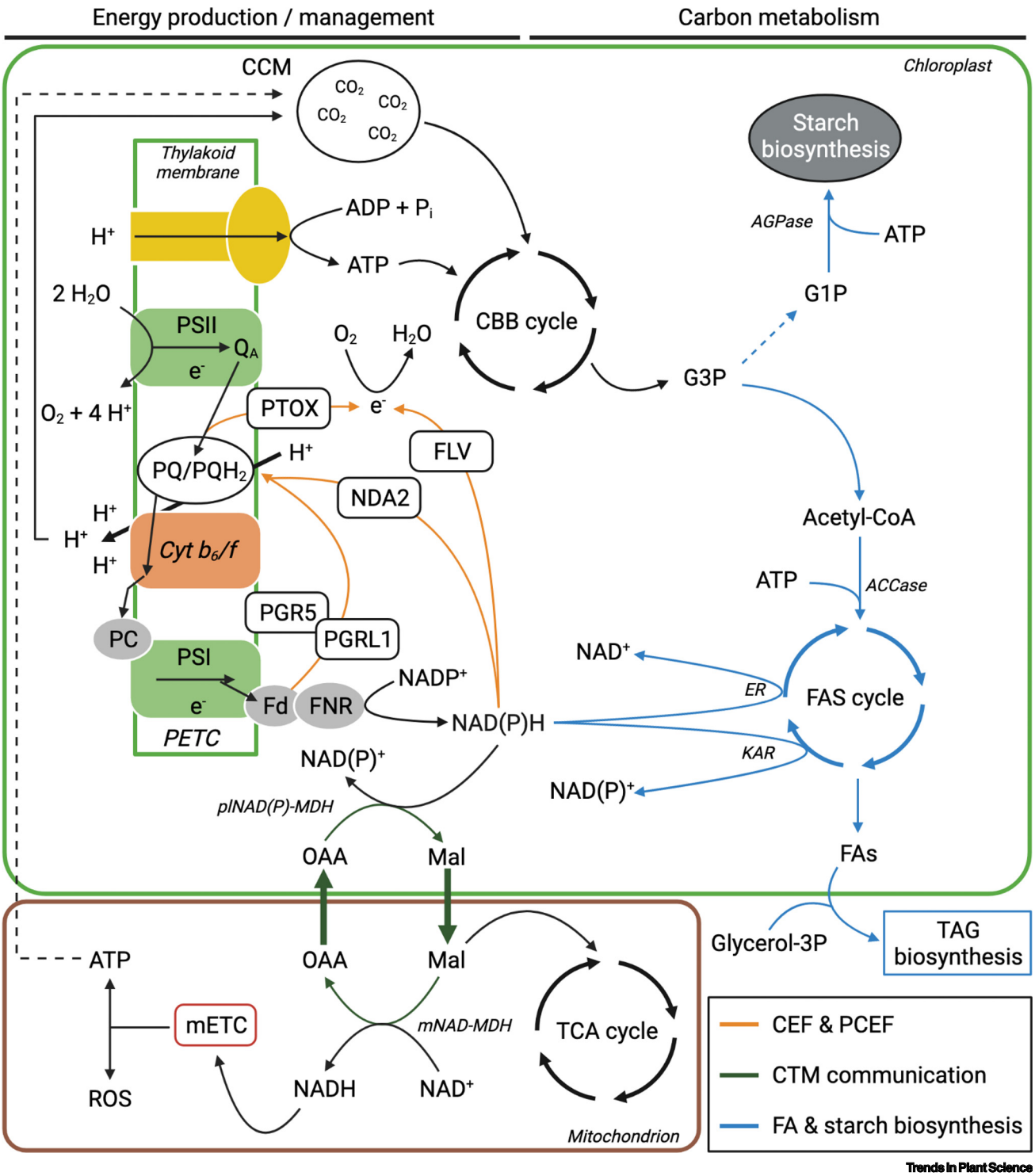

Figure 2. Photosynthetic redox production, management, and carbon metabolism in algae. The photosynthetic linear electron flow (LEF) occurring in the thylakoid membrane produces ATP and NADPH for the fixation of CO_2 in the Calvin–Benson–Bassham (CBB) cycle to yield carbohydrates. The G3P, ATP, and NAD(P)H are then used for starch and *de novo* fatty acid biosynthesis (blue arrows). Alternative electron pathways are represented by cyclic electron flow (CEF; orange

(Figure legend continued at the bottom of the next page.)

(FLV) proteins, also known as **pseudo-CEF**, or ATP import from mitochondria [17]. Conversely, NADPH dissipation can occur either through enhanced metabolic demand (e.g., boosting *de novo* fatty acid synthesis, which has a high need for reducing equivalents), or through its export to other subcellular compartments by the malate shuttle [18].

Despite having been hypothesized for several decades, the role of the malate shuttle in photosynthetic electron exportation (as a 'malate valve') has only recently been demonstrated. In arabidopsis, the knockout mutants in plastidial NADP-MDH (*nadp-mdh*) had a more reduced plastoquinone pool but with only slight impairment in growth under high light (HL) [19,20]. Strong growth defects, however, were observed under fluctuating light in a mutant in which one of its regulatory redox switches at its C terminus was deleted by genome editing [21]. The carboxylate translocator involved in the malate valve (AtOMT1) has also been characterized in arabidopsis. AtOMT1 is a high-affinity OAA translocator whose absence led to the hyperaccumulation of reducing power in the stroma, photoinhibition, and impaired growth under HL [22]. Taken together, the previously-mentioned studies demonstrate the essential role of the malate valve in chloroplast energy homeostasis, which is particularly important to balance the chloroplast redox status during fluctuating conditions.

In algae, none of the components of the chloroplast malate valve have been characterized at a physiological level. The chlamydomonas CrMDH5, the homolog of the arabidopsis NADP-MDH, has been studied *in vitro* [23]. The thioredoxin (TRX)-dependent redox regulation of the CrMDH5 activity is much faster (responding in an all-or-nothing way) than the plant homolog but the latter is fine-tuned due to the presence of an additional N-terminus disulfide regulatory domain. Even though its physiological function has not been shown directly, it was proposed that the malate valve may operate in the chlamydomonas mutant lacking the proton-gradient-like-1 (PGRL1)-mediated CEF in conditions of strong ATP/NADPH imbalance [24]. It was hypothesized that the malate valve helps to evacuate excess reducing equivalents towards cytoplasm and mitochondria. Nevertheless, there is no direct evidence of the operation of the malate valve in algae.

It is worth bearing in mind that the 'malate valve' in the chloroplast could also function in importing reducing power into the chloroplast. Responses of photosynthesis and chloroplast metabolism to changes in redox state in other subcellular compartments (mitochondria or the peroxisome) seem to support such a possibility [25,26]. Our understanding of redox homeostasis in the chloroplast is further complicated by the presence of both NAD^+- and $NADP^+$-dependent MDHs and the coexistence of two pyridine nucleotide pools (NADH versus NADPH) (discussed in Outstanding questions).

The role of the malate shuttle in plant photorespiration

Photorespiration, initiated by the oxygenation reaction of RuBisCo, is inevitable in an oxygen-containing atmosphere. The rate of photorespiration is reduced in land plants performing C4

arrows) and pseudo-CEF (PCEF; orange arrows). Chloroplast-to-mitochondrial (CTM) communication is represented in dark green arrows. Excess NAD(P)H is used by plNAD(P)-MDHs to reduce OAA to malate that is shuttled through chloroplastic malate translocators to fuel the mitochondrial electron transport chain (mETC) for the synthesis of ATP. The CO_2-concentrating mechanism (CCM) is represented in the chloroplast where atmospheric CO_2 is pumped then sequestrated into the pyrenoid. Part of energy needed for CCM comes from the proton motive force generated by photochemical reactions, and another part is likely to be derived from the mETC (dashed black arrow). Note that most of the reactions shown in this figure are also present in higher plants, except the algal CCM. Created with BioRender.com. ACCase, acetyl-CoA carboxylase; ADP, adenosine diphosphate; AGPase, ADP-glucose pyrophosphorylase; ATP, adenosine triphosphate; CBB, Calvin–Benson–Bassham cycle; CEF, cyclic electron flow; Cyt b_6f, cytochrome b_6f complex; ER, enoyl-acyl carrier protein (ACP) reductase; FA, fatty acid; FAS, fatty acid synthase; Fd, ferredoxin; FLV protein, flavodiiron protein; FNR, ferredoxin-$NADP^+$ reductase; G1P, glucose-1-phosphate; G3P, glucose-3-phosphate; KAR, ketoacyl-ACP reductase; Mal, malate; MDH, malate dehydrogenase; mETC, mitochondrial electron transport chain; NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); NDA2, NAD(P)H dehydrogenase 2; OAA, oxaloacetate; PC, plastocyanin; PCEF, pseudo-CEF; PETC, photosynthetic electron transport chain; PGR5, proton gradient regulation 5; PGRL1, proton-gradient-like-1; PQ/PQH_2, plastoquinone/plastoquinol; PTOX, plastoquinone terminal oxidase; PS, photosystem; Q_A, quinone A; ROS, reactive oxygen species; TAG, triacylglycerol; TCA cycle, tricarboxylic acid cycle.

photosynthesis and in algae having a CCM [27]. Photorespiration consists of multiple metabolic reactions distributed over four subcellular compartments: chloroplast, cytosol, peroxisome, and mitochondrion, and it therefore requires intimate interorganelle communication. Photorespiratory reactions have a strong impact on cellular energetics, producing NADH in the mitochondria where two glycine molecules are converted to serine, but consuming it in the peroxisome where hydroxypyruvate is converted to glycerate (Figure 1). Therefore, NADH homeostasis in mitochondria and the peroxisome is critical for optimal functioning of photorespiration. Further, changes in the leaf internal CO_2 concentration, as a consequence of an altered stomatal aperture, can impact redox status in the respective organelles through photorespiration. Indeed, in land plants, malate shuttle components (i.e., plNADP-MDH, plNAD-MDH, DiT1, and DiT2.1) are upregulated upon acclimation to low CO_2 and downregulated under elevated CO_2 (where photorespiration is suppressed), suggesting their possible implication in the adaptation of cellular metabolism in response to fluctuating CO_2 availability [28,29].

The malate shuttle contributes to photorespiration at multiple levels (Figure 1). It is involved in the transamination of glyoxylate into glycine coupled with the conversion of glutamate to 2-oxoglutarate in the peroxisome catalyzed by the glutamate:glyoxylate aminotransferase (GGT). The plastidial malate/2-oxoglutarate shuttle provides glutamate from the chloroplast that serves as a donor of NH_3 for the transamination of glyoxylate [30]. Arabidopsis DiT2.1, but not DiT2.2, is a glutamate/malate translocator involved in photorespiration [31]. And moreover, DiT2.1 and DiT1 (AtpOMT1) work in concert for the optimal functioning of photorespiration, and the lack of either DiT2.1 or DiT1 led to growth impairment under photorespiratory conditions [22,31,32].

The malate shuttle is further involved in the export of NADH produced during glycine decarboxylation in mitochondria, thus recycling NAD^+ and ensuring proper functioning of photorespiration. The decrease of mNAD-MDH activity in the *mmdh1* knockout mutant had a strong effect on photorespiratory flux, leading to reduced growth under photorespiration-promoting conditions (i.e., very low CO_2) [33]. In fact, the *mmdh1* mutant displayed a high glycine/serine ratio indicative of impaired glycine decarboxylation due to the lack of the electron acceptor NAD^+ and an over-reduction in the mitochondria. The homeostasis of NAD^+/NADH can be achieved either by increasing the consumption of NADH by mitochondrial reactions or by exporting the excess NADH to other compartments. A likely route for exporting NADH from glycine decarboxylation is through a malate/aspartate shuttle towards peroxisomes where NADH could be consumed by the hydroxypyruvate reductase (HPR), another key reaction of photorespiration (Figure 1). The aspartate/glutamate translocators involved in this malate/aspartate shuttle have been recently characterized in arabidopsis as belonging to the uncoupling protein (UCP) family [11]. Both AtUCP1 and AtUCP2 are able to efficiently counterexchange aspartate and glutamate [11]. Similar to *mmdh1*, mutants of AtUCP1 are impaired in photorespiration with a reduced rate of glycine oxidation in the mitochondria [11]. Therefore, AtUCP1 and 2 and mNAD-MDH1 and 2 work together in photorespiration by forming the malate/aspartate shuttle that connects glycine decarboxylation in the mitochondria with hydroxypyruvate reduction in the peroxisome. We note that redox equivalents derived from photorespiratory glycine decarboxylation in mitochondria may not be necessarily shuttled to the peroxisome but could potentially fuel nitrate and/or hydroxypyruvate reduction in the cytoplasm [34,35]. Antisense repression of plastidial OMT1/DiT1 in tobacco, however, indicates a prominent role of the plastidial malate shuttle in supplying nitrate reduction with NADH [36]. Therefore, the contribution of mitochondria-derived NADH in nitrate reduction in the cytosol could only be minor.

Finally, at the step of hydroxypyruvate reduction into glycerate by the peroxisomal HPR, pNAD-MDH can oxidize malate imported from the cytosol to provide additional reducing equivalents

needed for HPR. Double mutants of pNAD-MDH1 and 2 were, however, viable under photorespiratory conditions (i.e., ambient CO_2), but, the stoichiometry of photorespiratory CO_2 generated per RuBisCo oxygenation was increased by 50% as compared to the wild type [37]. This was explained by an alternative pathway which was thought to be a non-enzymatic conversion of hydroxypyruvate to glycolate, thereby releasing extra CO_2. Timm *et al.* [35] showed that *HPR2* encodes a cytosolic hydroxypyruvate reductase activity (i.e., not peroxisomal) and that reduction of hydroxypyruvate can flexibly shift between peroxisomes and the cytoplasm.

A putative role of the malate shuttle in the algal CCM

To cope with the low CO_2-to-O_2 ratio, unlike plants where photorespiration plays a significant role, microalgae frequently use a CCM. The algal biophysical CCM is an energetic mechanism that pumps and sequestrates atmospheric CO_2 into the pyrenoid close to the active site of RuBisCo, key to the proliferation of algae in their natural habitat where the CO_2 level could be extremely low [27]. In the past 10 years, enzymes (carbonic anhydrases), transcription factors (CCM1), and also several inorganic carbon (Ci) transporters involved in the CCM have been identified and characterized in chlamydomonas [38,39]. Known transporters include the HL-activated 3 (HLA3) protein that is localized at the plasma membrane, a low-carbon inducible protein (LCIA) in the chloroplast envelope, and recently the thylakoid-localized bestrophin-like (BST) transporters [40–43]. However, not until recently has the energization mechanism of the CCM begun to be unveiled.

By studying double mutants of chlamydomonas lacking both PGRL1 and FLV-B mediating the CEF and pseudo-CEF respectively, Burlacot *et al.* [15] showed that double mutants are impaired in growth under air (i.e., a low CO_2 condition) as was the reduced affinity of photosynthesis of the double mutants for Ci. The authors concluded that the pmf produced through the combined action of CEF and pseudo-CEF mechanisms favors the conversion of bicarbonate anions transported by the thylakoid-localized BSTs into CO_2 at the active site of the carboxylating enzyme RuBisCo. By showing that inhibitors of mitochondrial respiration also decrease the affinity of photosynthesis for Ci, Burlacot *et al.* [15] further proposed that mitochondrial respiration serves as an alternative source of energy powering distant transporters involved in the CCM (located either at the plasma membrane or at the chloroplast envelope). Considering that the CCM is a light-driven process, it is thus highly plausible that the malate shuttle contributes to the CCM through mediating redox exchange between chloroplast and mitochondria (Figure 2).

Furthermore, implication of the malate shuttle in the CCM is supported by RNAseq-based transcriptome analyses, revealing the upregulation of putative chloroplastic 2-OG/malate translocators and MDHs in chlamydomonas and also in four phytoplankton species when transferred from a high to a low CO_2 condition [44–46]. Nevertheless, molecular actors and experimental evidence for such a function is yet to come, for example, by investigating the Ci affinity in mutants impaired in malate shuttle mediating the redox exchange between chloroplast and mitochondria.

The malate shuttle mediates interorganelle signaling through ROS

Intercompartmental exchange of signals is key for cellular homeostasis and the acclimation of photosynthetic organisms in fluctuating environments [47]. ROS signaling has been considered a powerful system for the regulation of gene expression, leading to a cascade of physiological adjustments, such as induction of programmed cell death (PCD) [48,49]. ROS can be generated in four major subcellular compartments, that is, chloroplast, peroxisome, mitochondria, and cytosol. ROS are produced when O_2 serves as an alternative electron acceptor during photosynthesis or during mitochondrial electron transport chain and is intimately linked to peroxisome-based fatty acid β-oxidation or photorespiration. ROS signaling has therefore been found to connect organelle

redox state between chloroplast and mitochondria, and between chloroplast and peroxisome [19,49,50]. In the following section, we discuss such examples.

ROS generation in the mitochondria was shown to trigger PCD in the arabidopsis mutant *mod1* (for *mosaic death 1*) affected in the chloroplast enoyl-ACP reductase (ENR) catalyzing the NADH-dependent enoyl-ACP reduction in the fatty acid synthase (FAS) complex [51]. A screen for *mod1* suppressors identified various components of the malate shuttle involved in chloroplast-to-mitochondrion communication, including plNAD-MDH, DiT1, and mNAD-MDH1 [50]. Fatty acid biosynthesis is a major sink for reductant in the chloroplast [52]. Therefore, impairment in ENR leads to overaccumulation of NADH in the chloroplast. The plNAD-MDH, in conjunction with DiT1, allows the export of reducing power in the form of malate. The translocator responsible for malate import into mitochondria is still unknown. Nevertheless, once inside the mitochondrion, malate oxidation by the mNAD-MDH1 generates NADH to fuel mitochondrial complex 1, ultimately resulting in the production of ROS [50]. These mitochondria-generated ROS have been considered as the signaling molecules in triggering PCD in the *mod1* mutant cells [50].

Interorganelle communication from chloroplast to peroxisome has also been proposed to play a role in retrograde signaling in arabidopsis [19]. Under conditions where the photosynthetic chain becomes over-reduced, due to the limitations of the electron acceptor $NADP^+$, molecular oxygen (O_2) can be used to accept electrons coming from PSI [53]. Photoreduction of O_2 can occur via two routes, that is, the non-enzymatic reaction (also known as Mehler reaction) [54,55] and the enzymatic reduction of O_2 [56,57]. In the Mehler reaction, the transfer of electrons from the PSI acceptor side to O_2 is associated with the generation of superoxide O_2^- that could be converted to H_2O_2 by superoxide dismutase (SOD) and then detoxified by the peroxidase located in the chloroplast, such as ascorbate peroxidase (APX). H_2O_2 can also diffuse out into other compartments where it can be detoxified, for instance, in the peroxisome by catalase (CAT) [58]. H_2O_2 is a cellular messenger and has been shown to play an important role as a signaling agent [59]. However, to take up such a role, the intracellular concentration of H_2O_2 must be sufficient to induce the expression of H_2O_2-responsive nuclear genes. The reversible inactivation of CAT in the peroxisome, allowing a burst in H_2O_2, requires the import of reducing equivalents originating from the chloroplast through the malate shuttle to the peroxisome [19,60]. This reversible inactivation of CAT was abolished in the arabidopsis *nadp-mdh* mutants [19], indicating the important role of the light malate valve (i.e., plNADP-MDH) in the regulation of CAT activity by transmitting the redox state of the chloroplast to the peroxisome.

An H_2O_2-based signaling pathway from peroxisome to chloroplast has also been proposed recently in chlamydomonas. The chlamydomonas mutants deficient in the peroxisomal MDH2 exhibited a higher rate of photosynthesis, starch, and *de novo* fatty acid biosynthesis in the chloroplast during photoautotrophic nitrogen deprivation [25]. Communication between the two organelles is proposed to be mediated by an H_2O_2/malate-dependent signaling pathway as supported by an elevated amount of H_2O_2 and malate in the *mdh2* knockout mutants. Exogenously supplied H_2O_2 has a strong positive effect on the expression of starch- and lipid- related genes in wild-type cells of chlamydomonas [61], corroborating that the increase in starch and lipid in the *mdh2* mutants resulted from the increase in the H_2O_2 intracellular concentration [25]. Collectively, the malate shuttle represents a redox-poise system communicating the redox state between compartments involving an H_2O_2-mediated signaling pathway.

The malate shuttle connects fatty acid catabolism to chloroplast metabolism

Fatty acid β-oxidation, photorespiration, and the glyoxylate cycle occur either totally or partially in the peroxisome, making it a third subcellular compartment involved in energy metabolism after

the chloroplast and the mitochondrion [62–64]. In addition, peroxisomes house reactions that produce H_2O_2. Fatty acid degradation and glyoxylate cycle generate the reducing equivalents NADH inside the peroxisome, whereas photorespiration consumes it. Therefore, CO_2 availability, by modulating the activity of photorespiration, can potentially impact these metabolic pathways. Plants and algae have therefore evolved several mechanisms that regulate NADH homeostasis in the peroxisome. This could be achieved either indirectly through the use of metabolite translocators involving the malate shuttle or directly through the import of NAD^+ via the NAD^+ carrier or through NAD^+ regeneration by a local antioxidant system [7,65,66]. In germinating castor bean, the transfer of NADH generated by fatty acid β-oxidation from the peroxisome to the mitochondria was postulated to occur through a malate/aspartate shuttle [67]. In arabidopsis, both direct and indirect routes occur, which include a peroxisomal NAD^+ carrier (PXN) belonging to the mitochondria carrier family (MCF) [7], the two peroxisomal MDHs (pMDH1, pMDH2) [68], the photorespiratory HPR [69], or the components of the ascorbate peroxidase/monodehydroascorbate reductase (APX/MDAR) electron transfer system [70]. The occurrence of these multiple mechanisms suggests the importance of the redox balance control in peroxisomes. Indeed, mutants deficient in either of these pathways have difficulty in seed germination, seedling rigor, or optimal growth.

The occurrence of plant-like peroxisomes has initially been debated in chlamydomonas, and this was mostly built on two observations (i) the lack of a crystalloid core under electron microscopy, typically observed in plant- or animal-type peroxisomes; and (ii) the likely location of catalase in mitochondria based on organelle purification studies [71]. But recent data have firmly established that chlamydomonas does have classical peroxisomes, and the evidence is: (i) peroxisomes have been observed in chlamydomonas under both confocal and electron microscopy [72,73]; (ii) the typical peroxisomal targeting signal 1 or 2 (PTS1/2) is functional in chlamydomonas [73]; (iii) the subcellular localization studies for enzymes of the glyoxylate cycle [4]; (iv) the identification of an H_2O_2-producing activity, that is acyl-CoA oxidase in the peroxisome and its role in fatty acid β-oxidation [63]; (v) catalase 1 is found to be peroxisomal in chlamydomonas [74]; and finally (vi) genes encoding homologs of the arabidopsis proteins involved in redox homeostasis (MDH, PXN, HPR, and MDAR) or in fatty acid β-oxidation are present [75].

That said, except for MDH2, experimental evidence for the function of the other proteins is still lacking. The chlamydomonas *mdh2* mutants, similar to its arabidopsis counterpart (the *pmdh1pmdh2* mutants), were compromised in oil remobilization during nitrogen recovery following a period of N deprivation [25], firmly establishing its contribution to fatty acid β-oxidation in green algae. Interestingly, in addition to peroxisome metabolism, metabolic responses have also been observed in the chloroplast, that is, enhanced photosynthetic activity and enhanced starch and *de novo* fatty acid biosynthesis under high CO_2 conditions (2% CO_2 supplementation of the air). It is worth mentioning here that the phenotypes of the chlamydomonas *mdh2* mutants were suppressed under atmospheric CO_2 levels (photorespiratory conditions), pointing to the complex contribution of diverse pathways to redox management and homeostasis. Nevertheless, the exact route and the molecular identity of the peroxisomal translocators involved remain to be identified (see Outstanding questions).

Concluding remarks and future perspectives

Because variations in environmental parameters may differentially affect the different cellular functions involved in the bioenergetics of photosynthetic cells, which are located in different subcellular compartments, plants and algae have evolved efficient trafficking of reducing equivalents between subcellular compartments to maintain redox homeostasis. Among these mechanisms, the 'malate shuttle' enables efficient transport of reducing equivalents between chloroplasts,

Outstanding questions

Does redox exchange occur between NADH and NADPH pools in the chloroplast? Both plant and algal chloroplasts contain two pools of pyridine nucleotides (NADH versus NADPH). Possible interaction between the two redox pools (NADH versus NADPH) in the chloroplast is not known but is likely mediated by the $NAD(P)^+$ transhydrogenase.

Do chlamydomonas chloroplasts contain an NAD-MDH? CrMDH1 is found peroxisomal based on fusion of the first 25 amino acids at the N terminus to the yellow fluorescent protein, but it is predicted to be chloroplastidial by PredAlgo. A key question is the subcellular location(s) of CrMDH1. The location of this enzyme could have implications for our understanding of metabolism and redox homeostasis not only for the chloroplast but also for the peroxisome.

Is there any connection between the enzymatic and non-enzymatic function of the plastidial NAD-dependent MDH? It was recently shown that the NAD-dependent MDH plays a moonlighting function in chloroplast metabolism and biogenesis. The moonlighting function is an interesting feature of some proteins to have more than one function in the cell. In addition to its enzymatic function as malate dehydrogenase, the plastidial NAD-dependent MDH plays a central role in chloroplast biogenesis through its physical interaction with the FtsH12–FtsHi protease complex. However, it is still not clear whether these two functions are connected or what is the biological significance of grouping these two functions (enzymatic and structural) in one widespread protein such as MDH.

What is the identity of the peroxisomal malate translocator? This is probably one of the most elusive components of peroxisome redox metabolism. Putative candidates are emerging with modern proteomics analysis of isolated peroxisomes.

What is the interaction between redox trafficking through the malate shuttle and carbon storage in microalgae? Starch and fatty acid biosynthesis are a major sink of cellular carbon and energy. Their synthesis requires a

cytosol, mitochondria, and peroxisomes. Research in recent decades has shed light on the role of the malate shuttle in key subcellular pathways, including photosynthesis, photorespiration, fatty acid β-oxidation, nitrogen assimilation, and interorganelle signaling, but has also raised new questions (detailed in Outstanding questions). Beyond physiology and energy homeostasis, emerging literature suggests a critical role for the malate shuttle in the CCM [15] and carbon storage [25].

Future investigations towards a detailed understanding of the malate shuttle mechanism in plants and algae should involve the combination of cutting-edge molecular genetics tools for creating combinatory mutations in different genes, overexpressing multiple isoforms combined with the use of ^{13}C labeling techniques to monitor changes in metabolic fluxes. We envision that emerging genetically encoded biosensors to monitor real-time changes in subcellular redox and ATP status will be valuable to monitor in real time redox status in subcellular compartments [76,77]. Taken together, by connecting energy homeostasis to carbon and nitrogen metabolism, the malate shuttle represents an exciting target for engineering the cellular energetic status for better climate resilience and for improved production of plant and algal biomass for food, fuel, and biomaterials, key to a sustainable and greener future.

carbon source, ATP, and NAD(P)H but at different ratios. Alterations of redox trafficking and cell energetic metabolism could have direct consequences for biomass productivity and composition.

What is the nature of the malate uniport mechanism in C4 plants? Using isolated chloroplasts from a range of C4 plants, two types of malate uniporters have been found, one coupled to sodium symport (in most C4 species tested), the other coupled to proton symport (in maize, sorghum, and sugar cane). In contrast to the metabolite translocators, this type of malate transporter would allow for net malate transport for, for example, the malic enzyme reaction.

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Declaration of interests

No interests were declared.

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