2-Hydroxy Acids in Plant Metabolism

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Glycolate, malate, lactate, and 2-hydroxyglutarate are important 2-hydroxy acids (2HA) in plant metabolism. Most of them can be found as D- and L-stereoisomers. These 2HA play an integral role in plant primary metabolism, where they are involved in fundamental pathways such as photorespiration, tricarboxylic acid cycle, glyoxylate cycle, methylglyoxal pathway, and lysine catabolism. Recent molecular studies in *Arabidopsis thaliana* have helped elucidate the participation of these 2HA in in plant metabolism and physiology. In this chapter, we summarize the current knowledge about the metabolic pathways and cellular processes in which they are involved, focusing on the proteins that participate in their metabolism and cellular/intracellular transport in Arabidopsis.

INTRODUCTION

2-Hydroxy acids or α-hydroxy acids (2HA or AHA) are molecules comprising at least two carbon atoms, where one forms a carboxylic acid and the other binds a hydroxyl group (Figure 1). The second carbon, bound to the hydroxyl group, can be referred to as the α-position or the 2-position. The proton of the carboxylic acid typically has a pKa < 4, and the 2HA will thus overwhelmingly be present in the deprotonated (conjugate base) form in plants. In this book chapter we will therefore refer to the molecules as their conjugate bases (e.g., glycolate, lactate) and not as the acid form (e.g., glycolic acid, lactic acid). Furthermore, all 2HA longer than two carbon atoms have a stereocenter at the α position. Thus, most of them can be found as D- and L-stereoisomers, which are metabolized by stereoisomer-specific enzymes.

2HA are commonly oxidized by dehydrogenases or oxidases (which convert the α-hydroxy group into a keto group) to form α-keto acids (2KA or AKA) (Figure 1A). Conversely, 2KA can be
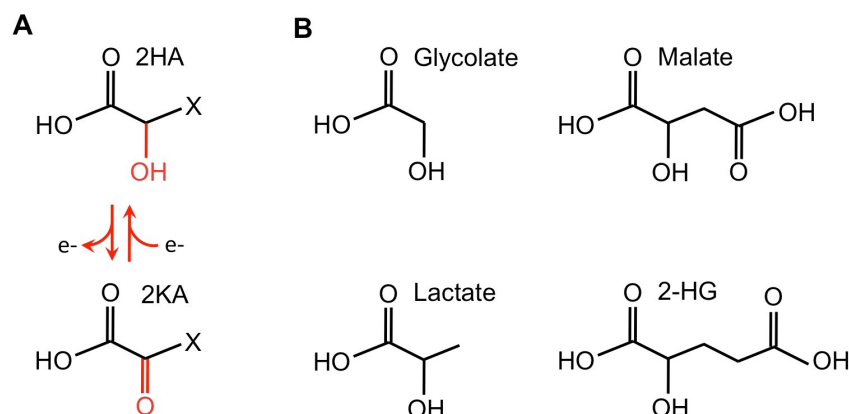

Figure 1.

A. Interconvertion of a 2-hydroxyacid (2HA) into a 2-ketoacid (2KA) **B.** Structure of the most relevant 2HA in plant metabolism. 2-HG, 2-hydroxyglutarate.

reduced to form chiral 2HA (Figure 1). 2HA and 2KA can thus be interconverted in a reversible manner. In reversible reactions the equilibrium constant (K_{eq}) of the reaction indicates in which direction the reaction is preferentially catalyzed. While K_{eq} close to 1 indicates a completely reversible reaction, very small or very large K_{eq} indicate that the reaction is essentially irreversible. However, a crucial feature of these reactions is often overlooked. The equilibrium constant is strongly influenced by the electron acceptor used in the reaction. Electron acceptors have varying affinity for electrons. This property is reported as the standard oxidation-reduction potential at pH 7 (E_0'). A negative E_0' number indicates poor affinity for electrons, while a positive number indicates high affinity for electrons. The redox potential of electron acceptors relevant to this chapter can be ordered from low to high (negative to positive): NAD(P)H < FAD << cytochrome c (cyt c)< O_2 (Figure 2). If an electron acceptor has poor affinity for electrons (negative numbers in Figure 2), it drives the reduction of the substrate in an enzymatic reaction. Conversely, if an electron acceptor has high affinity for electrons (positive numbers in Figure 2), it drives the oxidation of the substrate in an enzymatic reaction. Oxidases, which use O_2 as an electron acceptor, generate low 2HA to 2KA ratios when the reaction is at equilibrium. Conversely, dehydrogenases, which use NAD(P)H as a co-substrate, generate high 2HA to 2KA ratios when the reaction is at equilibrium. Dehydrogenases that use FAD or cyt c as an electron acceptor are between these two extremes. When interpreting the physiological function of redox active enzymes, it is important to consider these fundamental thermodynamic properties, as we highlight in this chapter.
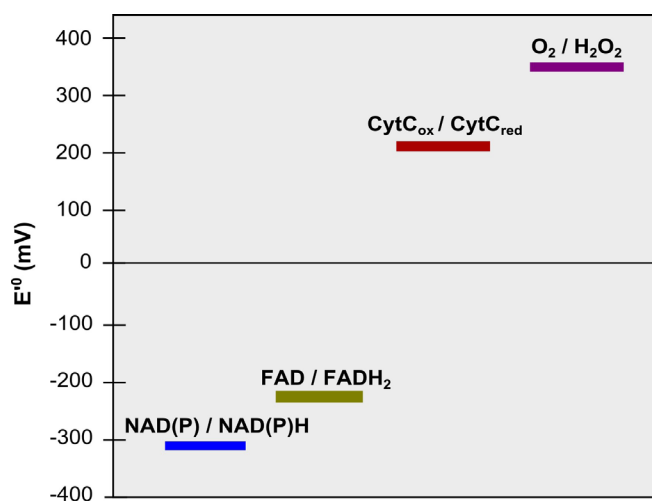

Figure 2. Overview of standard apparent reduction potential of important half-reactions.

Redox pairs that have very negative redox potentials (E'^0) have strong reducing power and pairs that have positive redox potentials have strong oxidizing power. The redox potentials strongly influence the substrate to product ratio at equilibrium in reactions involving interconversions of alpha-hydroxy and alpha-keto acids. The redox potentials were calculated at pH 7 and an ionic strength of 0.25 M except for the FAD/FADH$_2$ pair which was calculated at an ionic strength of 0.15 M.

2HA play an integral role in plant metabolism and form the branching points of many pathways. In this chapter, we review the current knowledge on some of the most important 2HA in plant primary metabolism: glycolate, lactate, malate, and 2-hydroxyglutarate (Figure 1B). Glycolate is the 2HA with the smallest molecular size and has no stereoisomers. Lactate is found in nature in both stereoisomeric forms, L- and D-lactate. These enantiomers participate in independent metabolic pathways. Malate is a dicarboxylic acid that has two stereoisomeric forms (L- and D-enantiomers), though only L-malate occurs naturally. 2-hydroxyglutarate participates in metabolism in two stereoisomeric forms, L- and D-2-hydroxyglutarate, which are involved in different metabolic pathways.

GLYCOLATE

Glycolate, a driver of photorespiration

During photosynthesis, glycolate is formed in the chloroplasts as a consequence of the oxygenase activity of ribulose bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) (Figure 3). In this reaction, ribulose-1,5-bisphosphate (RubP) is converted to 3-phosphoglycerate (3-PGA) and 2-phosphoglycolate (2-PG). This unavoidable biochemical side reaction accompanies the main activity of Rubisco, the carboxylation of RubP that forms 3-PGA. While 3-PGA is used in the synthesis of more complex sugars and other compounds, 2-PG is a dead-end metabolic product of high metabolic toxicity.

Plants evolved a complex metabolic pathway, the photorespiratory cycle, to convert 2-PG into 3-PGA (Maurino and Peterhansel, 2010). But this repair system is far from perfect: one of the four carbon atoms contained in two molecules of 2-PG entering the pathway is lost as CO_2, together with ammonia and energy (Figure 3), significantly limiting the biomass gains of C3 plants. During this carbon recovery system, dephosphorylation of 2-PGA in the chloroplasts produces glycolate by 2-PG phosphatase (PGLP). The glycolate formed does not undergo further reactions in the chloroplast; instead, it is transported to the peroxisome, where it is oxidized to glyoxylate and H_2O_2 by glycolate oxidase (GOX).

If 2-PG is a dead-end metabolic product, then why do plants produce it? When plants first evolved, the early atmosphere contained little oxygen (O_2) (Kasting and Howard, 2006; Kasting and Ono, 2006), so the inability of Rubisco to distinguish between O_2 and CO_2 was not a problem. As the O_2 level in the atmosphere gradually increased as a consequence of oxygenic photosynthesis by cyanobacteria, algae, and plants (Igamberdiev and Lea, 2006), the formation of 2-PG during photosynthesis began to rise, and this led to a problem which was solved by evolving the photorespiratory pathway. Seen in this context, the formation of 2-PG by Rubisco is a metabolic error, and its catabolism through the photorespiratory pathway is an evolutionary adaptation for making the best of a bad situation.

2-PG is toxic to plants since it inhibits glycolytic phosphofructokinase (Kelly and Latzko, 1976) and triose phosphate isomerase, which impairs the conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate, interfering with the regeneration of RubP (Figure 3) (Anderson, 1971). 2-PG also inhibits Rubisco activity (Quick et al., 1992). In this context, an Arabidopsis loss-of-function mutant of PLPG1 shows considerable growth only in
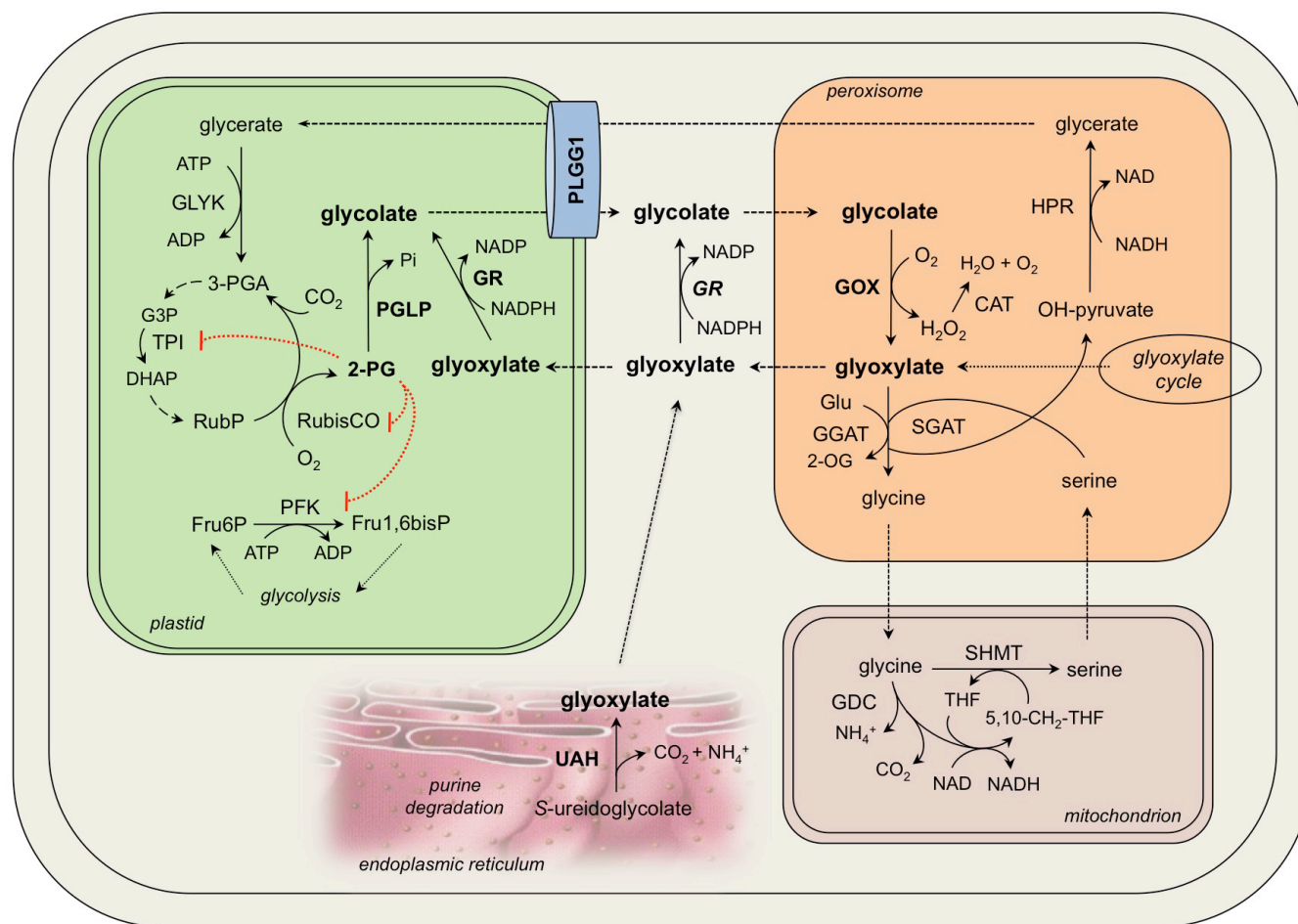
Figure 3. Participation of glycolate in plant metabolism (for a detailed explanation refer to the main text).

Metabolites and enzymes in bold type indicate the reactions directly involved in glycolate metabolism. Dotted lines represent connection to a metabolic pathway. Dashed lines represent transport processes. Red dotted lines represent inhibitory effects.

Abbreviations: CAT: catalase; 2; DiT1: and DiT2: dicarboxylate transporter 1 and 2; DHAP: dihydroxyacetone phosphate; Fru6P: fructose-6-phosphate; Fru1,6bisP: fructose-1,6-bisphosphate; G3P: glyceraldehyde-3-phosphate; GDC: glycine decarboxylase; GGAT: glutamate-glyoxylate aminotransferase; GLYK: glycerate kinase; GO: glycolate oxidase; GOGAT: glutamate-oxoglutarate aminotransferase; GR: glyoxylate reductase; HPR1: peroxisomal hydroxypyruvate reductase; PFK: phosphofructokinase; PGP: phosphoglycolate phosphatase; RubisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; RubP: ribulose-1,5-bisphosphate; SGAT: serine-glutamate aminotransferase; SHMT: serine hydroxymethyl transferase; THF: tetrahydrofolate; TPI: triose phosphate isomerase; UAH: ureidoglycolate amidohydrolase; 2-PG: 2-phosphoglycolate; 3-PGA: 3-phosphoglycerate; 5,10-CH$_2$-THF: 5,10-methylene-THF.

air containing more than 1% CO_2, while a complete restoration of growth occurs in 2% CO_2 (Schwarte and Bauwe, 2007). This high sensitivity of the plants to O_2 highlights that even the accumulation of small amounts of 2-PG due to the existence of small photorespiratory fluxes at 1% CO_2 are enough to impair plant development. Also, a PLPG mutant of the green alga *Chlamydomonas reinhardtii* is not viable in ambient air (0.04% CO_2), where it accumulates high amounts of 2-PG (Suzuki et al., 1990).

The accumulation of glycolate leads to the inhibition of metabolic processes that impair CO_2 assimilation, although the exact mechanism is not clear (González-Moro, 1997). Glycolate added to leaf extracts leads to the inhibition of Rubisco activity. The

highly toxic metabolic effects of glycolate were recently elegantly demonstrated *in planta* using a GOX loss-of-function mutant of the C4 specie maize (*Zea mays*) (*go1*) (Zelitch et al., 2009). C4 plants have low rates of photorespiration and GOX is confined to bundle sheath cell peroxisomes. In *go1*, GOX activity is extremely low, and glycolate accumulates at rates equal to the rate of 2-PG formation. These plants cannot survive under ambient CO_2 conditions and present impaired CO_2 assimilation in 1% O_2. The importance of an efficient elimination of glycolate is also evidenced in algae and cyanobacteria. *C. reinhardtii* growing in CO_2 limiting conditions, as well as many cyanobacteria strains growing under ambient air, excrete high amounts of glycolate formed during pho-

torespiration, even though they possess a fully functional carbon concentrating mechanism (Kaplan and Berry, 1981; Renstrom, 1989). The cyanobacterium Synechocystis sp. strain PCC 6803 possesses three alternative routes to metabolize glycolate, and only mutants deficient in all three pathways accumulate high levels of glycolate and present a high-CO_2-requiring phenotype (Eisenhut et al., 2008).

All these examples indicate that regardless of the mechanisms of 2-PG/glycolate toxicity, even minute traces of them exert cumulative detrimental effects and only the existence of efficient degradation pathways of 2-PG/glycolate can maintain normal development in organisms carrying on oxygenic photosynthesis.

Characterized proteins involved in plant glycolate metabolism

2-PG phosphatase (PGLP) converts 2-PG into glycolate

PGLP (EC 3.1.3.18) belongs to the family of the hydrolases and catalyzes the dephosphorylation of 2-PG (2-phosphoglycolate + H_2O = glycolate + phosphate) to generate glycolate (Figure 3). Arabidopsis possesses two genes encoding active PGLPs, At5g36700 (*PGLP1*) and At5g47760 (*PGLP2*) (Schwarte and Bauwe, 2007). As these isozymes were only partially purified from a heterologous expression system, comparative kinetic constants describing their biochemical behaviors are still lacking. Nevertheless, biochemical studies performed with the enzyme from other species indicated that spinach (*Spinacia oleracea*) and cyanobacterial PGLP have a broad pH optimum between 5.0 and 8.0, are specific for the hydrolysis of 2-PG, and have a requirement of Mg^{2+} and Cl^- for the enzymatic activity (Husic and Tolbert, 1984; Norman and Colman, 1991). In addition, ribose-5-P acts as an allosteric inhibitor of the enzyme from spinach and tobacco (*Nicotiana tabacum*) and as activator of the enzymes from *C. reinhardtii* (Husic and Tolbert, 1984; Mamedov et al., 2001). The activation of PGLP by both divalent cations and anions and the species-dependent inhibition or activation by ribose 5-P may be involved in the enzymatic mechanism and the *in vivo* regulation of the enzymatic activity. Experimental evidence indicates that Cl^- acts after the transfer of the phosphate group from 2-PG to PGLP. It is proposed that Cl^- assists the phosphoryl transfer from the phosphoenzyme, which is an intermediate in the reaction mechanism, to water by binding to the carboxyl site of the substrate (Seal, 1987).

In Arabidopsis only PGLP1, which localizes to chloroplasts, participates in photorespiration (Schwarte and Bauwe, 2007). PGLP1 knock-out mutants have very low leaf PGLP activity (97% reduction of total leaf PGLP activity compared to wild type) and cannot survive in ambient air; primary leaves become chlorotic very soon after germination, and the plants die within 3 weeks. However, such plants grow well in a CO_2-enriched atmosphere, where photorespiration is low (Somerville, 1979; Schwarte and Bauwe, 2007). This phenotypic feature results from the harmful effects of 2-PG accumulation described above, which reduce the synthesis of RuBP and the photosynthetic rate (Anderson, 1971; Kelly and Latzko, 1976). Deletion of PGLP2 does not result in a visible phenotype, and total leaf PGLP activity is unaltered (Schwarte and Bauwe, 2007). In line with these activity assays, PGLP1 shows 20-fold higher transcript levels in leaves than PGLP2, and much lower levels in non-photosynthetic organs. The

subcellular localization, substrate specificities, and physiological function of PGLP2 have not yet been experimentally clarified. Nevertheless, PGLP2 is not predicted to contain any organellar signal sequence, and it is thus most probably cytosolic.

The plastidal glycolate/glycerate translocator 1 (PLGG1)

Glycolate is exported from the chloroplasts through the glycolate/glycerate translocator (PLGG1; At1g32080) (Figure 3) (Pick et al., 2013). The *Arabidopsis* T-DNA insertion mutant, *plgg1*, is no longer able to transport glycolate and glycerate across the chloroplast envelope and has a mild photorespiratory phenotype (Pick et al., 2013). Under ambient CO_2 conditions, the mutant slowly develops bleached lesions on the leaf lamina, most probably due to the accumulation of toxic concentrations of glycolate and glycerate. This phenotype can be suppressed by growth under non-photorespiratory conditions. This transport of glycolate/glycerate was also characterized in isolated intact pea (*Pisum sativum*) chloroplasts (Howitz and McCarty, 1986) and in reconstituted vesicles containing chloroplast inner envelope membranes of spinach (Howitz and McCarty, 1991).

Glycolate oxidase (GOX) converts glycolate to glyoxylate

The oxidation of glycolate to glyoxylate in higher plants occurs in the peroxisomes and is catalyzed by GOX (EC 1.1.3.15), an octameric flavin-containing protein (Esser et al., 2014; Engqvist et al., 2015). During this reaction, O_2 is converted to H_2O_2, which *in vivo* is further dismutated by catalase (Figure 3) (Foyer et al., 2009). The reaction catalyzed by GOX is the only known route for metabolizing glycolate in plants (Zelitch et al., 2009). However, no mutant lacking GOX was ever recovered in classical screens. The presence of five distinct *GOX* loci in the Arabidopsis genome was hypothesized to be responsible for the failure to recover conditional *GOX* mutants. However, recent work clearly demonstrated that Arabidopsis possess only two GOX genes involved in the photorespiratory pathway, GOX1 (At3g14420) and GOX2 (At3g14415), while other members of the (L)-2-hydroxyacid-oxidase gene family, GOX3 (At4g18360), IHAOX1 (At3g14130), and IHAOX2 (At3g14150), are involved in non-photosynthetic functions (Esser et al., 2014; Engqvist et al., 2015). Moreover, only GOX1 and GOX2 are highly expressed in green organs (Reumann et al., 2004) and are coexpressed with other photorespiratory genes (Pick et al., 2013).

The oxidation of glycolate to glyoxylate requires O_2 as an electron acceptor. Arabidopsis GOX displays a sigmoidal kinetic dependence on O_2, with $S_{0.5}$ values of approximately 200 µM, and positive cooperativity for the binding (Engqvist et al., 2015). GOX1, GOX2, and GOX3 contain FMN as a prosthetic group and do not accept cytochrome c, NAD^+, or $NADP^+$ as electron acceptors. The enzymes show a Ping-Pong Bi-Bi kinetic mechanism, which was elucidated using human GOX (Pennati and Gadda, 2009). In this mechanism, the oxidized enzyme and the substrate form a reversible Michaelis complex (E-FMN_{ox}·S). Then, the enzyme reacts with glycolate irreversibly to generate glyoxylate with concomitant reduction of the enzyme-bound flavin (E-FMN_{red}·P). The catalytic cycle is completed with the release of glyoxylate

from the active site of the enzyme, followed by the second-order reaction of the reduced enzyme-bound FMN with O_2 to generate H_2O_2 and an oxidized enzyme. The availability of the three dimensional structure of spinach GOX allowed the construction of a homology model of Arabidopsis GOX1 and the identification of active site amino acid residues involved in substrate binding and substrate specificity (Lindqvist and Branden, 1985, 1989; Stenberg and Lindqvist, 1997; Esser et al., 2014).

All three purified recombinant isozymes from Arabidopsis have a pH optimum of 7.5 and a narrow substrate specificity. They display the highest activities using glycolate, followed by L-lactate (Engqvist et al., 2015). The relative activities with L-lactate are 46%, 48%, and 76% in the case of GOX1, GOX2 and GOX3, respectively. On the other hand, all isoforms can additionally utilize other long- and medium-chain 2-hydroxy acids, although with much lower activities (e.g., 2-hydroxyoctanoic acid, leucic acid) (Engqvist et al., 2015).

The photorespiratory isoforms, GOX1 and GOX2, use glycolate with much higher efficiencies than L-lactate, while GOX3 is able to use L-lactate with a similar efficiency as it uses glycolate. GOX1, GOX2, and GOX3 have similar high catalytic efficiencies (k_{cat}/K_m) for the oxidation of glycolate, with values of 911, 744, and 725 min^{-1} mM^{-1}, respectively. With L-lactate as substrate, GOX1 and GOX2 have 22- and 31-fold lower catalytic efficiencies than with glycolate, which results from low affinities towards L-lactate (Engqvist et al., 2015). In contrast, GOX3 has a catalytic efficiency only 3.8-fold lower than with glycolate, which results from a much higher affinity to L-lactate in comparison to GOX1 and GOX2 (Engqvist et al., 2015). None of the isozymes use D-lactate as substrate, nor do they catalyze the reverse reaction starting from glyoxylate with either NADH or NADPH.

As expected from the redundant biochemical behavior and expression pattern of the photorespiratory GOX1 and GOX2, Arabidopsis knock-out mutants of single enzymes do not present a visible phenotype; double knock-out mutants are very difficult to generate, as both genes are close to each other on the same chromosome. Knowledge of the consequences of a loss-of-function of photorespiratory GOX was obtained with a maize mutant caused by an *Activator* insertion in the photorespiratory GOX gene (Zelitch et al., 2009). These plants had greatly decreased glycolate oxidase activity and were nonviable at ambient CO_2 concentrations. Under high CO_2 conditions, GOX deficiency had no visible adverse effects on plant growth. However, when the mutants were transferred from growth in high CO_2 to ambient air in the light, CO2 assimilation was rapidly and linearly diminished and glycolate accumulated linearly for 6 h to levels 7-fold greater than the initial concentration. Also in rice and tobacco, GOX moderately silenced knock-down lines present reduced growth and increased glycolate accumulation in ambient air, and highly silenced lines are not viable (Yamaguchi and Nishimura, 2000; Xu et al., 2009).

The GOX of plantae is an ancient peroxisomal eukaryotic enzyme recruited into photorespiration. Previous speculations suggested that plant GOX was derived from a cyanobacterial L-lactate oxidase (LOX) acquired through primary endosymbiosis (Hackenberg et al., 2011; Kern et al., 2013), and that the GOX activity evolved only when land plants appeared (Bauwe et al., 2012). A more comprehensive phylogenetic analysis showed, however, that the biological role of GOX in photorespiration evolved by co-opting an existing peroxisomal protein of the common eukaryotic ances-

tor, which already possessed GOX activity (Esser et al., 2014). An additional LOX sequence was indeed acquired by the common ancestor of plants and algae from the cyanobacterial endosymbiont. While most algal lineages (glaucophyta, rhodophyta, and charophyta) independently lost the cyanobacterial LOX, chlorophyta replaced eukaryotic GOX with cyanobacterial LOX. Duplication and diversification of an ancestral GOX occurred independently in ancestral animalia and vascular plants (tracheophyta), giving rise to the lHAOX subfamilies (Esser et al., 2014).

Do plants possess a glycolate dehydrogenase? An evolutionary view

As described above, enzymatic removal of glycolate is an essential part of photorespiration, a pathway that takes place in all organisms that drive oxygenic photosynthesis. In archaeplastida, the oxidation of glycolate is catalyzed by two phylogenetically unrelated enzymes: GOX and glycolate dehydrogenase (GlcDH; EC 1.1.99.14).

Land plants and the algal lineages glaucophyta, rhodophyta, and charophyta have GOX activity, which produces H_2O_2 in peroxisomes (Frederick et al., 1973; Stabenau; Stabenau and Winkler, 2005), and was inherited from the eukaryotic ancestor of the primary endosymbiosis (Esser et al., 2014). These species do not possess a GlcDH activity; instead, they possess a homolog of cyanobacterial GlcDH that functions in mitochondria as a D-lactate dehydrogenase, and not as GlcDH as previously proposed (Bari et al., 2004; Engqvist et al., 2009; Wienstroer et al., 2012); see next section). In contrast, in the green alga lineage chlorophyta and as well as in cyanobacteria, glycolate oxidation is catalyzed by GlcDH (Nelson and Tolbert, 1970; Beezley et al., 1976; Nakamura et al., 2005; Stabenau and Winkler, 2005; Eisenhut et al., 2008). These organisms do not possess GOX. Instead, they possess a bacterial homolog with LOX activity (Hackenberg et al., 2011; Esser et al., 2014). Chlorophyta replaced the ancestral eukaryotic GOX with a LOX obtained from cyanobacteria through the primary endosymbiosis (Esser et al., 2014).

The presence of GOX in charophytes may have been crucial for the evolution of land plants, as the high turnover rate of this enzyme would have enabled their evolution in the presence of substantial atmospheric O_2 concentrations. In contrast, the use of GlcDH in the mitochondrial glycolate pathway is energetically favorable (it produces ATP), but GlcDH also has a lower turnover rate for glycolate in comparison to GOX (Stabenau and Winkler, 2005). Such a low capacity system for the detoxification of glycolate is sufficient in cyanobacteria and chlorophyta due to their lower rates of photorespiratory glycolate synthesis owing to the presence of a carbon concentrating mechanism, and to the fact that they can excrete glycolate under conditions promoting its high formation (Giordano et al., 2005; Stabenau and Winkler, 2005; Huege et al., 2011).

Glyoxylate reductase (GR): a recycling enzyme

In case any glyoxylate would leak out of the peroxisome, the cytoplasmic rescue enzyme glyoxylate reductase (GR; EC 1.1.1.79)

would reduce glyoxylate to glycolate using NADPH (Figure 3) (Allan et al., 2009). The glycolate produced in the reaction must then be transported back into the peroxisome to be fed back into the photorespiratory pathway, or to be converted into serine, which serves as a buffer for one-carbon units (Figure 3). GR is a member of the ß-hydroxyacid dehydrogenase protein family (Hoover et al., 2013). In Arabidopsis, cytosolic GR1 (At3g25530) and plastidial GR2 (At1g17650) catalyze the essentially irreversible conversion of glyoxylate to glycolate with K_m values of 4.5 and 34 µM, respectively (Hoover, 2007; Simpson et al., 2008). Both isozymes can also convert succinic semialdehyde (SSA) - an intermediate in GABA metabolism - into γ-hydroxybutyrate. These enzymes have a 250- to 350-fold higher preference for glyoxylate than SSA based on the catalytic efficiency (K_{cat}/K_m) (Hoover, 2007; Simpson et al., 2008). The action of GR may be a means to recycle glyoxylate derived from purine degradation.

Glycolate in non-photosynthetic tissues

As described above, in green tissues glycolate is produced as a consequence of the oxygenase activity of Rubisco. Nevertheless, this 2HA is also present in non-photosynthetic plant tissues, such as roots and seeds ((Engqvist et al., 2015); Schmitz and Maurino, unpublished results). What is the source and what is the fate of glycolate in these tissues? In plants, the sequential action of Rubisco and PGLP is the only known pathway for the formation of glycolate. Furthermore, the only known mechanism for the metabolism of glycolate is its conversion to glyoxylate by GOX in peroxisomes (Zelitch et al., 2009).

Although there are no reports on the production of glycolate via Rubisco in heterotrophic tissues, this cannot be ruled out; proteomic studies revealed the presence of the small and large subunits of Rubisco (At1g67090 and AtXg00490) in heterotrophic tissues, albeit at much lower amounts in comparison with green tissues (Baerenfaller et al., 2008). An alternative way to produce glycolate might be from glyoxylate through the action of glyoxylate reductase (Hoover, 2007; Allan et al.; Simpson et al.; Allan et al., 2009), where glyoxylate can arise through the degradation of purines or the glyoxylate cycle (see below).

These processes appear incompatible with the high amounts of glycolate found in non-photosynthetic tissues or may result in a futile cycle. Moreover, the glyoxylate cycle is not always active, especially in mature organs such as roots. Thus, most probably glycolate is transported from source to sink organs. The expression of all GOX genes in Arabidopsis in non-photosynthetic organs (Esser et al., 2014), such as roots and endosperm, strengthens the hypothesis that in these organs glycolate is converted to glyoxylate for its further integration into plant metabolism through the synthesis of serine, which is essential for the biosynthesis of biomolecules and for one-carbon (C1) metabolism (Cossins, 1967; Kalhan and Hanson, 2012).

Fates of glyoxylate

Glyoxylate is a very reactive aldehyde that forms adducts with free amines and sulfhydryl groups and forms Schiff bases with lysyl residues of proteins. It has the potential to react with DNA, oxidize membrane lipids, and modify proteins, thereby causing cellular and developmental problems. Glyoxylate inhibits CO_2 assimilation through a reduction of the activation state of Rubisco (Cook et al., 1985; Chastain, 1989; Wendler, 1992). Moreover, *in vivo* accumulation of this metabolite induces a decline in RuBP accumulation (Chastain, 1989).

In plants, glyoxylate is not only produced and further metabolized in the peroxisome during photorespiration (i); it is also an intermediary in the glyoxylate cycle (ii) and originates as a product of purine degradation in the endoplasmic reticulum (iii) (Figure 3).

(i) Photorespiratory produced glyoxylate is converted to glycine through the action of glutamine:glyoxylate aminotransferase (GGAT) and serine-glyoxylate aminotransferase (SGAT). Glycine is then transported to the mitochondria and further converted to serine through the successive action of glycine decarboxylase and serine hydroxymethyl transferase (Figure 3). The cycle is closed by the transport of serine back to the peroxisome, where it is converted to hydroxypyruvate and further to glycerate by the successive actions of SGAT and hydroxypyruvate reductase. Glycerate is finally transported to chloroplasts, where glycerate kinase phosphorylates it to 3P-glycerate, which can enter the Calvin Benson cycle (Figure 3).

(ii) The glyoxylate cycle is active during seed germination and allows the formation of 4-carbon units from acetyl-CoA, which derives from ß-oxidation of fatty acids or the degradation of amino acids (see below). In the glyoxylate cycle, cleavage of isocitrate by isocitrate lyase (ICL) generates glyoxylate and succinate. The resulting glyoxylate is further condensed with acetyl-CoA into malate by malate synthase (MS) (Wanders and Waterham, 2006; Theodoulou and Eastmond, 2012).

(iii) Glyoxylate can also be derived from purine ring degradation (Werner and Witte, 2011). In the last step of this pathway, ureidoglycolate amidohydrolase (UAH) hydrolyzes *S*-ureidoglycolate to glyoxylate, CO_2, and two molecules of NH_3 in the endoplasmic reticulum (Werner et al., 2010) (Figure 3). The produced glyoxylate would be metabolized to glycine and serine, presumably by enzymes homologous to those of the photorespiratory pathway, or condensed with acetyl-CoA providing malate. It is not known if glyoxylate is further metabolized in the endoplasmic reticulum. If this is not the case, it has not yet been determined how glyoxylate leaves the endoplasmic reticulum and enters the peroxisomes. This pathway would take place in green tissues, as *UAH* is expressed in leaves (Werner et al., 2010).

Manipulation of glycolate metabolism

Modifications of intracellular glycolate metabolism by genetic engineering have been used both as tools to understand cellular processes as well as to improve growth and water use efficiency of C3 plants.

Fahnenstich et al. (2008) produced Arabidopsis plants that overproduce photorespiratory GOX in the chloroplasts (GO plants). In GO plants, glycolate produced by Rubisco is converted to glyoxylate and H_2O_2 in chloroplasts. The production of H_2O_2 in GO plants can be modulated by light intensity and air CO_2 con-

centrations. These plants thus provide a tunable genetic system to perturb H_2O_2 levels in the chloroplasts and to study the resulting effects. GO plants grow like wild-type plants under non-photorespiratory conditions, but develop oxidative stress lesions under high photorespiratory fluxes (Fahnenstich et al., 2008). H_2O_2 is responsible for the GO phenotype, as GO plants coexpressing enzymes that further metabolize glyoxylate still accumulate H_2O_2 and show all features of the GO phenotype. A similar phenotype is present in catalase loss-of-function mutants (*cat2* plants), which cannot detoxify H_2O_2 produced in peroxisomes by GOX. These genetically modified plants were used to perform a comparative analysis of peroxisomal H_2O_2 signalling effects (Balazadeh et al., 2012; Sewelam et al., 2014).

Photorespiration lowers photosynthetic efficiency, as CO_2 and ammonia are lost and must be re-assimilated. This leads to consumption of ATP and reducing power. In higher plants, photorespiration may result in a loss of at least 25% of the carbon fixed, with enhanced losses at high temperatures and low stomata aperture (Leegood, 1995). Thus, a reduction of the flux through photorespiration should enhance CO_2 fixation and plant growth (Maurino and Weber, 2013). One way to achieve this is by increasing the metabolism of glycolate formed by the oxygenase activity of RubisCO directly in the chloroplasts (Peterhansel and Maurino, 2011). The feasibility of this strategy was recently shown through the introduction of two independent intrachloroplastic glycolate oxidation pathways in Arabidopsis. In one of the approaches, the glycolate catabolic pathway of *E. coli*, which converts glycolate into glycerate, was expressed in Arabidopsis chloroplasts (Kebeish et al., 2007). In an alternative approach, a glycolate catabolic cycle designed *de novo*, which completely oxidizes glycolate to CO_2, was engineered into Arabidopsis chloroplasts (Maier et al., 2012). Both these synthetic pathways led to plant growth improvements, indicating that they are beneficial under the conditions studied.

LACTATE

Lactate is a 2HA with a single methyl group at the α carbon in addition to the alcohol Figure 1B). It is thus the smallest chiral 2HA. The L- and D-forms of the molecule have completely different metabolic origins in the cell, and are metabolized in different subcellular compartments. L-lactate is generated through reduction of pyruvate in the cytosol, and is re-converted to pyruvate in peroxisomes. D-lactate is generated as the end product of the methylglyoxal detoxification pathway and is oxidized to pyruvate in mitochondria. The metabolism of L- and D-lactate is complex and plants make use of redox enzymes with different electron acceptors to control the directionality of lactate and pyruvate interconversions.

L-lactate

L-lactate is the stereoisomer of lactate that most people are "familiar" with and typically refer to when talking about lactate or lactic acid. It is the form that accumulates in yoghurt and other dairy products during bacterial fermentation of lactose and other

sugars, and it is also the form of lactate that accumulates in human muscles during strenuous exercise. In plants, L-lactate functions as an electron sink during microoxic conditions (hypoxia) (Dolferus et al., 2008; Pucciariello et al., 2014), but is also involved in plant defense against pathogens (Winter et al., 2007).

Biosynthesis of L-lactate: L-lactate dehydrogenase (L-LDH)

Microoxic conditions are mainly encountered in plant roots during waterlogging (flooding of the roots) (Bailey-Serres et al., 2012). Cellular catabolism of glucose reduces NAD^+ to NADH. NADH is then re-oxidized in oxidative phosphorylation in the mitochondrion. However, in the absence of oxygen - the terminal electron acceptor - NADH accumulates. Reactions that require NAD^+ are then unable to proceed or can only proceed at very low rates.

To alleviate the over-reduction of the NAD^+ pool, plants respond by producing L-lactate, ethanol, and alanine through fermentation (Dolferus et al., 1997). Ethanol is generated through the concerted action of pyruvate decarboxylase (PDC, EC 4.1.1.1) and alcohol dehydrogenase (ADH, EC 1.1.1.1). L-lactate is produced from pyruvate by the cytosolic NAD-dependent L-lactate dehydrogenase (L-LDH, EC 1.1.1.27, At4g17260) (Paventi et al., 2007; Dolferus et al., 2008; Passarella et al., 2008). L-LDH uses the electrons of NADH to reduce pyruvate to L-lactate, regenerating $NAD(P)^+$ in the process (Dolferus et al., 2008). The reaction is thermodynamically driven towards L-lactate production by the intrinsically low redox potential of the NAD^+/NADH cofactor as well as the high availability of NADH substrate vs. NAD^+. Plants lacking L-LDH completely lose the ability to generate L-lactate (Dolferus et al., 2008). Conversely, over-expressing L-LDH can protect Arabidopsis root tissue during extensive waterlogging (Dolferus et al., 2008). The production of L-lactate is transient and precedes a more sustained ethanol production (Dolferus et al., 1997). Consistent with its role in hypoxia, the expression of L-LDH is induced 10-fold in hypoxic roots, and only 2- to 3-fold in hypoxic leaves (Mustroph et al., 2009). Increased lactic acid fermentation, due to L-LDH over-expression, also leads to increased levels of PDC. Furthermore, ADH and L-LDH expression in roots are both stimulated by abiotic stresses, such as mechanical wounding, drought and cold stress (Dolferus et al., 1994; Dolferus et al., 2008).

L-lactate is not only produced in roots, and its physiological role is not restricted to being an electron sink during hypoxia. L-lactate is continuously produced also in leaf tissue by L-LDH. The aerobic lactate production is due to a basal expression of L-LDH, as demonstrated by Northern bot analysis, β-glucuronidase (GUS) reporter lines, and by a basal L-LDH activity in normoxia (Dolferus et al., 2008). L-LDH expression levels are moderately induced during infection with *Botrytis cinerea* (Winter et al., 2007). Furthermore, mechanical wounding of leaves results in L-LDH expression in cells a few cell layers away from the wounding site (Dolferus et al., 2008). The role of L-LDH in leaves has not yet been completely elucidated, but it may be important in plant defense against pathogens (see below).

Excretion of L-lactate to the rizosphere, and catabolism through GOX3, a peroxisomal short-chain (L)-2-hydroxyacid-oxidase with high affinity for L-lactate

As mentioned above, plant L-LDH reduces pyruvate to L-lactate. However, this enzyme does not efficiently catalyze the oxidation of L-lactate to pyruvate, as NAD^+, the redox partner of L-LDH, has a relatively low affinity for electrons. The reaction catalyzed by L-LDH thus results in a high L-lactate to pyruvate ratio at equilibrium (Halprin and Ohkawara, 1966).

What then is the fate of L-lactate formed in different plant tissues? Arabidopsis excretes most L-lactate formed during microoxic conditions into the surrounding medium (Dolferus et al., 2008; Engqvist et al., 2015). This excretion is facilitated by NIP2.1 (Nodulin 26 intrinsic proteins; At2g34390), a L-lactate transporter that is highly sensitive to anaerobiosis (Choi and Roberts, 2007). In roots of plants subjected to waterlogging, transcript levels of NIP2.1 increase rapidly during the first hour of hypoxia and then decline to steady state levels (Choi and Roberts, 2007; Mustroph et al., 2009). A further indication that L-LDH does not re-oxidize L-lactate to pyruvate is that L-LDH loss-of-function mutants take up and metabolize externally added L-lactate at the same rate as wild-type plants (Dolferus et al., 2008).

In Arabidopsis, L-lactate is also oxidized to pyruvate by glycolate oxidase 3 (GOX3, EC 1.1.3.15), a peroxisomal enzyme that uses oxygen as an electron acceptor (Engqvist et al., 2015). In contrast to NAD^+, O_2 has a very high affinity for electrons; thus, GOX3 very efficiently drives L-lactate oxidation, resulting in decreased physiological L-lactate to pyruvate ratios (Engqvist et al., 2015). It is not clear how L-lactate enters the peroxisome or how pyruvate exits it. In humans, a peroxisomal monocarboxylate transporter is responsible for this exchange (McClelland et al., 2003).

In Arabidopsis, the gene At4g18360 encodes GOX3. This enzyme and the two homologs GOX1 and GOX2 all have activity towards both glycolate and L-lactate (see glycolate section). While the three enzymes have similar catalytic efficiencies towards glycolate, GOX3 has higher affinity for L-lactate, with a K_m 5-10 times lower than that of GOX1 and GOX2, while the associated catalytic efficiency is 5-10 times higher (Engqvist et al., 2015). In roots, GOX3 ensures the sustainment of low levels of L-lactate after its formation under normoxia. In plants grown under normoxic conditions, loss-of-function of *GOX3* induces metabolic rearrangements that mirror wild-type responses under hypoxia (Engqvist et al., 2015).

In roots, the combined expression of GOX1 and GOX2 is five times lower than the expression of GOX3. Conversely, the combined expression of the GOX1 and GOX2 genes in Arabidopsis leaves is 300 times higher than that of GOX3 (Schmid et al., 2005; Winter et al., 2007). These expression data fit well with the roles of GOX1 and GOX2 in photorespiratory glycolate metabolism in green tissues and that of GOX3 in root L-lactate metabolism. However, in some conditions the expression levels of the GOX isoforms are significantly modified. For instance, 48 hours after inoculation of Arabidopsis leaves with *Botrytis cinerea,* the combined expression of GOX1 and GOX2 in leaves is reduced 3-fold, whereas the expression of GOX3 increases almost 30-fold (Winter et al., 2007). Therefore, in these conditions, the combined expression level of GOX1 and GOX2 in leaves becomes comparable to that of GOX3. Arabidopsis thus responds to *B. cinerea* infection by decreasing its glycolate metabolic capacity and increasing its L-lactate metabolic capacity.

GOX3 is also involved in non-host resistance in Arabidopsis, by regulating plant defense responses through the production of H_2O_2 (Rojas and Mysore, 2012; Rojas et al., 2012). The substrate for GOX3 in these conditions are likely L-lactate and glycolate, since the enzyme has activity towards both substrates (Engqvist et al., 2015). Furthermore, mechanical wounding of leaf tissue results in L-LDH expression in cells a few cell layers away from the wounding site (Dolferus et al., 2008). This increased expression should lead to greater availability of L-lactate substrate for GOX3 in these conditions. This role for GOX3 in plant pathogen resistance is further substantiated by co-expression data showing GOX3 co-expression with several chitinases and glucanases, which play an important role in plant non-adaptive resistance (Sawaki et al., 2009). Furthermore, in Arabidopsis cell cultures the oxidative burst coincides with a 50% decrease in L-lactate levels (O'Brien et al., 2012). It was hypothesized that L-LDH oxidizes L-lactate stored in leaves to pyruvate to reduce NAD^+ to NADH. The NADH could in turn be used by NADPH oxidase to produce H_2O_2 (O'Brien et al., 2012). However, the equilibrium of the reaction catalyzed by L-LDH lies firmly towards L-lactate formation and not L-lactate consumption. A more likely candidate for the L-lactate consumption is GOX3, which can generate H_2O_2 directly (Engqvist et al., 2015).

A model therefore emerges where L-LDH and GOX3 form a futile cycle, which cycles between L-lactate and pyruvate while consuming NADH and generating the highly oxidizing agent H_2O_2 (GOX3: L-lactate → pyruvate + H_2O_2 and L-LDH: pyruvate + NADH → L-lactate + NAD^+) (Engqvist et al., 2015).

D-lactate

In contrast to L-lactate, D-lactate is not a fermentation product in eukaryotes. Instead, D-lactate is the end product of the glyoxalase pathway (Thornalley, 1990; Atlante et al., 2005). This detoxification pathway removes methylglyoxal (Figure 4). The fact that D-lactate is the end product of a detoxification pathway has been used to engineer a system for antibiotic-independent selection in plants. Transgenic plants that over-express a D-lactate dehydrogenase (D-LDH) are more resistant to externally added D-lactate and can thus be discriminated from non-transgenic plants (Wienstroer et al., 2012).

Biosynthesis of D-lactate: the methylglyoxal pathway

In plants, D-lactate is produced during the detoxification of methylglyoxal, a highly reactive, cytotoxic compound formed as side product of primary metabolism (Mustafiz et al., 2014; Shimakawa et al., 2014). Methylglyoxal is formed enzymatically as well as non-enzymatically through phosphate elimination of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Figure 4) (Kalapos, 1999; Chen and Thelen, 2010). The enzymatic phosphate elimination is catalyzed as an undesirable side reac-
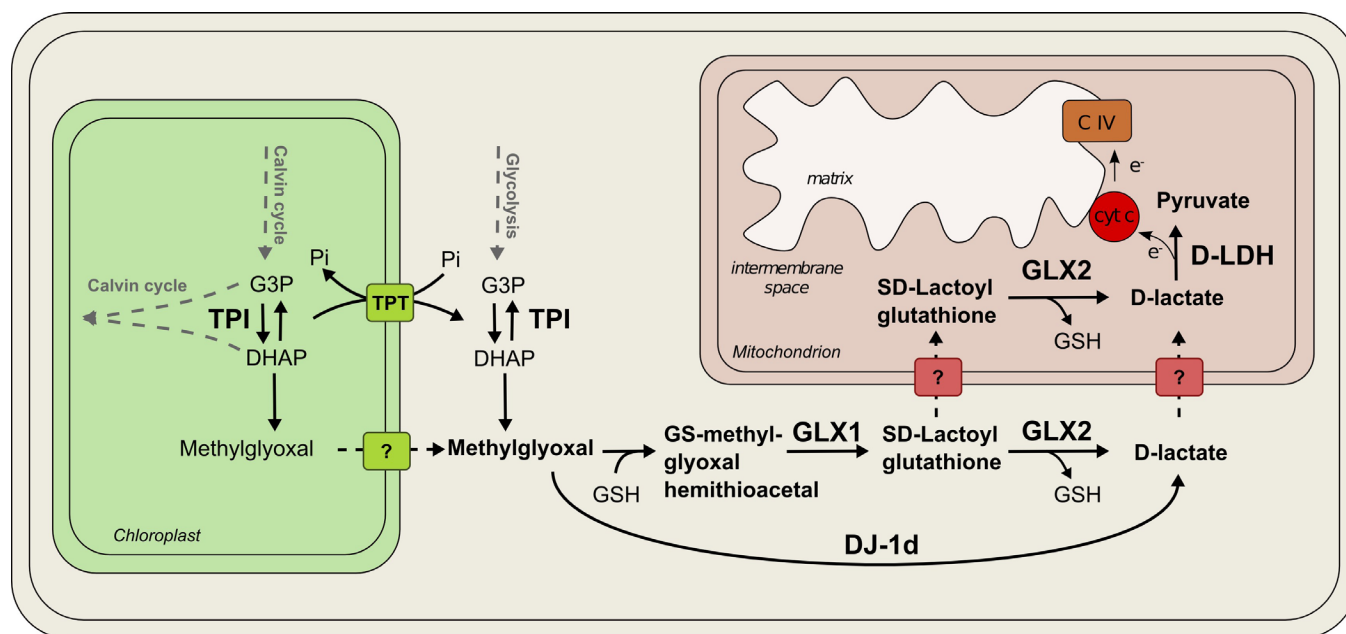
Figure 4. Generation of D-lactate by metabolism of methylglyoxal through the glyoxalase system (GLX1 and GLX2) or the DJ-1d enzyme (for a detailed explanation refer to the main text).

Dashed grey lines represent connections to metabolic pathways. Dashed black lines represent transport processes. In the mitochondrion D-lactate is metabolized to pyruvate by D-LDH. Cytochrome c acts as an electron acceptor for this enzyme.

Abbreviations: TPI: triose phosphate isomerase, GLX1: glyoxalase I, GLX2 glyoxalase II, DJ-1d: glyoxalase III, D-LDH: D-lactate dehydogenase, TPT: triose phosphate translocator, cyt c: cytochrome c, C IV: complex IV, GSH and GS: glutathione, G3P: Glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate.

tion of cytosolic triose phosphate isomerase (cTPI, EC 5.3.1.1, At3g55440) as well as by its plastidic isozyme (pdTPI, At2g21170) (Phillips and Thornalley, 1993; Takagi et al., 2014). Production of methylglyoxal is higher during abiotic stresses such as salt, drought, cold, and heavy metal stress (Yadav et al., 2005; Xu and Chen, 2006) and may contribute to the formation of reactive oxygen species (ROS) (Saito et al., 2011; Hoque et al., 2012). The detoxification of methylglyoxal is important for plant stress resistance. Arabidopsis has several enzymatic pathways to detoxify this compound; at least two of them convert methylglyoxal to D-lactate (Figure 4).

First, methylglyoxal is metabolized to D-lactate through the glyoxalase pathway, which comprises the enzymes glyoxalase I (GLX I, EC 4.4.1.5) and glyoxalase II (GLX II, EC 3.1.2.6) (Mustafiz et al., 2014). There are eleven genes which encode putative GLX I enzymes in Arabidopsis and five genes which encode putative GLX II enzymes (Mustafiz et al., 2011). The glyoxalase pathway is initiated through a non-enzymatic step where methylglyoxal spontaneously reacts with glutathione to form glutathione-methylglyoxal hemithioacetal. GLX1 then catalyzes the conversion of this hemithioacetal to S-D-lactoylglutathione. Subsequently, GLX2 hydrolyzes S-D-lactoylglutathione to regenerate glutathione and to release D-lactate. In Arabidopsis, GLX I was only found in the cytosol, whereas GLX II localizes to the cytosol and mitochondria (Maiti et al., 1997; Atlante et al., 2005).

Second, methylglyoxal can also be metabolized to D-lactate in a single step through the glutathione-independent cytosolic enzyme glyoxalase III (DJ-1d, EC 4.2.1.130, At3g02720) (Kwon et al., 2013). This enzyme is a newly discovered hydro-lyase that requires no cofactors (Misra et al., 1995; Subedi et al., 2011). Its catalytic mechanism is not yet fully understood, but seems to rely on two conserved Cys and Glu residues (Choi et al., 2014). In addition to DJ-1d, there are at least five other predicted members of the DJ-1 family in Arabidopsis, but these have very low activity with glyoxylate *in vitro* and likely use a different substrate *in vivo* (Kwon et al., 2013).

Catabolism of D-lactate: D-lactate dehydrogenase

D-lactate is metabolized in the mitochondrial intermembrane space by a cytochrome c-dependent D-lactate dehydrogenase (D-LDH, EC 1.1.2.4, At5g06580) (Figure 4) (Engqvist et al., 2009; Wienstroer et al., 2012). In addition to D-lactate, D-LDH accepts other 2HA substrates, such as D-2-hydroxybutyrate (D-2HB), glycolate, and glycerate. However, the catalytic rates with D-glycerate (k_{cat} = 6 min^{-1}) and glycolate (k_{cat} = 0.1 min^{-1}) are low, making them unlikely *in vivo* substrates. In contrast, the enzyme oxidizes D-lactate (k_{cat} = 65 min-1) and D-2HB (k_{cat} = 88 min-1) at 10-fold higher rates. These higher rates are combined with high affinities,

making the catalytic efficiency of D-LDH for these two substrates very high. D-2HB is not known to be involved in plant metabolism, thus making D-LDH the most likely physiologically relevant substrate for D-LDH (Engqvist et al., 2009).

D-LDH has a short N-terminal hydrophobic sequence. This structural feature likely anchors the enzyme to the outside of the mitochondrial inner membrane, where it may interact with the cyt c electron acceptor (Figure 4) (Engqvist et al., 2009). The use of cyt c as a cofactor ensures that the reaction is thermodynamically driven forward. Despite D-LDH's role in methylglyoxal detoxification, D-LDH loss-of-function plants do not display any phenotype under normal growth conditions. It may be that the AKR enzymes can compensate for the loss, or that another enzyme may metabolize some of the formed D-lactate. However, the loss-of-function plants showed increased sensitivity to D-lactate and methylglyoxal (the precursor of D-lactate, see section above) supplied to the growth medium (Engqvist et al., 2009).

MALATE

Participation of malate in plant metabolism

The metabolism of malate occupies a central role in plants, as it is involved in a whole range of processes. Malate is intimately associated with mitochondrial energy metabolism as an intermediate of the tricarboxylic acid (TCA) cycle. Malate is also the origin of carbon skeletons exported from the mitochondrion in support of amino acid biosynthesis. Moreover, it is an important form of fixed carbon that can be rapidly metabolized, is involved in regulating stomatal aperture, plays a role in nutrient uptake as a component of root exudates, and also functions as a reducing equivalent that is shuttled between subcellular compartments (Fahnenstich et al., 2007; Fernie and Martinoia, 2009; Zell et al., 2010). In addition to these roles in plant growth and development, malate has been co-opted as a key metabolite in the photosynthetic process in species that use C4 or Crassulecan acid metabolism (CAM) photosynthesis (Maier et al., 2011). In this section, we review the roles of malate in plant metabolism, focusing on the enzymes and transporters involved.

Carbon storage & respiratory substrate

Malate and fumarate constitute a significant fraction of the fixed carbon in Arabidopsis and other C3 plants, functioning as temporary carbon storage molecules (Chia et al., 2000; Fahnenstich et al., 2007). Malate and fumarate are synthesized from the triosephosphates formed during carbon fixation through a sequence of reactions involving the intermediates phosphoenolpyruvate (PEP) and oxaloacetate (OAA) (Figure 5). In Arabidopsis, malate levels increase during the day and decrease during the night, similarly to those of starch and sucrose (Fahnenstich et al., 2007; Zell et al., 2010). The turnover (measured in μmol g fresh weight^{-1} h^{-1}) of these organic acids in Arabidopsis growing in a 12 h photoperiod is equivalent to approximately 20-30% of that of starch (Gibon et al., 2009).

Arabidopsis plants grown in short day conditions partition more assimilates to the formation of starch than those grown in long days, thereby providing carbon skeletons for prolonged dark periods (Gibon et al., 2004). Despite the greater starch partitioning, the respiratory quotient at the end of the night indicated a shift from carbohydrates to organic acids as the main substrates for respiration (Zell et al., 2010). The roles of malate and fumarate as essential carbon stores in Arabidopsis were further demonstrated using plants with sustained highly reduced levels of malate and fumarate due to the overexpression of a plastidic NADP-malic enzyme (NADP-ME) (Fahnenstich et al., 2007). These plants use fatty acids and proteins to fuel mitochondrial respiration by the end of the night. When grown in short day conditions, they develop a carbon starvation phenotype; plants are pale green with decreased biomass and lower photosynthetic performance (Zell et al., 2010). Starch synthesis is stimulated in these plants (Fahnenstich et al., 2008), although the rate of utilization of organic acids and starch is usually tightly coordinated (Gibon et al., 2009). Moreover, they showed an accelerated dark-induced senescence that could be rescued by supplying glucose, sucrose, or malate. In line with these findings, malate and fumarate were the only two metabolites whose levels were significantly decreased after dark incubation and whose levels recover to values similar to wild type after incubation with glucose (Fahnenstich et al., 2007).

During the day, malate accumulates in the vacuole (Gout et al., 1993) (Figure 5). Upon demand, malate is utilized to refuel the TCA cycle pool. This function is fulfilled through the concerted action of malate dehydrogenase (MDH) and NAD-malic enzyme (NAD-ME) (Figure 5). By the action of NAD-ME, malate is decarboxylated to pyruvate and this, in turn, is converted to acetyl-CoA, which by condensation with OAA by citrate synthase forms citrate, allowing repeated cycling of carbon skeletons through the TCA cycle. It has been suggested that MDH and NAD-ME manage the flux of malate through the TCA cycle differentially during a diurnal cycle. MDH would have a prevalent role during the light period, while NAD-ME would be more important during the night period (Tronconi et al., 2008). While diurnal or circadian changes in transcript abundance for most of the TCA enzymes have been observed (Blasing et al., 2005; Giraud et al., 2010), for the more short-term adjustments of the dark-light transitions, it is unlikely that dynamic adjustments in gene expression alone can provide sufficiently rapid control, especially since changes in the abundances of mitochondrial proteins have been found to be relatively slow and modest (Okada and Brennicke, 2006; Lee et al., 2010; Nelson et al., 2014). It is proposed that light effects on the TCA cycle activities act primarily post-transcriptionally and putative phosphorylation and acetylation sites on these proteins habe been described in Arabidopsis (Ito et al., 2009; Konig et al., 2014). Nevertheless, experimental evidence of the functional effect of these putative posttranslational modifications is still lacking. However, the expression of the genes encoding the mitochondrial MDH (mMDH) isoforms in Arabidopsis are induced by light (Thum et al., 2004; Rasmusson and Escobar, 2007). In contrast, *NAD-ME* expression and activity is higher during the night (Tronconi et al., 2008). Major modifications occur in the metabolic profile of Arabidopsis NAD-ME loss-of-function mutants during this period. In these plants, the excess of mitochondrial malate occurring in the night period is diverted to the synthesis of amino acids from intermediates of the TCA cycle (Tronconi et al., 2008).

In plants that associate with symbiotic nitrogen-fixing bacteria, malate is also used as a primary carbon source to support respiration of the bacteroid and the fixation of N_2 by nitrogenase
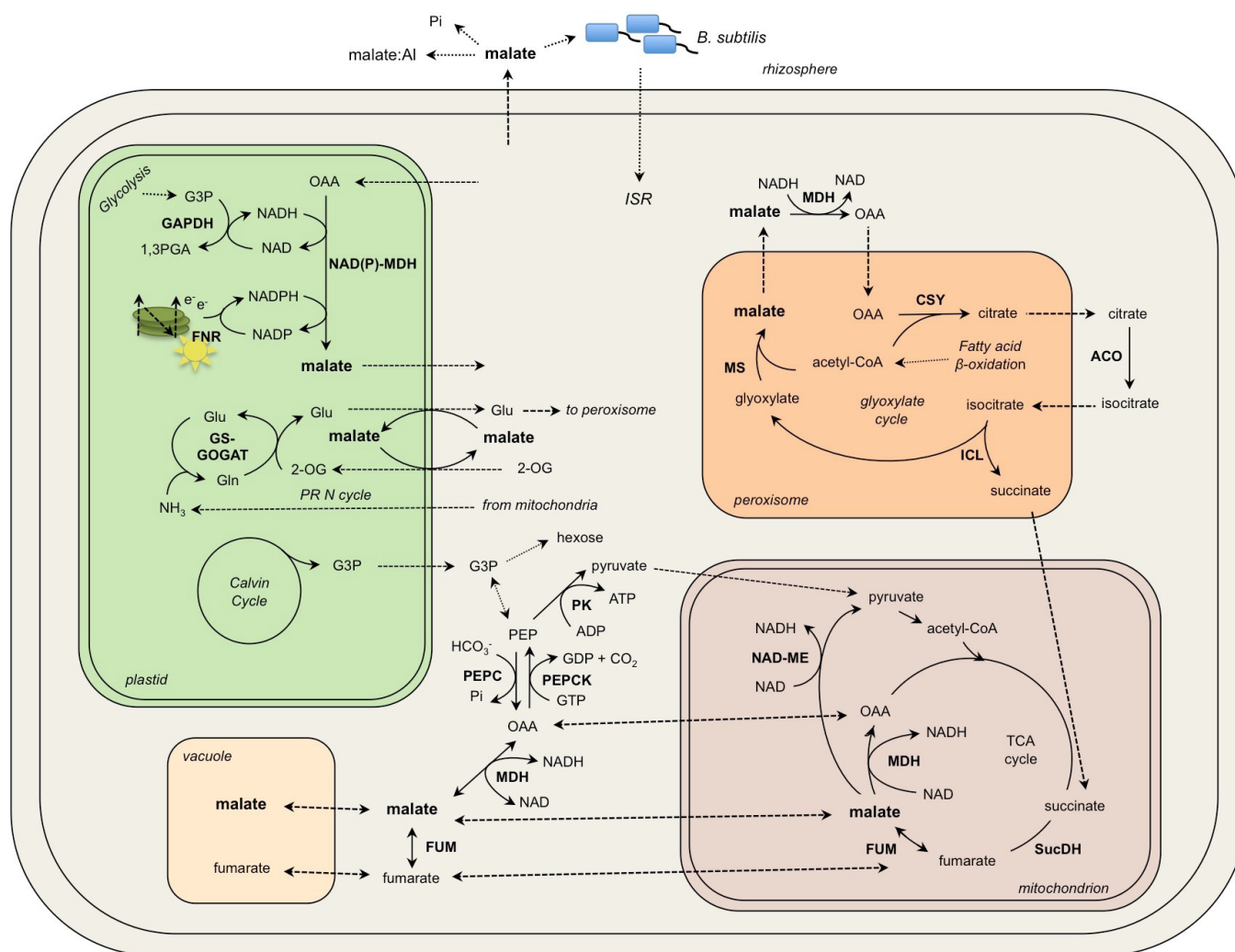
Figure 5. Involvement of malate in plant metabolism (for a detailed explanation refer to the main text).

Dotted lines represent connection to a metabolic pathway. Dashed lines represent transport processes.

Abbreviations: ACO: aconitase; CSY: citrate synthase; e⁻: electrons; FNR: ferredoxin-NADP reductase; FUM: fumarase; GAPDH: NAD(P)-glyceraldehyde-3-P dehydrogenase; Gln: glutamine; Glu: glutamate; GS-GOGAT: glutamine synthetase-glutamate synthase; G3P: glyceraldehyde-3-phosphate; ICL: isocitrate synthase; ISR: induced-systemic resistance; MDH: malate dehydrogenase; MS: malate synthase; NAD-ME: NAD-dependent malic enzyme; OAA: oxaloacetate; PEP: phosphoenolpyruvate; PEPC: phosphoenolpyruvate carboxylase; PEPCK: phosphoenolpyruvate carboxykinase; PK: piruvate kinase; PR N cycle: photorespiratory nitrogen cycle; SucDH: succinate dehydrogenase; TCA cycle: tricarboxylic acid cycle; 1,3PGA: 1,3-bisphosphoglycerate; 2-OG: 2-oxoglutarate.

(Rosendahl et al., 1990; Miller et al., 1998; Rocha et al., 2010). Malate is highly abundant in the root nodules and is transported to the bacteroids through a high affinity dicarboxylic acid transporter (Udvardi et al., 1988).

Glyoxylate cycle

Malate is an intermediate of the glyoxylate cycle, which provides the means to convert fatty acids into gluconeogenic intermediates during germination (Kunze et al., 2006).

The end product of β-oxidation, acetyl-CoA, is partially used for biosynthesis via the glyoxylate cycle, and partially for energy production via the TCA cycle (Figure 5). The glyoxylate cycle is a variation of the TCA cycle that bypasses the decarboxylation steps by using ICL to convert isocitrate into glyoxylate and succinate (Kornberg and Krebs, 1957; Kunze et al., 2006). In the next steps, glyoxylate condenses with acetyl-CoA through the action of MS to produce malate, which is further oxidized to OAA by MDH. Succinate is released as a net product of the cycle, which can be oxidized to fumarate by mitochondrial succinate dehydrogenase; it can further be used to replenish the

TCA cycle or as a precursor for amino acid synthesis. Fumarate and/or malate can also be exported to the cytosol, where they are converted into malate, OAA and finally PEP, which serves as a precursor for carbohydrate biosynthesis (Kunze et al., 2006). The importance of the glyoxylate pathway is observed in Arabidopsis loss-of-function mutants of *ICL*. These plants grow poorly, as they are unable to convert acetyl-CoA from fatty acid β-oxidation into sugars (Eastmond et al., 2000). Instead, the seedlings slowly utilize their fatty acids by transferring products of fatty acid metabolism, most probable in the form of citrate, from the peroxisome to the mitochondrion, where the carbon can be subsequently used for respiration. Without ICL, the TCA cycle cannot be supplied with succinate or with malate produced by MS, and OAA cannot be generated for gluconeogenesis (Eastmond and Graham, 2001).

The synthesis of malate by MS occurs in the peroxisomes, but peroxisomal MDH does not participate in the glyoxylate cycle (Pracharoenwattana et al., 2007). Instead, malate is transported to the cytosol, where cytosolic MDH (cytMDH) produces OAA (Figure 5). OAA is then transported back to the peroxisome. As aconitase is also located in the cytosol, citrate should also be transported out of the peroxisome, with isocitrate being returned to the organelle (Figure 5). The OAA produced by cytMDH can also serve as a substrate for phosphoenolpyruvate carboxykinase (PEPCK) (Kunze et al., 2006) in the first step of gluconeogenesis. An explanation for the peroxisomal location of MS and citrate synthase could be the direct utilization of acetyl-CoA generated by β-oxidation, and a rationale for the cytosolic location of some glyoxylate cycle enzymes might be their sensitivity towards H_2O_2 (Verniquet et al., 1991; Yanik and Donaldson, 2005).

Excretion of malate into the soil: role in plant nutrition and immunity

Malate and citrate are the major carboxylates found in root exudates, where they have a role in plant nutrition and communication with microorganisms (Schulze et al., 2002; Bais et al., 2006). These functions are ascribed, in part to the release of protons, a process that leads to acidification of the surrounding area, and in part to the use of these organic anions as carbon sources by root-associated bacterial communities.

Approximately 50% of the world's potentially arable lands are acidic. Acid soils impose an important constraint to agriculture, as they possess high levels of aluminum (Al^{3+}), which inhibit root growth and development, as well as suboptimal levels of phosphorous (Kochian et al., 2004).

It is likely that multiple Al^{3+} tolerance mechanisms are employed by different plant species. However, experimental evidence indicate root Al^{3+} exclusion based on exudation of Al^{3+}-activated organic anions (malate, citrate, and oxalate) from the root apex as a main mechanism of tolerance in numerous plant species. The excretion of these carboxylates results in chelation of Al^{3+} in the rhizosphere, diminishing the uptake of high quantities of this toxic element into the roots (Figure 5) (Kinraide et al., 2005; Delhaize et al., 2007; Zhou et al., 2014). Al^{3+}-activated malate release is very rapid and localized very specifically to the first few millimeters of the root apex (Delhaize et al., 2007). Malate reaches the rhizosphere through a malate channel, the Al^{3+}-activated malate transporter, ALMT (see below) (Liu et al., 2009). Al^{3+} activates ALMT activity from the apoplastic side and at least in Arabidopsis it also upregulates its transcription (Sasaki et al., 2004; Hoekenga et al., 2006). Work performed in wheat suggests that protein phosphorylation may be required for activation of ALMT by Al^{3+} (Osawa and Matsumoto, 2001). Overexpression of citrate synthase and malate dehydrogenase in transgenic tobacco, *Arabidopsis*, and alfalfa (*Medicago sativa*) resulted, in some cases, in increased root organic anion content and exudation, as well as enhanced Al^{3+} tolerance (de la Fuente et al., 1997; Koyama et al., 2000; Tesfaye et al., 2001).

Roots also secrete malate together with citrate to release unavailable forms of phosphorus, an essential mineral nutrient. These organic anions can desorb phosphate (Pi) from mineral surfaces, solubilizing it from associations with Al, Fe, and Ca oxides and hydroxides via metal complexation. Malate and citrate are responsible for the capture of inorganic phosphate (Pi) from the soil by plants species that do not form mycorrhiza symbioses, as in the case of Arabidopsis (Figure 5) (Ryan et al., 2001; Dong et al., 2004). Some plant species even increase the root surface by developing cluster or proteoid roots, which show a prolific development of root hairs and release large amounts of malate and citrate to improve the extraction of Pi from the soil (Johnson et al., 1996; Shane and Lambers, 2006).

Malate excretion by roots is induced by upregulation of the transcription of ALMT1 (see below) upon infection of Arabidopsis leaves with *Pseudomonas syringae* (Pst DC 3000) (Rudrappa et al., 2008). The malate released from Arabidopsis roots via ALMT induces the chemiostatic motility of *Bacillus subtilis* FB17, which colonize the rhizosphere and the root surface, reducing the plant's susceptibility to further pathogen attacks (Figure 5) (Rudrappa et al., 2008).

Stomata function

Malate, together with some other factors such as abscisic acid (ABA), redox signals, K^+ and Cl^- fluxes, is involved in the regulation of guard cell turgor pressure, mediating guard cell opening and closure (Vavasseur and Raghavendra, 2005). During stomatal closure, malate is partially converted to osmotically inactive starch and is released from guard cells to the apoplast (Roelfsema and Hedrich, 2005). The presence of external malate shifts the voltage gate of the rapid (R-type) anion channel towards more negative membrane potentials, favoring channel opening at the resting state, and in turn depolarization of guard cells (Raschke et al., 2003; Konrad and Hedrich, 2008). Additionally, apoplastic malate is required for efficient stomatal opening (Lee et al., 2008).

The activity of the malate channels AtABCB14, SLAC1 and AtALMT12 (see Transport of malate), which mediate the transfer of this organic acid between the guard cell cytosol and the apoplast, induces stomata closure. Plants lacking ABCB14 showed reduced stomatal aperture compared to the wild type after transfer to low CO_2 atmosphere (Lee et al., 2008). On

the other hand, plants lacking SLAC1 exhibited enhanced stomatal aperture under high CO_2 atmosphere compared to the wild-type, which was further increased under low CO_2 (Negi et al., 2008; Vahisalu et al., 2008); plants lacking AtALMT12 are impaired in dark- and CO_2-induced stomatal closure (Meyer et al., 2010).

Indirect but valuable evidence for the participation of malate in stomatal function is found in several reports on mutant plants of tricarboxylic acid cycle enzymes that are involved in malate metabolism. Some examples are tomato (*Solanum lycopersicum*) plants partially deficient in fumarase, which are defective in stomata opening (Nunes-Nesi et al., 2007) and tomato and Arabidopsis mutants partially deficient in succinate dehydrogenase, which have increased stomatal aperture and density (Araujo et al., 2011; Fuentes et al., 2011).

Photosynthesis

Malate also has a specialized role in many plants performing C4 and CAM photosynthesis, transiently storing carbon from CO_2 (Holtum et al., 2005; Drincovich et al., 2011; Maier et al., 2011). These plants have evolved biochemical pumps to concentrate CO_2 at the site of Rubisco and thus have lower photorespiratory fluxes. CO_2 concentration is achieved by using PEP carboxylase (PEPC), a more efficient enzyme than Rubisco, to fix CO_2 in the cytosol and producing an organic acid, malate and/or aspartate, to store and transport CO_2.

C4 plants developed a spatial separation of the biochemical components of the CO_2 pump. In these plants, PEPC is located in the cytosol of mesophyll cells, and the C4 acid formed (malate or OAA) is shuttled to the bundle sheath cells (BSC) (Drincovich et al., 2011). The release of CO_2 from the C4 organic acids in BSCs can be mediated by three different decarboxylases: NADP-ME, NAD-ME, and PEP carboxykinase (PEPCK). C4 plants have been traditionally grouped into three biochemical subtypes depending on the major decarboxylase used (Maier et al., 2011). It is now suggested that only two subtypes exist, NADP-ME and NAD-ME, which both inherently involve a supplementary PEPCK cycle (Wang et al., 2014). In the NADP-ME subtype, malate is transported to the chloroplasts of the BSC, where NADP-ME is located, whereas in the NAD-ME subtype, malate is decarboxylated in the mitochondria through NAD-ME (Drincovich et al., 2011). The PEPCK activity decarboxylates OAA forming pyruvate in the cytosol of BSC (Drincovich et al., 2011).

CAM plants use a temporal separation of the components of the CO_2 pump. In these plants, CO_2 diffuses into the leaf during the night, when stomata are opened, and combines with PEP to form OAA through the action of PEPC, and is further converted to malate through the action of a cytosolic NAD-MDH (Black and Osmond, 2003). Malate is then stored in a large central vacuole. During the day, malate is released from the vacuole and decarboxylated via NADP-ME.

The specific enzymes that metabolize malate (MDH, NAD-ME, and NADP-ME) during C4 photosynthesis did not evolve *de novo*. Instead, they were recruited from existing housekeeping isoforms, whose functions are described below (Monson, 2003; Maier et al., 2011).

Enzymes involved in malate synthesis

Fumarase

Fumarase (EC 4.2.1.2) catalyzes the reversible hydration/dehydration of fumarate to/from malate. In Arabidopsis, there are two genes encoding fumarase, *FUM1* (At2g47510) and *FUM2* (At5g50950). The FUM1 protein is found in mitochondria (Heazlewood and Millar, 2005), whereas the location of FUM2 is cytosolic (Pracharoenwattana et al., 2010) (Figure 6). Other plant species only possess a unique mitochondrial fumarase (Tuskan et al., 2006; Nunes-Nesi et al., 2007). It has been suggested that a cytosolic fumarase might be associated specifically with plants that accumulate large amounts of organic acids during photosynthesis and nitrate assimilation (Chia et al., 2000; Tschoep et al., 2009).

FUM1 is an essential enzyme of the TCA cycle. Homozygous *fum1* knock-out plants are lethal (Pracharoenwattana et al., 2010). FUM2 is required for the massive accumulation of fumarate in leaves of plants growing in the presence of high nitrogen (Pracharoenwattana et al., 2010). This implies that FUM2 would function in the direction of malate dehydration during the day, but kinetic evidences for this assumption is still lacking.

Malate synthase

Malate synthase (MS, EC 2.3.3.9) is localized to peroxisomes, where it catalyzes the irreversible production of malate from acetyl-CoA and glyoxylate (Figure 6). It is a key enzyme in the glyoxylate cycle, which enables the conversion of acetyl-CoA from fatty acid ß-oxidation into sugars to support seedling growth.

The expression of *MS* is activated in cotyledons during postgerminative growth and then repressed as the cotyledons become photosynthetic (Eastmond et al., 2000; Lingard et al., 2009). The expression is also activated in senescing organs (Gut and Matile, 1988; Graham et al., 1992) and under anaerobiosis caused by submergence (Lu et al., 2005).

Arabidopsis possesses one gene encoding MS (At5g03860). *MS* loss-of-function mutants seedlings differ very little from wild type; the mutants have only a slightly stunted phenotype when grown in the absence of exogenous sugar in the light. Thus, MS is partially dispensable for lipid utilization and gluconeogenesis in Arabidopsis seedlings (Cornah et al., 2004). The phenotype is more obvious in the dark where the hypocotyl elongation is inhibited, and little root development occurs. MS loss-of-function seedlings are able to convert acetate into sugars (Cornah et al., 2004). In these plants, the glyoxylate produced by ICL can be converted to glycine and serine, hydroxypyruvate, glycerate, and ultimately sugars.

Malate dehydrogenase

Malate dehydrogenase (MDH) catalyzes the reversible reduction of OAA to malate. Plants possess MDHs with different coenzyme specificity and subcellular localizations (Figure 6). Chlo-
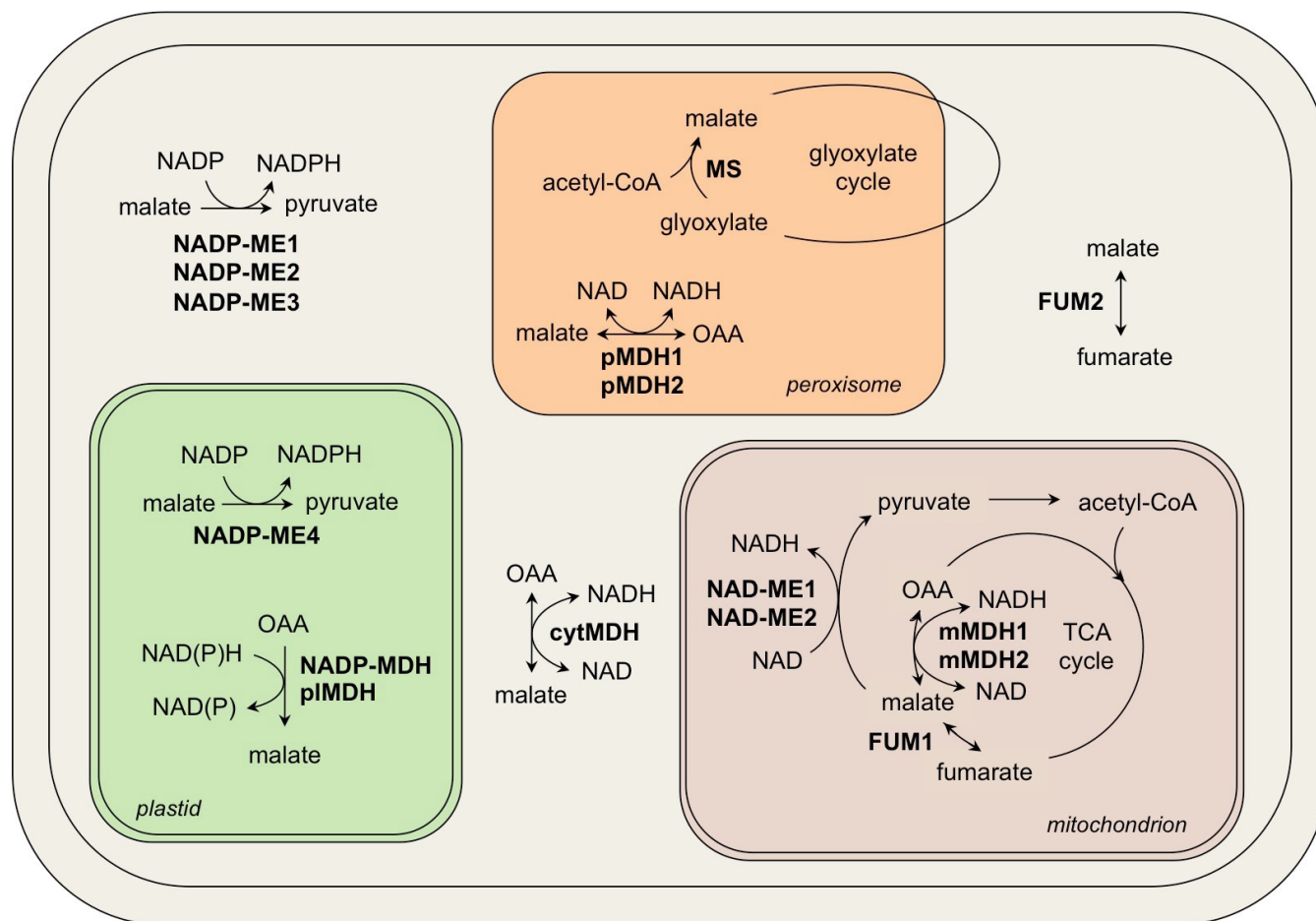
Figure 6. Subcellular compartmentalization of the enzymes involved in malate production and degradation in Arabidopsis.

Enzymes are abbreviated as given in the text. OAA: oxaloacetate; TCA cycle: tricarboxylic acid cycle.

roplasts contain a unique NADP-dependent MDH (NADP-MDH; EC 1.1.1.82), with a subunit mass of 42 kDa. NAD-dependent MDHs (NAD-MDH; EC 1.1.1.37) are found in mitochondria, peroxisomes, plastids, and cytosol. MDHs are homodimers with a subunit mass of 33-36 kDa and share amino acid identities of at least 20% (Berkemeyer et al., 1998; Miller et al., 1998). A general function of MDH is the indirect transport of reducing equivalents between cellular compartments (Scheibe, 2004).

Chloroplastic NADP-MDH

NADP-MDH participates in the "malate valve", a mechanism that allows chloroplast reducing equivalents, produced in excess during the light reactions of photosynthesis, to be transferred to the cytosol (Figure 5-7) (Scheibe, 2004). In the light, excess NADPH induces the conversion of OAA to malate, by the activated form of NADP-MDH, resulting in the regeneration of the electron acceptor NADP. Malate is transported to the cytosol via the plastidic transporter OMT1 (also known

as DiT1; see below) (Taniguchi et al., 2002), where cytMDH converts malate back to OAA, with NAD$^+$ acting as an oxidant to form NADH. The resulting NADH is then used as reducing equivalents in the cytosol or is transferred into other organelles. Malate exported to the cytosol can also serve other purposes; it can be transported to the vacuole for storage, it can be transported to mitochondria for metabolization, or it can support photorespiration.

NADP-MDH evolved from a duplicate of an original NAD-MDH, and presents additional N- and C-terminal extensions, both of which harbor a redox-sensitive cysteine pair involved in enzyme activation/inactivation (Scheibe et al., 1990). NADP-MDH is subjected to post-translational light/dark modulation through the ferredoxin-thiorredoxin system (Kromer and Scheibe, 1996; Scheibe, 2004). It is regulated by the NADPH/NADP ratio, being completely inactivated upon oxidation of the regulatory cystein residues in darkness (Edwards et al., 1985).

Only one gene, At5g58330, encodes NADP-MDH in Arabidopsis. Nevertheless, loss-of-function plants of NADP-MDH
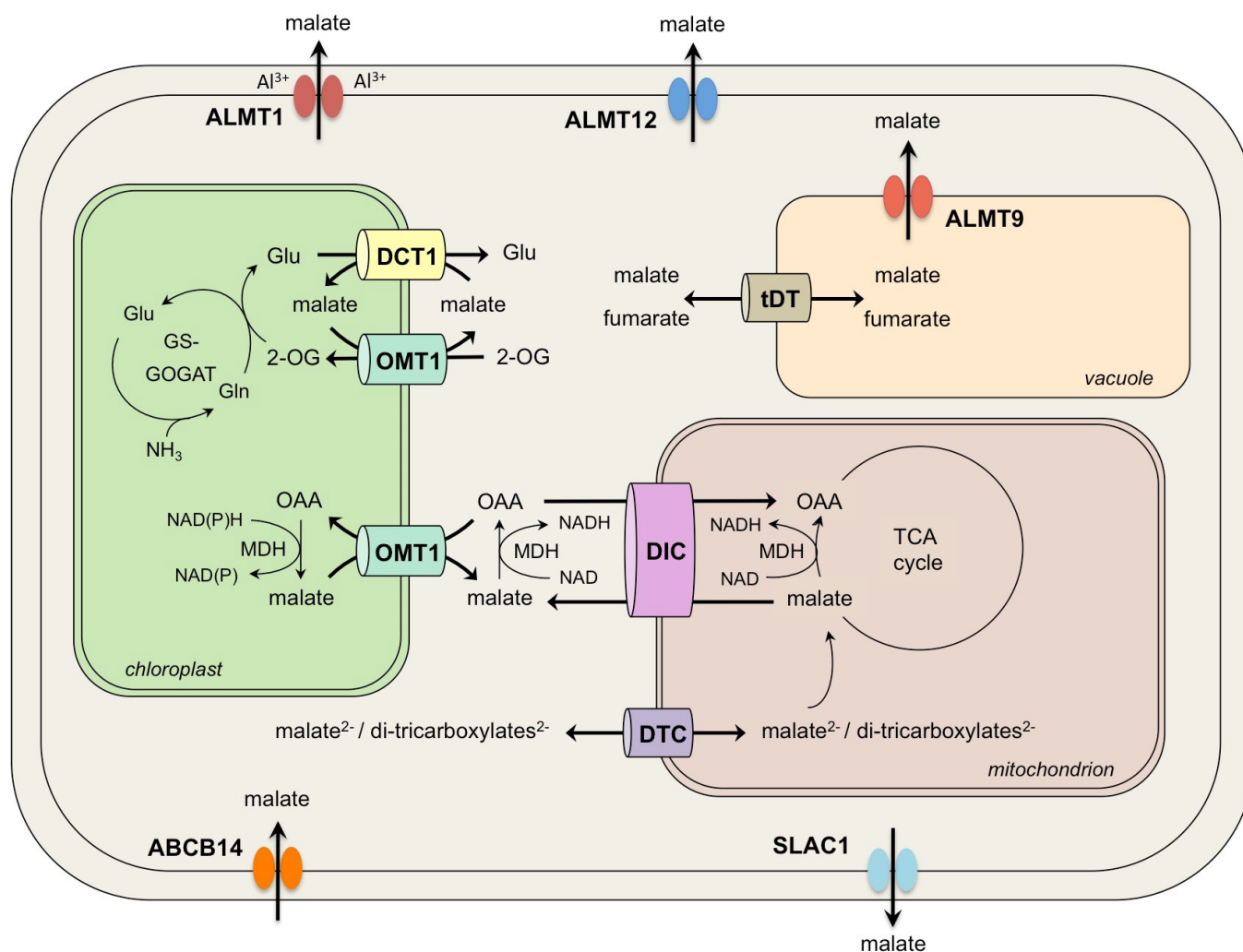
Figure 7. Transport systems for malate in Arabidopsis (for a detailed explanation refer to the main text).

Transport proteins are abbreviated as given in the text.

Abbreviations: Gln: glutamine; Glu: glutamate; GS-GOGAT: glutamine synthetase-glutamate synthase; MDH: malate dehydrogenase; OAA: oxaloacetate; TCA cycle: tricarboxylic acid cycle.

resemble the wild-type even in high light conditions (Hebbel-mann et al., 2012). In these plants, compensatory mechanisms such as a stimulated chloroplast NADPH-thioredoxin reductase (NTRC)/2-Cys peroxidredoxin system, adjustments in photores-piration, and proline biosynthesis, act to maintain a balanced redox state in absence of an active malate valve (Hebbelmann et al., 2012).

Most other plants also possess only one NADP-MDH, with the exception of some C4 plants (Rondeau et al., 2005). In these species, the NADP-MDH involved in C4 photosynthesis is highly expressed in mesophyll cells, where it catalyzes the formation of malate from the primary CO_2 fixation product OAA (Maier et al., 2011).

Cytosolic MDH (cytMDH)

cytMDH is involved, together with an OAA/malate antiporter (OMT or DIC; see below) and a chloroplastic- or mitochondrial-localized MDH in transporting reducing equivalent (NAD^+/NADH) between the cytosol and the organelle (Figure 6 and 7) (Pastore et al., 2003). In the cytosol, OAA is reduced to malate by electrons from NADH. Malate is then transported into the organelle via the OMT. Inside the organelle, malate is oxidized by NAD^+ back to OAA, forming NADH. Finally, OAA is transported back to the cytosol by the OMT. In this way, NADH is transferred from the cytosol into different organelles. cytMDH also works in conjunction with PEPC to generate malate from PEP formed during carbon fixa-

tion, or through glycolysis in the dark or in heterotrophic tissues (see below) (Figure 5).

Arabidopsis contains three genes encoding cytMDHs, At1g04410 (*cytMDH1*), At5g43330 (*cytMDH2*), and At5g56720 (*cytMDH3*). Although the kinetic parameters of these Arabidopsis isozymes were not investigated so far, those of cytosolic MDHs from other species indicate that the enzyme mainly catalyzes the formation of malate *in vitro* (Miller et al., 1998; Ding and Ma, 2004; Yao et al., 2011). This is also supported by an enhanced accumulation of malate in cytMDH overexpressors (Yao et al., 2011).

Plastidic MDH (plMDH)

As in the case of illuminated chloroplasts, in heterotrophic plastids or in chloroplasts during the night there is a need to regenerate NADH, which is produced during glycolysis. The generation of NADH is catalyzed by the bi-specific NAD(P)-glyceraldehyde-3-P dehydrogenase (GAPDH) - which uses NAD^+ during the night - or by NAD-GAPDH in non-green plastids (Figure 5). NADH requires a continuous regeneration, which is achieved via a "dark-malate valve" that involves the plMDH instead of NADP-MDH (Scheibe, 2004; Selinski et al., 2014).

plMDH is present in all plastid types, where it is permanently active (Backhausen, 1998). Arabidopsis possesses one gene, At3g47520, encoding a plMDH (Berkemeyer et al., 1998). This plMDH is essential for maintaining redox homeostasis in the dark and in non-green plastids, and for embryo viability (Selinski et al., 2014). While a null-mutant in *plMDH* is embryo lethal (Beeler et al., 2014; Selinski et al., 2014), knock-down plants are small, pale green, show a reduction in seed yield, and reduced respiration rates at night due to lower rate of starch mobilization (Beeler et al., 2014). A plMDH is also abundant in nodules of soybean (*Glycine max*) and pea, where it would be responsible for the synthesis of malate to provide a source of carbon and reducing power for the bacteroid (Imsande et al., 2001).

plMDH preferable catalyzes the reduction of OAA, as it has a much higher affinity for OAA and NADH than for malate and NAD^+, and as the turnover rate highly favors malate synthesis (Appels and Haaker, 1988; Miller et al., 1998; Cvetic et al., 2008).

Peroxisomal MDH (pMDH)

Arabidopsis possesses two genes encoding pMDH, At2g22780 (pMDH1) and At5g09660 (pMDH2). *pMDH1* is preferentially expressed in seedlings, while *pMDH2* is preferentially expressed in leaves (Pracharoenwattana et al., 2007). Both pMDH proteins contain type-2 peroxisomal targeting sequences (Pracharoenwattana et al., 2007). pMDH produces malate from OAA to reoxidize NADH for continued fatty acid ß-oxidation (Figure 6). Arabidopsis mutants lacking both isoforms have a sucrose-independent germination but a sucrose-dependent seedling growth (Pracharoenwattana et al., 2007). Moreover, the seedlings mobilize triacylglycerols very slowly and contain high amounts of lipid bodies (Pracharoenwattana et al., 2007). In these plants, the glyoxylate cycle functions normally, thus a participation of pMDH in this pathway is ruled out (Pracharoenwattana et al., 2007). In addition to this, pMDH, predominantly pMDH2, is involved in maintaining the

stoichiometry of CO_2 released during photorespiration, although it is not essential for the operation of the pathway (Cousins et al., 2008). The *pmdh* null-mutants have greater rates of photorespiratory CO_2 release than the wild type and only slightly reduced photosynthetic rates. This indicates that pMDH is not essential for supplying NADH for the peroxisomal hydroxypyruvate reductase (pHPR) reaction as suggested (Reumann and Weber, 2006). Indeed, the activity of the pHRP can be bypassed by the cytosolic HPR activity (Timm et al., 2008).

Mitochondrial MDH (mMDH)

mMDH is involved in two major processes in C3 plants (Figure 5-6): (i) as a standard TCA-cycle enzyme, mMDH oxidizes malate from the fumarase reaction to OAA to form citrate; (ii) in the conversion of glycine to serine, mMDH reduces OAA to malate and provides NAD^+ for the glycine decarboxylase reaction (Journet et al., 1981). While the MDH reaction is reversible, the affinity is higher for OAA and NADH than for malate and NAD^+, favoring the synthesis of malate *in vitro*, a direction that is also thermodynamically favorable (Hameister et al., 2007; Hüdig et al., 2015). Arabidopsis possesses two genes encoding mMDH, At1g53240 (mMDH1) and At3g15020 (mMDH2). The activity rate of mMDH2 is only 28% of that of mMDH1 when acting on OAA, while the mMDH1 activity rate is 67% of that of mMDH2 when acting on malate (Hüdig et al., 2015). Both isoforms showed normal hyperbolic saturation kinetics when using OAA as substrate. mMDH1 has a 4.7-fold higher overall catalytic rate (K_{cat}) than mMDH2 and both isoforms possess low K_{mOAA} values, indicating high affinities for this substrate (K_{mOAA} of 0.27 and 1.0 mM for mMDH1 and mMDH2, respectively) (Hüdig et al., 2015).

Although mMDH1 is the dominant form in mitochondria (Millar et al., 2001; Lee et al., 2008) and accounts for approximately 30% of the total NAD-MDH activity in Arabidopsis leaves, growth is unaffected in single loss-of-function mutants (Tomaz et al., 2010). In contrast, growth is highly reduced in the double mutant, which presents decreased net CO_2 assimilation rate but higher respiration rates. Thus, mMDH exerts metabolic control in leaves, as it imposes some degree of limitation of the respiratory rate (Tomaz et al., 2010). This could be due to the fact that normally mMDH uses OAA from the cytosol to produce malate competing with the electron transport chain (ETC) for NADH and slowing the TCA flux by competing with citrate synthase for OAA (Tomaz et al., 2010). The mmdh null-mutants may drive maximal malate-dependent respiration flux through the ETC using NAD-ME. The activity of mMDH also influences photorespiration mainly through a limitation on glycine oxidation rate (Tomaz et al., 2010).

PEP carboxylase

Phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) catalyzes the irreversible β-carboxylation of PEP in the presence of HCO_3^- and Mg^{2+} to yield OAA and Pi. The cytosolic production of OAA by PEPC coupled to the action of cytMDH yields malate.

PEPC is a cytoplasmic enzyme composed of four identical subunits with monomeric molecular mass of about 110 kD. Arabidopsis possesses three plant-type PEPC genes, AtPPC1

(At1G53310), AtPPC2 (At2G42600), and AtPPC3 (At3G14940), and one bacterium-type PEPC gene, AtPPC4 (At1G68750). PPC1 and PPC2 are highly expressed in leaves. A double mutant of these genes (ppc1/ppc2) exhibited severe growth-arrest, reduced synthesis of malate and suppression of ammonium assimilation, indicating that in Arabidopsis PEPC plays a crucial role in modulating the balance of carbon and nitrogen metabolism (Shi et al., 2015).

Apart from this, PEPC plays a wide range of metabolic roles in higher plants: in heterotrophic tissues and in the leaves of C3 plants, the primary function of PEPC is anaplerotic, replenishing the TCA cycle with intermediates that are withdrawn for nitrogen assimilation and other biosynthetic pathways (Schuller et al., 1990; Chollet et al., 1996); it also plays role in the provision of malate in guard cells and legume root nodules (Outlaw; Wadham et al.), and in C4 and CAM plants the reaction catalyzed by PEPC is the primary fixation step of photosynthetic CO_2 assimilation (Andreo et al., 1987). This initial carboxylation reaction takes place in mesophyll cells in the light in C4 plants or in the dark in CAM plants.

The activity of PEPC is subjected to allosteric control and post-translational regulation. Glucose 6-P (G6P) and triose-P are positive metabolite effectors, while malate and aspartate are negative ones (Doncaster and Leegood, 1987; Outlaw, 1990; Duff and Chollet, 1995; O'Leary et al., 2011). Plant cytosolic PEPC undergoes activation through reversible phosphorylation of a conserved Ser residue near the N terminus, which changes it sensitivity to allosteric effectors: it becomes less sensitive to its feedback inhibitor malate and more sensitive to the activator G6P (Jiao and Chollet, 1991; Le Van et al., 1991; Ogawa et al., 1992; Pacquit et al., 1993; Schuller and Werner, 1993; Wang and Chollet, 1993). In maize, the phosphorylation of leaf PEPC occurs prior to dawn and decreased phosphorylation begins well before dark. This suggests circadian and/or metabolic control mechanisms in addition to light regulation (Ueno et al., 2000).

Enzymes that act on malate

Malate dehydrogenase

As mentioned above, the mMDH reaction is reversible. Nevertheless, the direction of the reaction *in vivo* depends on the physiological conditions. For example, removal of OAA by citrate synthase, coupled with the removal of NADH by the respiratory chain, causes the mMDH reaction to function in the direction of malate oxidation as part of the TCA cycle.

NAD-Malic enzyme

The NAD-dependent malic enzyme (NAD-ME; EC 1.1.1.39) catalyzes the oxidative decarboxylation of malate, producing pyruvate, CO_2, and NADH. NAD-ME is exclusively present in mitochondria, where it is involved in malate respiration, a role shared with mMDH (Figure 5-6) (Grover et al., 1981).

Plant NAD-MEs are generally composed of two dissimilar subunits, which are separated into two related groups in a phylogenetic tree: a-subunits with molecular masses of ~65 kDa and b-subunits with molecular masses of ~58 kDa (Maier et al., 2011). The Arabidopsis genome contains two genes encoding NAD-MEs (*NAD-ME1*, At4g13560, and *NAD-ME2*, At4g00570). Arabidopsis NAD-ME1 belongs to the a-subunits group, whilst NAD-ME2 groups with the known b-subunits. NAD-ME1 and NAD-ME2 share about 65% sequence identity at the protein level. In line with their universal role in malate respiration, NAD-ME1 and NAD-ME2 are expressed constitutively in Arabidopsis, showing high level of expression in cells around the vascular system (Tronconi et al., 2008; Brown et al., 2010). It was suggested that these decarboxylases may also release CO_2 from malate for photosynthesis in these type of cells (Hibberd and Quick, 2002). However, recent work demonstrated that NAD-ME is not involved in the fixation of carbon from the xylem stream in Arabidopsis (Brown et al., 2010).

Arabidopsis NAD-ME assembles as hetero- and homodimers, although *in vivo* it functions mostly as a heterodimer (NAD-MEH) (Tronconi et al., 2008). NAD-ME2 is the only form of the enzyme present in anthers (Tronconi et al., 2010b). The separated recombinant homodimers and the reconstituted heterodimer present similar catalytic efficiencies, but differential kinetic mechanisms and regulation by metabolic effectors (Tronconi et al., 2008; Tronconi et al., 2010a; Tronconi et al., 2010b). The proteins have a pH optimum of about 6.5, a K_m for malate of 2.7 to 3.0 mM, and K_{cat} values of 31 to 44 s^{-1}. NAD-ME1 is strongly activated by fumarate and succinate and shows cooperativity for the binding of malate, indicating that NAD-ME1 is best suited for physiological situations where organic acids are the main substrates for mitochondrial respiration (Tronconi et al., 2010b). On the other hand, NAD-ME2 responds to the glycolytic intermediate PEP and CoA, suggesting a concerted function with PEPC under situations where the pyruvate supply to the TCA cycle by the pyruvate kinase reaction is diminished (Tronconi et al., 2010b). Finally, NAD-MEH is a form that can integrate the several forms of regulation and respond synergistically to activators of NAD-ME1 or NAD-ME2 (Tronconi et al., 2010b). The properties of the different enzymatic entities produced by alternative association of the subunits suggest that NAD-ME activity may be regulated by variations in the native association *in vivo*. In other plant species, no activity was associated with the separated subunits, but activity could be found in a reconstituted system (Willeford and Wedding, 1987). In these cases, the b-subunit would play a regulatory role (Long et al., 1994).

Double loss-of-function mutant in *NAD-ME1* and *NAD-ME2* show no NAD-ME activity, but the single mutants have residual NAD-ME activities, indicating that both proteins are also active homodimers *in vivo*. NAD-ME activity is not essential for normal autotrophic development, as neither single nor double loss-of-function mutants showed a growth or a developmental phenotype. Nevertheless, a highly modified metabolic profile of leaves of plants completely lacking NAD-ME activity during the night period in comparison to the light period indicates a major participation of NAD-ME during nocturnal metabolism (Tronconi et al., 2008). In line with this, NAD-ME expression and activity are lower during the day than during the night period (Tronconi et al., 2008).

Apart from its universal role in malate respiration, NAD-MEs provide CO_2 for the Calvin cycle during photosynthetic metabolism in some C4 and CAM plants, where they function in BSC mitochondria (Maier et al., 2011). The presence of NAD-ME in

cells surrounding the vasculature in C3 plants such as Arabidopsis may have been the starting point for the evolution of the C4 form of the enzyme (Brown et al., 2010). Interestingly, there exists a unique NAD-ME in C4 plant mitochondria, which has been evolutionarily adapted to perform a dual function, combining the respiration of malate (performed in all plant mitochondria) with malate decarboxylation to release CO_2 at the site of Rubisco in C4 photosynthetic BSC mitochondria (Maier et al., 2011).

NADP-Malic enzyme

NADP-malic enzyme (NADP-ME; EC 1.1.1.40) catalyzes the oxidative decarboxylation of malate, producing pyruvate, CO_2, and NADPH in the presence of divalent cations such as Mg^{2+} or Mn^{2+}. In Arabidopsis, four genes encode NADP-MEs (*NADP-ME1*, At2g19900; *NADP-ME2*, At5g11670; *NADP-ME3*, At5g25880; and *NADP-ME4*, At1g79750) (Gerrard Wheeler et al., 2005; Maurino et al., 2009). NADP-ME1-3 are localized to the cytosol, while NADP-ME4 is localized to plastids (Figure 6) (Gerrard Wheeler et al., 2005; Gerrard Wheeler et al., 2009). Arabidopsis NADP-MEs show amino acid identities between 78% and 90%; despite their highly similar sequences, they differ in their biochemical properties and expression patterns (Gerrard Wheeler et al., 2005; Gerrard Wheeler et al., 2008; Gerrard Wheeler et al., 2009; Maurino et al., 2009).

NADP-ME1 shows the lowest catalytic efficiency (K_{cat}/K_m) for both malate and $NADP^+$. NADP-ME2 has the highest specific activity and is the most regulated isoform, being activated by succinate and fumarate (Gerrard Wheeler et al., 2008; Gerrard Wheeler et al., 2009). The activation of NADP-ME2 by fumarate may be relevant *in vivo,* as Arabidopsis accumulates high amounts of this organic acid during the day and use it for transport of carbon to non-photosynthetic organs and as energy source upon demand (Chia et al., 2000; Fahnenstich et al., 2007; Zell et al., 2010). NADP-ME3 and NADP-ME4 have the highest affinities for $NADP^+$ and malate, respectively. NADP-ME3 is the only isoform that is inhibited by malate at pH 7,0 (Gerrard Wheeler et al., 2009), a feature that was only associated to the C4 photosynthetic NADP-ME. The kinetic properties of NADP-ME4 resemble those of the plastidic photosynthetic and non-photosynthetic NADP-ME from the C4 plant maize (Detarsio et al., 2003; Saigo et al., 2004; Gerrard Wheeler et al., 2005; Maier et al., 2011), suggesting that most probable a C3 plastidic isoform is the ancestor of the plastidic isoforms found in C4 plants (Maier et al., 2011). In addition, all Arabidopsis NADP-MEs are able to catalyze the reductive carboxylation of pyruvate *in vitro*. This reverse reaction may be relevant *in vivo*, at least in some particular physiological situation or subcellular context, as the values of K_m for pyruvate are in the physiological range (Gerrard Wheeler et al., 2008).

NADP-ME1 is expressed in the embryo only at the latest stages of embryogenesis, in root tips during germination, and in some secondary roots in adult plants. In a phylogenetic tree, this isoform groups together with other cytosolic NADP-MEs with similar kinetic properties, such as a low catalytic efficiency (Gerrard Wheeler et al., 2005; Detarsio et al., 2008).

NADP-ME2 is constitutively expressed in vegetative organs and is responsible for the majority of NADP-ME activity in leaves (Gerrard Wheeler et al., 2005). The expression and activity of this isoform rise after treatment with pathogen-associated molecular patterns and after pathogen infection. In line with this, Arabidopsis mutants lacking NADP-ME2 showed enhanced susceptibility during infection with the hemibiotrophic fungal pathogen *Colletotrichum higginsianum* (Voll et al., 2012). Moreover, these plants showed depressed transient apoplastic ROS production after elicitation, and callose-papillae formation after infection. Thus, this isoform plays a role during the basal defense response, where it is required for the production of ROS following pathogen recognition (Voll et al., 2012). It was suggested that NADP-ME2 could also be involved in meeting the demand of reducing power for the increased synthesis of building blocks and for the production of superoxide and H_2O_2 through NADPH oxidase and/or cell-wall peroxidases (Voll et al., 2012).

The expression of NADP-ME3 is high and restricted to trichomes, stipules, and pollen grains in the latest maturation stages (Gerrard Wheeler et al., 2005). This isoform might be involved in providing NADPH for detoxification reactions (Gutierrez-Alcala et al., 2000) and could generate substrates for cellular respiration during male gametogenesis (Gerrard Wheeler et al., 2005).

NADP-ME4 is constitutively expressed in vegetative and reproductive organs (Gerrard Wheeler et al., 2005). Similarly to the plastidic NADP-ME of *Ricinus communis* (Smith et al., 1992; Shearer et al., 2004), NADP-ME4 may participate in plastidic lipid metabolism, where pyruvate and NADPH are sources of carbon and reducing power to support fatty acid biosynthesis. Consistent with this role, NADP-ME4 is highly expressed during developmental stages in which fatty acid biosynthesis is very active, such as embryogenesis and germination (Gerrard Wheeler et al., 2005).

NADP-ME2 and NADP-ME4 are highly expressed in the vasculature of vegetative organs (Gerrard Wheeler et al., 2005), where the cytosolic isoform is responsible for 80% of NADP-ME activity (Brown et al., 2010). Mid-veins of Arabidopsis are able to use malate present in the xylem stream for photosynthesis (Brown et al., 2010). In cells around the vascular bundles of the C3 plant tobacco, NADP-ME is supposed to participate in the decarboxylation of malate derived from the respiratory activity of heterotrophic tissues (Hibberd and Quick, 2002). In a similar way, malate transported through the vasculature in Arabidopsis could be decarboxylated by NADP-ME2 and/or NADP-ME4 in the neighboring cells, where the CO_2 released would be used for the production of carbohydrates (Brown et al., 2010).

Transport of malate

Fluxes of malate between the different subcellular compartments are fast (Kalt et al., 1990). In plants, transport systems involved in malate translocation have so far been identified in the plasma membrane, chloroplast, the mitochondria, and the vacuole (Figure 7).

Plasma membrane transport

ALMT: Aluminum-activated malate transporter

ALMT received this name because the first transporter identified (the one of wheat, ALMT1) facilitates Al^{3+}-activated malate efflux

(Sasaki et al., 2004; Hoekenga et al., 2006). The protein has six transmembrane domains with the amino and carboxyl termini located on the extracellular side of the plasma membrane of root apices (Motoda et al., 2007). In Arabidopsis, two known ALMTs, ALMT1 and ALMT12, belong to a small family composed of 14 members in this specie (Figure 7).

The gene *ALMT1* (At1g08430) encodes a plasma membrane Al^{3+}-activated malate efflux transporter (Figure 7) (Hoekenga et al., 2006). ALMT1 transport activity is activated by Al^{3+} from the apoplastic side, and it is also transcriptionally upregulated by Al^{3+} (Sasaki et al., 2004; Hoekenga et al., 2006). ALMT1 expression in Arabidopsis is increased by indole acetic acid, ABA, and flagellin 22, as well as low pH, which indicates that ALMT is potentially involved in a wide array of biological functions in addition to Al^{3+} tolerance (Kobayashi et al., 2013). Expression of ALMT1 requires STOP1, a putative zinc finger transcription factor involved in low pH resistance and Al^{3+} tolerance in Arabidopsis (Iuchi et al., 2007; Sawaki et al., 2009). Loss-of-function mutants of ALMT1 have a reduction of approximately 60% in Al^{3+} tolerance compared to the wild-type (Hoekenga et al., 2006); the expression of wheat ALMT1 in tobacco and transgenic barley plants increased Al^{3+} tolerance (Delhaize et al., 2004; Sasaki et al., 2004).

The Arabidopsis gene *ALMT12* (At4g17970) encodes a component of a guard cell plasma membrane R-type anion channel, permeable to malate, chloride, and nitrate, which is not induced by Al^{3+} (Meyer et al., 2010). The activity of the heterologously expressed ALMT12 depends on extracellular malate, which shifts the threshold for voltage activation of ALMT12 towards more hyperpolarized potentials (Meyer et al., 2010). Thus, malate functions as a gating modifier as well as a permeating substrate of ALMT12. Loss-of-function mutants of *ALMT12* are impaired in malate-dependent R-type channel activity and in dark- and CO_2-induced stomatal closure, as well as in response to abscisic acid (Meyer et al., 2010; Sasaki et al., 2010).

ABCB14: ABC transporter B family member 14

In Arabidopsis, the gene At1g28010 encodes the ABC transporter B family member 14. *ABCB14* is strongly expressed in guard cell plasma membranes. It catalyzes the transport of malate from the apoplast into guard cells (Figure 7), where it acts as an osmoticum and induces guard cell swelling (Lee et al., 2008). ABCB14 reduces stomatal closure on transition to elevated CO_2, a function that depends on the presence of apoplastic malate (and fumarate) (Lee et al., 2008). The ABCB14 uptake mechanism allows recycling of malate and possibly fumarate released in response to high CO_2 levels or during normal stomatal closure. ABCB14-mediated malate uptake across the plasma membrane has a major effect on plant development under stress conditions; ABCB14 overexpressing plants have a retarded floral induction in comparison to wild type under conditions of combined high CO_2 and drought (Lee et al., 2008).

SLAC1: Slow Anion Channel-Associated 1

The Arabidopsis gene At1g12480 encodes the slow, deactivating, weak voltage-dependent anion channel *SLAC1*, which provides a gate for malate^{2-} transport across the guard-cell plasma membrane (Figure 7) (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009). The SLAC1 is a ~63kDa protein with 10 predicted transmembrane helices. It is a distant homolog of bacterial and fungal C4-dicarboxylate transporters (TDT) (Saier et al., 1999).

SLAC1 seems to be required to drive the long-term efflux of osmotically active anions from guard cells and stomatal closure; it thus appears necessary for an effective decrease in transpiration when soil water is limiting (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009).

SLAC1 is posttranscriptionally controlled by phosphorylation/dephosphorylation through components of the abscisic acid (ABA) signaling pathway: the protein kinase OST1 (open stomata 1) and the protein phosphatase ABI1 (ABA insensitive 1) (Geiger et al., 2009). SLAC1 is activated by the protein kinase OST1, while the protein phosphatase ABI1 functions as a negative regulator of OST1-dependent phosphorylation of SLAC1 by targeting OST1 activation rather than the channel dephosphorylation.

Mutations in *SLAC1* impair slow (S-type) anion channel currents that are activated by cytosolic Ca^{2+} and ABA, but do not affect R-type anion channel currents or Ca^{2+} channel function (Vahisalu et al., 2008). SLAC1 loss-of-function mutants have a markedly reduced anion efflux from the guard cells, which causes a constitutive stomatal opening phenotype.

Plastidic transport

The plastidic 2-oxoglutarate (2-OG)/malate transporter (OMT) and the general dicarboxylate transporter (DCT) participate in a double transporter system at the inner chloroplast membrane (Figure 7). In this transport system, there is no net transport of malate, as OMT imports 2-OG in exchange for stromal malate, and DCT exports glutamate in exchange for cytosolic malate. OMT and DCT are thus involved in ammonia assimilation and re-cycling, as 2-OG is the substrate of the stromal glutamine synthetase-glutamate synthase (GS-GOGAT) cycle; this cycle synthesizes glutamate that is exported for further use in the photorespiration pathway and in amino acid metabolism (Figure 5 and 7). Apart from this role, OMT has a second function as an OAA/malate transporter in the "malate valve" (Figure 5 and 7).

Plastidic 2-oxoglutarate/malate transporter (OMT1 or DiT1)

OMT1 or DiT1 is a 50 kDa protein that contains 12 putative transmembrane segments in an α-helical conformation and intervening hydrophilic loops (Taniguchi et al., 2002; Weber, 2005). OMT1 transports 2-OG in exchange with malate, with a $K_{mMalate}$ of 0.70 mM (Taniguchi et al., 2002). The protein also transport OAA with a high affinity (K_{mOAA} of 0.042 mM). OAA binds at the same active site as malate and 2-OG (Taniguchi et al., 2002).

In Arabidopsis, OMT is encoded by the single copy gene At5g12860. This gene is expressed in many tissues, and the expression in leaves is induced by nitrate and light (Taniguchi et al., 2002). Loss-of-function mutants of *OMT1* have a delayed growth and are more susceptible to photo-inhibition under high-light stress than the wild-type; they also have an impaired co-ordination of carbon and nitrogen assimilation (Kinoshita et al., 2011). OMT1 functionally integrates carbon/nitrogen metabolism

and the malate valve, as it has a dual function as a 2-OG/malate transporter and as an OAA/malate transporter (Weber, 2005; Kinoshita et al., 2011).

General dicarboxylate transporter (DCT1 or DiT2)

The DCT1 or DiT2 protein is localized to the plastid envelope membrane and contains 12 transmembrane helices. *In vitro* it behaves as a general dicarboxylate transporter with broad substrate specificities for dicarboxylic acids and the amino acids glutamate and aspartate. It transports 2-OG and glutamate with similar affinities (K_m of 2.5 and 1.6 mM, respectively) in exchange with malate, and can also transport OAA with a K_{mOAA} of 0.23 mM (Taniguchi et al., 2002; Renne et al., 2003). But, when considering kinetic constants and the subcellular substrate concentrations, transport of 2-OG and OAA *in vivo* is likely negligible. Thus, DiT2 operates as a malate/glutamate antiporter, exporting glutamate to the cytosol and importing malate to the stroma (Figure 7).

In Arabidopsis, DCT1 is encoded by At5g64280. Transcripts of *DCT1* accumulate in all organs, and the expression is induced by light (Taniguchi et al., 2002). Loss-of-function mutants of *DCT1* grow normally under high CO_2 but are non-viable under normal atmospheric conditions, as re-assimilation of ammonia generated by the photorespiratory cycle is blocked (Taniguchi et al., 2002; Renne et al., 2003).

Mitochondrial transport

Malate is transported through the inner mitochondrial membrane through carriers with broad substrate spectra: the dicarboxylate carrier (DIC) and the dicarboxylate-tricarboxylate carrier (DTC) (Figure 7). These carriers belong to the Arabidopsis mitochondrial carrier family (MCF) (Picault et al., 2002). The structure of the individual members of this family is tripartite, consisting of three tandemly repeated sequences. Each repeat is ~100 amino acids in length and contains two hydrophobic stretches that span the membrane separated by a hydrophilic loop. MCFs operate as homodimers of ~32 kDa per subunit (Picault et al., 2004).

Dicarboxylate carrier (DIC)

In Arabidopsis, three genes encode DICs, At2g22500 (*DIC1*), At4g24570 (*DIC2*), and At5g09470 (*DIC3*). DIC1 and DIC2 proteins share 70% amino acid identity, whereas the protein sequence of DIC3 is only 55-60% identical to those of DIC1 and DIC2. While DIC1 and DIC2 are present in all plant organs at comparable levels, DIC3 is present at low levels in flower buds and siliques (Palmieri et al., 2008).

The Arabidopsis DICs transport malate, OAA, succinate, maleate, and malonate in exchange for phosphate, sulfate, and thiosulfate at high rates, whereas 2-OG is a very poor substrate (Palmieri et al., 2008). All three DICs have similar K_m values for malate (0.4-0.79 mM). Although the Arabidopsis DIC isoforms have many properties in common, they show some differences. Relative to sulfate transport, dicarboxylates, phosphate and arsenate are transported more efficiently by DIC1 and DIC2 than

by DIC3, whereas thiosulfate and OAA are more efficiently transported by DIC3 than by DIC1 and DIC2 (Palmieri et al., 2008).

Because the DICs transport a broad spectrum of dicarboxylates and they show differential expression levels (DIC1 and DIC2 are expressed in almost all tissues at comparable levels, while the expression of DIC3 is very low), they may potentially play a role in a number of important metabolic functions that require organic acid flux to or from mitochondria (Palmieri et al., 2008). First, it is very likely that the DICs play an anaplerotic role by transporting dicarboxylic acids into the mitochondria (in exchange for phosphate or sulfate) to be used as respiratory substrates. Secondly, the malate/OAA exchange catalyzed by the DICs (import of OAA to the mitochondria and export of malate to the cytosol) coupled with the cytosolic and mitochondrial NAD-MDH activities represents the most important way to transfer reducing equivalents between the mitochondria and the cytosol (Figure 5-7). For example, the production of NADH in the cytosol is vital to fuel nitrate reductase for the reduction of nitrate to nitrite (Palmieri et al., 2008). Finally, by transporting succinate and malate in cotyledons, the DICs can play a role in the mobilization of storage lipids during germination and/or in gluconeogenesis.

Dicarboxylate-tricarboxylate carrier (DTC)

The DTC is encoded by a single gene in Arabidopsis (At5g19760) (Picault et al., 2004). DTC transports dicarboxylates, such as malate, 2-OG, OAA, and succinate, and tricarboxylates, such as citrate, isocitrate, and cis- and trans-aconitate, by a counter exchange mechanism. DTC accepts only the single protonated form of tricarboxylates (e.g. H-citrate^{2-}) and the unprotonated form of dicarboxylates (e.g. malate^{2-}), and catalyzes an obligatory, electroneutral exchange of substrates (Picault et al., 2002) (Figure 7).

DTC is expressed in many plant tissues (Picault et al., 2002) and transports a broad spectrum of dicarboxylates. Thus, it may play a role in a number of plant metabolic functions that require organic acid flux to or from the mitochondria. The malate/OAA exchange catalyzed by DTC can enable the export of redox equivalents from the mitochondrial matrix. The citrate exported from the mitochondria to the cytosol in exchange for OAA can be cleaved by citrate lyase to acetyl-CoA and OAA and used for fatty acid elongation and isoprenoid synthesis. In addition, DTC would have a role in nitrogen assimilation, as the export of citrate or 2-OG from the mitochondria has been proposed as a means to transport ammonium for assimilation by the plastidic GS-GOGAT (Hodges, 2002). Moreover, DTC expression is induced in nitrogen-starved tobacco plants along with other genes known to be involved in nitrogen assimilation (Picault et al., 2002).

Vacuolar transport

Plants can maintain constant malate cytosolic concentrations (1 to 3.5 mM) (Chang and Roberts, 1991; Heineke et al., 1991) by accumulating malate to very high concentrations in the vacuole (up to 300 mM). This is possible through the action of the tonoplast dicarboxylate transporter (tDT) and at least one malate channel (ALMT) (Figure 7).

Tonoplast dicarboxylate transporter (tDT)

The tDT is a 60 kDa protein that localizes to the tonoplast and contains 12 transmembrane domains (Emmerlich et al., 2003). The tDT imports and exports malate and fumarate (Figure 7). Arabidopsis contains a single copy gene encoding the tDT (At5g47560) (Emmerlich et al., 2003). Loss-of-function mutants of tDT exhibited no apparent phenotype, but contain 25% less malate in leaves. These plants have a residual vacuolar malate transport activity of about 30% of that observed with vacuoles isolated from WT plants. This is due to the presence of the vacuolar malate channel (ALMT) activity (Hurth et al., 2005). Due to the reduced malate levels in leaves, the *tdt* mutant is severely compromised in overcoming cellular acidification, implying that the TDT activity is critical for regulation of pH homeostasis (Hurth et al., 2005).

Vacuolar malate channel (ALMT)

The Arabidopsis gene At3g18440 encodes the vacuolar malate channel, ALMT9, that belongs to the ALMT protein family. ALMT9 contains two transmembrane domains, one localized to the N-terminus, consisting of between five and seven transmembrane α-helices, and one localized to the C-terminus that spans the tonoplast only once (Kovermann et al., 2007). *ALMT9* is expressed in nearly all organs of the plant, and in leaves specifically in mesophyll cells.

Deletion mutants for *ALMT9* exhibit strongly reduced vacuolar malate currents, but only slightly reduced malate content and no visible phenotype (Kovermann et al., 2007). In these mutant plants, tDT and a residual malate channel activity (still unknown) are sufficient to sustain the transport activity necessary to regulate the cytosolic malate homeostasis.

2-HYDROXYGLUTARATE

2-hydroxyglutarate is a five-carbon dicarboxylic acid with a single hydroxyl group at the α-position (Figure 1B). This hydroxyl forms a single stereocenter in the molecule. Both stereoisomers, D-2-hydoxyglutarate (D-2HG) and L-2-hydroxyglutarate (L-2HG), participate in Arabidopsis metabolism: D-2HG is involved in lysine catabolism, whereas L-2HG is involved in a metabolic repair mechanism (Figure 8). Both D- and L-2HG are oxidized to 2-oxoglutarate (2-OG) through the action of two mitochondrial stereospecific enzymes (Figure 8) (Engqvist et al., 2014). In some plant species, 2-HG has functions beyond being an intermediate in metabolism. In *Mercurialis perennis L.*, for example, 2-HG forms condensation products with caffeic and p-coumaric acid to form a class of molecules known as depsides (Lorenz et al., 2012).

D-2-Hydroxyglutarate

The presence of D-2HG in plants is known since at least 1969, when it was identified in extracts of the succulent plant *Euphorbia resinifera* (Boe et al., 1969). Despite this early identification of the molecule, it is only recently that the elucidation of its role in plant metabolism has received serious attention.

Biosynthesis of D-2HG

The current body of literature strongly suggests that lysine catabolism is the source of D-2HG in Arabidopsis (Araujo et al., 2010; Engqvist et al., 2011; Kirma et al., 2012; Boex-Fontvieille et al., 2013; Engqvist et al., 2014). However, the complete sequence of enzyme reactions that generate D-2HG from L-lysine is currently not known. Isotope tracer experiments performed on Arabidopsis plants lacking a functional D-2HG dehydrogenase (D-2HGDH) - the enzyme that metabolizes D-2HG (see next section) - using labeled L-lysine showed accumulation of lysine breakdown products as well as D-2HG (Araujo et al., 2010). Similarly, isotope-tracer experiments performed on isocitrate dehydrogenase mutants of Arabidopsis showed that 13C-labelled Lysine was recycled to 2-OG and subsequently to proline (Boex-Fontvieille et al., 2013). Furthermore, Arabidopsis D-2HGDH loss-of-function plants grown on agar plates supplemented with L-lysine accumulate D-2HG in a dose-dependent manner (Engqvist et al., 2011).

The initial steps of the catabolic pathway entail the conversion of L-lysine to saccharopine and subsequently to 2-aminoadipic-6-semialdehyde (Figure 8). These reactions are catalyzed by two functionally independent domains in the bi-functional cytosolic enzyme lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH, EC 1.5.1.8; At4g33150) (Epelbaum et al., 1997). Alternatively, L-lysine may be converted directly to 2-aminoadipic-6-semialdehyde by the AGD2-like defense response protein 1 (ALD1, EC 2.6.1.83; At2g13810) (Figure 8), which participates in the not yet fully elucidated L-pipecolate pathway (Navarova et al., 2012). The subsequent step comprises 2-aminoadipic-6-semialdehyde reduction to L-2-aminoadipate. No enzyme has yet been assigned to this reaction in Arabidopsis. However, the enzyme aldehyde dehydrogenase 7B4 (ALDH7B4, EC 1.2.1.3), which has >70% amino acid identity with the human enzyme antiquitin and converts 2-aminoadipic-6-semialdehyde to L-2-aminoadipate (Struys and Jakobs, 2010), is a likely candidate for this function (Engqvist et al., 2014). ALDH7B4 is encoded by a single gene, At1g54100. This gene is in the same co-expression network as those encoding D-2HGDH and SDH (ATTED-II database), indicating that the encoded enzymes may act together (Engqvist et al., 2014).

The subsequent enzymatic steps are not known, but have been predicted based on isotope-tracer experiments performed with whole cell extracts of *Pseudomonas putida* (Kopchick and Hartline, 1979; Engqvist et al., 2014). In these experiments, CoA-dependent conversion of labeled 2-aminoadipate to D-2HG was observed (Kopchick and Hartline, 1979). However, it cannot be said with certainty that this proposed metabolic pathway is indeed the exact route of D-2HG production from L-lysine in plants, since no enzymes that catalyze these reactions are currently known (Figure 8) (Engqvist et al., 2014).

Catabolism of D-2HG

D-2HG is oxidized to 2-OG in the mitochondrial matrix through the action of a D-2HG dehydrogenase (D-2HGDH, EC 1.1.99.39) (Figure 8), which is encoded in Arabidopsis by a single gene
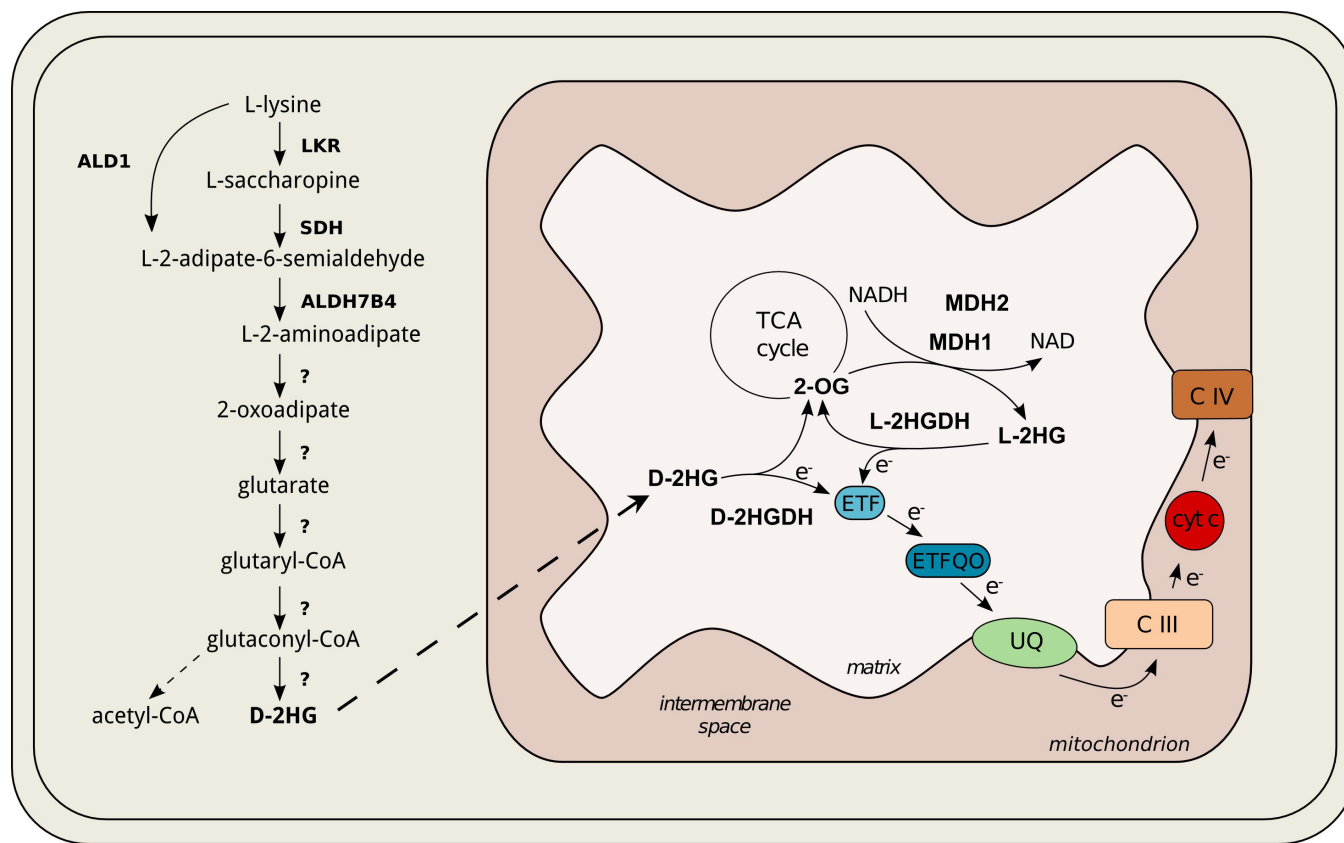
Figure 8. D-2HG and L-2HG metabolism (for a detailed explanation refer to the main text).

Dotted line represents several enzymatic steps. Dashed line indicates a possible transport process. Questionmarks indicate predicted enzymatic steps for which no enezymes have been identified.

Abbreviations: ALD1: AGD2-like defense response protein 1, LKR: lysine-ketoglutarate reductase, SDH: saccharopine dehydrogenase, ALDH7B4: aldehyde dehydrogenase 7B4, D-2HG: D-2-hydroxyglutarate, D-2HGDH: D-2-hydroxyglutarate dehydrogenase, 2-OG: 2-oxoglutarate, L-2HG: L-2-hydroxyglutarate, L-2HGDH: L-2-hydroxyglutarate dehydrogenase, MDH1: malate dehydrogenase 1, MDH2: malate dehydrogenase2, ETF: electron transfer flavoprotein, ETFQO: electron-transfer flavoprotein:ubiquinone oxidoreductase, UQ: ubiquinone, C III: complex III, cyt c: cytochrome c, C IV: complex IV.

(At4g36400) (Engqvist et al., 2009; Engqvist et al., 2011). At the time of characterization, this enzyme was added to the EC group 1.1.99.2, as this class covered all 2-hydroxyglutarate dehydrogenases. However, in 2013 the EC numbers were updated such that 1.1.99.2 is now specific for 2HGDHs that take the L-form, and the new EC number 1.1.99.39 was assigned to those enzymes that are specific for the D-form.

D-2HGDH is not known to take any alternate substrates in addition to D-2HG (Engqvist et al., 2009). Despite this, the affinity for D-2HG is fairly low, with a K_m of 584 µM (Engqvist et al., 2009). Since D-2HGDH catalyzes the oxidation of D-2HG to 2-OG, an electron acceptor is needed as a redox partner in the reaction. *In vitro*, the purified enzyme has no activity with either NAD(P)$^+$ or cyt c. All evidence to date indicates that the *in vivo* electron acceptor of D-2HGDH is the EFT/ETFQO enzyme complex (Engqvist et al., 2009; Araujo et al., 2010; Engqvist et al., 2011). This complex localizes to the matrix side of the inner mitochondrial membrane and collects electrons from other soluble mitochondrial dehydrogenases, which are passed on to the respiratory chain (Parker and Engel, 2000; Frerman, 2001). The EFT/ETFQO complex comprises the soluble electron transfer flavoprotein (ETF) and the membrane-associated electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO) (Araujo et al., 2010; Engqvist et al., 2011; Kleessen et al., 2012). Arabidopsis ETF is active as a heteromer of α- and β-subunits (the corresponding genes are At1g50940 and At5g43430, respectively). D-2HG accumulates in an Arabidopsis ETF β-subunit loss-of-function mutant under normal growth conditions (Hüdig et al., 2015). Upon incubation in the dark, 2-HG (the D- and L- stereoisomers were not separated in this experiment) accumulates in an ETFQO loss-of-function mutant (Araujo et al., 2010). The use of a flavoprotein electron acceptor results in the reaction proceeding almost exclusively in the direction of D-2HG oxidation.

The importance of D-2HG oxidation - and therefore D-2HG-DH activity - during normal growth conditions seems limited.

In these conditions, D-2HGDH loss-of-function plants do not display any phenotype, and there is only minor accumulation of D-2HG in plant tissue (Araujo et al., 2010; Engqvist et al., 2011). However, if such plants are subjected to long periods of extended darkness, the total amount of leaf D-2HG increases rapidly (Araujo et al., 2010; Engqvist et al., 2011). Such plants may also display increased rates of senescence and decreased leaf quantum yield (Araujo et al., 2010). However, an attempt to reproduce this phenotype was unsuccessful (Engqvist et al., 2011). Massive accumulation of D-2HG was also observed during developmental senescence in D-2HGDH loss-of-function plants. During this process, total leaf D-2HGDH activity increases in WT plants (Engqvist et al., 2011).

D-2HG is linked to nitrogen metabolism

2-OG is critical for plant nitrogen assimilation, as it is a substrate for glutamine synthetase (GS; EC 6.3.1.2). The exact enzymatic origin of this 2-OG has long been unknown, as several 2-OG generating enzymes exist in different subcellular compartments (Galvez et al., 1999). Some 2-OG is generated by the action of isocitrate dehydrogenase, but there also seem to be other sources (Galvez et al., 1999). Mutants with drastically decreased levels of mitochondrial isocitrate dehydrogenase do not have significantly impaired nitrogen metabolism (Boex-Fontvieille et al., 2013). The explanation to both the lysine breakdown and 2-OG generation is a metabolic bypass, where lysine is synthesized *de novo* from PEP and pyruvate, and immediately catabolized to 2-OG (Boex-Fontvieille et al., 2013).

Since D-2HGDH likely catalyzes the final step of this catabolic pathway (see section above), this also links D-2HGDH to nitrogen metabolism. Another link between D-2HGDH and nitrogen metabolism was provided in a study of an Arabidopsis mutant deficient in mitochondrial serine hydroxymethyltransferase 1 (SHMT1, EC 2.2.2.1; At4g37930) (Kuhn et al., 2013). In this classical photorespiratory mutant (Somerville and Ogren, 1981; Voll et al., 2006), D-2HG also accumulates under photorespiratory conditions (Kuhn et al., 2013). SHMT1 localizes to mitochondria, where it works in conjunction with glycine decarboxylase to convert two molecules of glycine to one molecule of serine, NH_3, and CO_2 (Somerville and Ogren, 1981; Voll et al., 2006). The released NH_3 is toxic, and 2-OG is used to reassimilate it to glutamate in a reaction catalyzed by GOGAT (EC 1.4.1.13; At1g23310) (Somerville, 1980; Jamai et al., 2009). The accumulation of D-2HG might occur through inhibition of D-2HGDH activity by 2-OG, which accumulates to high levels in the *shm1* loss-of-function mutant (Kuhn et al., 2013). Yet another example of the connection between D-2HG and nitrogen metabolism comes from maize. In a mutant of cytosolic GS, 2-HG accumulates five-fold compared to wild-type levels (Amiour et al., 2014). It is currently not known whether Arabidopsis GS mutants also accumulate D-2HG. In summary, there are tantalizing hints at an important role for D-2HG in plant lysine catabolism and nitrogen metabolism. However, the picture is far from complete and studies to elucidate the metabolic and regulatory connections should be undertaken.

L-2-Hydroxyglutarate

Until very recently, nothing was known about L-2HG metabolism in plants. The first indication that this metabolite was even present in plant extracts was published in 2011 (Engqvist et al., 2011). Meanwhile, both the metabolic origin and catabolism of L-2HG have been elucidated in Arabidopsis (Hüdig et al., 2015).

Involvement of L-2HG in plant metabolism

L-2HG is produced by Arabidopsis mMDH1 and mMDH2 through the NADH-dependent reduction of 2-OG in a side reaction (Figure 8) (Hüdig et al., 2015). The thermodynamic equilibrium of this reaction is strongly biased towards L-2HG formation, and the two mMDH isoforms cannot re-oxidize L-2HG to 2-OG at significant rates (Hüdig et al., 2015). In contrast to D-2HG, L-2HG does not appear to be involved in lysine or nitrogen metabolism. Indeed, L-2HG is not known to participate in any known pathways in plants and therefore appears to be a metabolic dead end. L-2HG is re-oxidized by the mitochondrially localized FAD-containing enzyme L-2HGDH (At3g56840, EC 1.1.99.2) (Engqvist et al., 2014; Hüdig et al., 2015). L-2HGDH has a turnover rate or K_{cat} of 613 s^{-1} and an affinity constant for L-2HG of 0.24 mM. Similar to D-2HGDH, L-2HGDH is not active with $NAD(P)^+$ or cyt c as electron acceptors. Instead, L-2HGDH uses ETF as an electron acceptor, as Arabidopsis ETF loss-of-function mutants as well as L-2HGDH loss-of-function mutants accumulate L-2HG to levels much higher than in the wild-type plant (Hüdig et al., 2015). Both L-2HGDH and D-2HGDH are specific for their respective stereoisomers of 2-HG. L-2HG does not accumulate in D-2HGDH loss-of-function plants (Engqvist et al., 2011), and D-2HG does not accumulate in L-2HGDH loss-of-function plants (Hüdig et al., 2015).

The Arabidopsis L-2HG metabolic repair mechanism is completely analogous to that characterized in humans (Figure 8) (Rzem et al., 2004; Rzem et al., 2007; Hüdig et al., 2015). In both humans and Arabidopsis, L-2HG is generated from 2-OG by a side reaction of mitochondrial MDH. In both types of organisms, L-2HG is re-oxidized to 2-OG by a flavin-containing L-2HGDH enzyme, and the electrons are passed on to an ETF protein (Figure 8). Furthermore, mutations of L-2HGDH or ETF lead to accumulation of L-2HG in both humans and Arabidopsis. The major difference between the two organisms is the physiological effect of high L-2HG levels. In humans, high L-2HG levels lead to severe detrimental effects in the form of a neurodegenerative disease known as L-2HG aciduria (Rzem et al., 2007). In contrast, high L-2HG levels do not adversely affect Arabidopsis development under a range of tested conditions (Hüdig et al., 2015). However, orthologs of L-2HGDH are found in all examined genomes of viridiplantae (Engqvist et al., 2014), indicating that this damage-repair path makes an essential contribution to plant fitness in as yet unidentified conditions in the wild (Hüdig et al., 2015).

FUTURE PROSPECTS

As described in this chapter, 2HA are involved in fundamental pathways of plant primary metabolism. Many enzymes involved

in the biosynthesis and degradation of the major 2HA, glycolate, malate, lactate, and 2-hydroxyglutarate, have been characterized in *Arabidopsis thaliana*. However, still little is known about factors influencing the transcriptional regulation of the genes coding for these enzymes and 2HA transport systems. More important, post-translational modifications of proteins provide a direct and rapid means to respond to alterations of cellular homeostasis by acting as switches of enzyme activities or by regulating substrate affinities or allosteric effects on enzymes. Thus, studies focusing on understanding *in vivo* allosteric and post-translational regulation of the enzymes, pathways, and transporters that participate in 2HA metabolism will be needed before our overall understanding of plant 2HA metabolism is complete. Future research will likely see progress on these fundamental issues.

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