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REVIEW PAPER

The role of proteins in C_3 plants prior to their recruitment into the C_4 pathway

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Abstract

Our most productive crops and native vegetation use a modified version of photosynthesis known as the C_4 pathway. Leaves of C_4 crops have increased nitrogen and water use efficiencies compared with C_3 species. Although the modifications to leaves of C_4 plants are complex, their faster growth led to the proposal that C_4 photosynthesis should be installed in C_3 crops in order to increase yield potential. Typically, a limited set of proteins become restricted to mesophyll or bundle sheath cells, and this allows CO_2 to be concentrated around the primary carboxylase RuBisCO. The role that these proteins play in C_3 species prior to their recruitment into the C_4 pathway is addressed here. Understanding the role of these proteins in C_3 plants is likely to be of use in predicting how the metabolism of a C_3 leaf will alter as components of the C_4 pathway are introduced as part of efforts to install characteristics of C_4 photosynthesis in leaves of C_3 crops.

Key words: C_3 photosynthesis, C_4 photosynthesis, *Cleome*, evolution, protein function.

C_4 photosynthesis

Plant species can be classified as using C_3 photosynthesis, C_4 photosynthesis, or Crassulacean acid metabolism depending on whether the primary product of photosynthesis contains three or four carbons (Sage, 2004). C_3 photosynthesis is considered ancestral, and the C_4 pathway is estimated to have evolved from C_3 plants at least 62 times in 18 separate families of plants (Sage *et al.*, 2011). The efficiency of photosynthesis, especially in warmer climates, can be enhanced in C_4 species, and this allows growth rates to be up to 50% higher than those of C_3 plants. Both the most productive native vegetation and crops use C_4 photosynthesis (Brown, 1999). C_4 plants also have higher water and nitrogen use efficiencies, and because of all these characteristics it has been proposed that it would be desirable to integrate characteristics of C_4 photosynthesis into C_3 crops (Matsuoka *et al.*, 2001; Hibberd *et al.*, 2008).

While a small number of species have developed a C_4 pathway that operates within single cells (Reiskind and Bowes, 1991; Voznesenskaya *et al.*, 2001, 2002), in most C_4 plants the photosynthetic apparatus is partitioned between bundle sheath (BS) and mesophyll (M) cells that are arranged concentrically around veins (Hatch, 1987). Both M and BS cells exist in C_3 species (Kinsman and Pyke, 1998), but they have become more specialized in C_4 plants with changes in both cell biology and biochemistry. In M cells of most C_4 plants, after conversion of CO_2 to HCO_3^- by carbonic anhydrase (CA), phospho*enol*pyruvate carboxylase (PEPC) acts as the initial carboxylase to produce oxaloacetate (OAA), which is then reduced to either malate or aspartate. These C_4 acids then diffuse to the BS where a C_4 acid decarboxylase releases CO_2 and so concentrates CO_2 around RuBisCO, favouring the carboxylation reaction and decreasing the oxygenation reaction. There are three C_4 acid decarboxylases known to have been recruited into C_4 photosynthesis, NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME), and phospho*enol*pyruvate carboxykinase (PEPCK) (Furbank, 2011). These three decarboxylases are associated with modified versions of the basic C_4 cycle (Fig. 1). To maintain the C_4 cycle, the three-carbon compound generated during decarboxylation diffuses back to the M, and the presence of

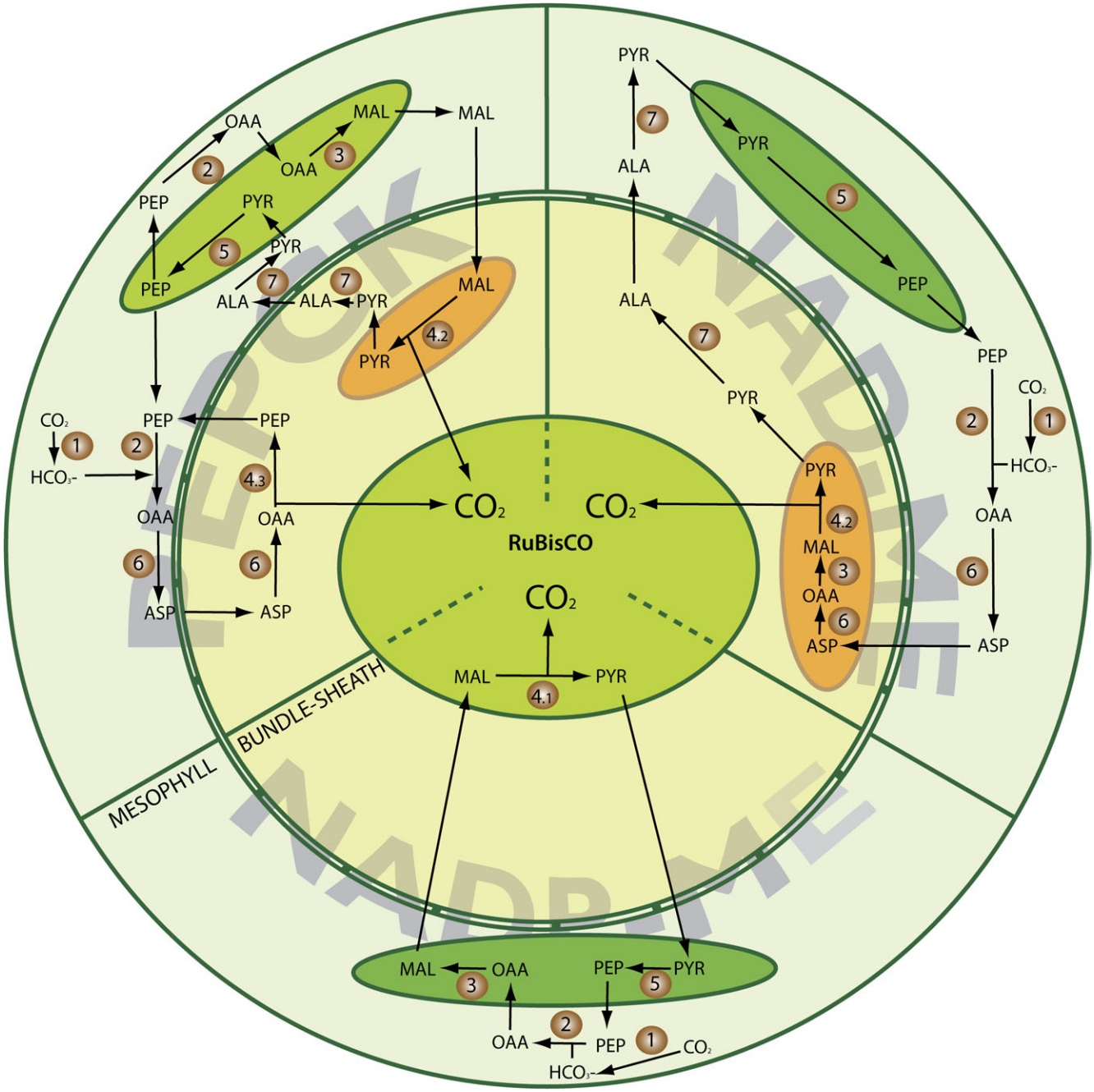

Fig. 1. Schematic representation of C4-related reactions in the three known subtypes, NAD-ME, NADP-ME and PEPCK. Mesophyll surrounding bundle-sheath cells. Chloroplasts are in green and mitochondria in orange. MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; ASP, aspartate; ALA, alanine; PYR, pyruvate. 1. Carbonic anhydrases. 2. Phosphoenolpyruvate carboxylase, 3. NAD/P-Malate dehydrogenase, 4 Decarboxylases, 5. Pyruvate,orthophosphate dikinase, 6. Aspartate amino acids transferases. 7. Alanine amino acids transferases.

pyruvate,orthophosphate dikinase (PPDK) in M chloroplasts allows regeneration of phospho*enol*pyruvate (PEP), the initial acceptor of HCO_3^-. Extensive reviews have been published in recent years describing the compartmentation of metabolism (Majeran and van Wijk, 2009) and regulation of gene expression (Hibberd and Covshoff, 2010) associated with alterations to photosynthesis in C_4 leaves.

All enzymes required for the C_4 pathway are present in C_3 plants. As a consequence, if characteristics of C_4 photosynthesis are integrated into C_3 crops such as rice, the activity of many proteins that form networks associated with central metabolism will be dramatically altered, and this may lead to pleiotropic effects. Here, recent advances in understanding of enzymes of the C_4 pathway prior to their recruitment into C_4 photosynthesis are discussed. A better understanding of the role of proteins in C_3 plants that are recruited into the highly efficient C_4 pathway may inform attempts to convert rice from C_3 to C_4 photosynthesis.

The function of proteins in C_3 plants that are recruited into the C_4 pathway

If proteins recruited into C_4 photosynthesis fulfil conserved roles in distantly related C_3 plants, then the limited number of studies done in specific C_3 species are useful to predict how the role of these proteins has altered. However, this has currently not been established. In addition, in C_3 species, many of the proteins recruited into the C_4 pathway are encoded by multigene families (Hibberd and Covshoff, 2010), implying that each isoform may be carrying out specific roles. To understand how the role of these proteins has altered as they are recruited into C_4 photosynthesis, it would therefore be desirable to identify orthologues in C_3 species, and then determine the role of proteins encoded by these orthologues. Unfortunately this has been difficult to do with many of the genes studied, because most of the C_3 and C_4 models are distantly related. It is however possible to take this phylogenetically informed approach with species in genera such as *Flaveria* or *Cleome* because functional analysis of proteins could be undertaken in the C_3 models tobacco and *Arabidopsis* that are relatively closely related to *Flaveria* and *Cleome*, respectively. Additionally, both *Flaveria* and *Cleome* possess C_4 and C_3 species, allowing a comparative approach (Akyildiz *et al.*, 2007; Brown *et al.*, 2005; Brautigam *et al.*, 2010). Therefore, where possible, the role of proteins in closely related C_3 and C_4 models is compared. The C_4 pathway also relies on a significant increase in the amount of inter- and intracellular metabolite exchange, and the function of transporters can alter as they are recruited into C_4 photosynthesis (Huber and Edwards, 1977; Day and Hatch, 1981).

Carbonic anhydrase (CA)

Plants possess three classes of CA known as the α-, β-, and γCAs. βCAs have been recruited into the C_4 pathway, and so their role in C_3 plants is specifically addressed next. Functions appear diverse, ranging from supplying CO_2 to photosynthesis (Price *et al.*, 1994) to roles in non-leaf tissue, such as lipid biosynthesis in dark-grown cotton seeds (Hoang *et al.*, 1999; Hoang and Chapman, 2002) and nodules of legumes (Kavroulakis *et al.*, 2000; Flemetakis *et al.*, 2003). In tobacco, although antisense-mediated reductions of CA to <2% of the levels in the wild type produced no significant reduction in the rate of photosynthesis, analysis of carbon isotope discrimination supported the proposal that CA facilitates the supply of CO_2 to the site of carboxylation (Price *et al.*, 1994).

Translational fusions between cDNAs of each βCA from *Arabidopsis* and the green fluorescent protein indicated that AtβCA1 and AtβCA5 localize to chloroplasts, AtβCA2 and AtβCA3 localize to the cytosol, AtβCA4 localizes close to the plasma membrane, and AtβCA6 localizes to mitochondria (Fett and Coleman, 1994; Fabre *et al.*, 2007). The phenotype of the two strongest *Arabidopsis* antisense lines for *CA1* and insertional mutants is stronger than those reported in tobacco, at least at the seedling stage, because plants lacking CA1 failed to develop when transferred into light (Ferreira *et al.*, 2008). Subsequent analysis of AtβCA1 indicated that in addition to its localization to the chloroplast it is also found close to the plasma membrane (Hu *et al.*, 2010). Both the presence of βCA1 in the chloroplast and its association with the plasma membrane imply multiple functions for this protein, and analysis of plants lacking this protein support this view. The reason for the differences between tobacco and *Arabidopsis* is not clear. Interestingly, it appears that in *Arabidopsis*, CA1 also fulfils a separate role in sensing CO_2. Analysis of double insertional mutants for *CA1* and *CA4* indicated that they function cooperatively in sensing and regulating the response of guard cell aperture to CO_2 (Hu *et al.*, 2010). This is thought to be due to a sensing role at the plasma membrane of guard cells. Double insertional mutants for *CA1* and *CA4* also had increased stomatal density.

In the C_4 *Flaveria bidentis*, βCA3 has been recruited into the C_4 cycle (Tanz *et al.*, 2009). In the closely related C_3 species, *F. pringlei*, CA3 is targeted to chloroplasts, while in *F. bidentis*, loss of the chloroplast targeting peptide leads to its localization in the cytosol (Tanz *et al.*, 2009). This indicates that it has been recruited from an original chloroplastic function. In C_4 *Cleome gynandra*, which is closely related to *Arabidopsis*, genes encoding five βCAs have been identified by RNA-seq, and it appears that orthologues to *Arabidopsis* βCA2 and βCA4 are recruited into the C_4 pathway (Brautigam *et al.*, 2010). This implies that the role of βCA4 has been extended from being involved in CO_2 sensing in guard cells in *Arabidopsis* to a role in supplying HCO_3^- to PEPC in leaves of *C. gynandra*. At present it is not clear whether these roles are important when trying to manipulate the accumulation and activity of these proteins in C_3 plants. Overall, CAs have been shown to play a range of functions in different compartments of C_3 cells, and this redundancy may have in fact helped in their reductions in the BS and increase in M cells of C_4 plants (Tanz *et al.*, 2009).

Phosphoenolpyruvate carboxylase (PEPC)

In C_3 plants PEPC is thought to carry out various functions depending on the tissue and stage of development. These include supplying carbon skeletons to the tricarboxylic acid (TCA) cycle, operating in malate homeostasis during drought stress, supplying carbon skeletons to allow ammonium assimilation, and regulating stomatal conductance. For example, replenishment of the TCA cycle with OAA would allow carbon skeletons to be withdrawn for biosynthesis of amino acids (Miyao and Fukayama, 2003). This role is supported by the fact that overexpression of genes encoding PEPC leads to increases in respiration in potato, rice, and tobacco (Hausler *et al.*, 2001; Fukayama *et al.*, 2003; Miyao and Fukayama, 2003). PEPC also appears to play a role in the extension of cotton fibres

(Li et al., 2010), and it is proposed that PEPC activity allows malate production and therefore increased turgor that is required for fibre elongation. In wheat, PEPC is relatively abundant in the meristematic and vascular cells, and the abundance of transcripts encoding PEPC increased during salt and drought stress (Gonzalez et al., 2003). Of the six genes predicted to encode PEPC in rice, *Osppc4* generates a protein that is targeted to chloroplasts. When *Osppc4* is knocked-down, plants are stunted and are particularly compromised under conditions in which ammonium is the main source of nitrogen. It appears that reducing the amount of PEPC in M chloroplasts of rice decreases the ability to supply carbon skeletons to allow assimilation of ammonium (Masumoto et al., 2010). In seed pods of rice, $^{14}CO_2$ labelling experiments showed that PEPC participates in the fixation of respired CO_2 (Imaizumi et al., 1997).

In *Arabidopsis*, salt and drought stress lead to increased abundance of transcripts encoding PEPC in roots, and, of the four genes encoding PEPC in *Arabidopsis*, semi-quantitative reverse transcription-PCR (RT-PCR) indicated that transcripts derived from *Atppc4* increased in abundance markedly in response to both stresses (Sanchez et al., 2006). In *C. gynandra*, orthologues to AtPPC1 and AtPPC2 have been recruited into the C_4 pathway (Bräutigam et al., 2010), but as their roles in *Arabidopsis* have not been clearly defined it is difficult to draw conclusions about how their function has altered.

Phosphoenolpyruvate carboxylase kinase (PPCk)

Phosphorylation activates PEPC by simultaneously lowering its K_m for PEP and its sensitivity to L-malate, and enhancing activation by glucose-6-phosphate. Phosphorylation of a serine residue towards the N-terminus of PEPC is catalysed by PPCk (Jiao and Chollet, 1991; Nimmo, 2003; Gregory et al., 2009). In *Flaveria*, only one *PPCk* gene has been isolated, and transcripts respond strongly to light and dark in C_4 *F. trinervia* compared with C_3 *F. pringlei* (Tsuchida et al., 2001; Furumoto et al., 2007).

In *Arabidopsis*, *PPCk1* transcripts are relatively abundant in rosettes (https://www.genevestigator.com/gv/index.jsp), and the insertional mutant *ppck1* shows significant alterations to the abundance of intermediates of both the TCA cycle and photorespiration, as well as soluble sugars and some secondary metabolites (Sullivan et al., 2004; Meimoun et al., 2009). Although knocking out PPCk in *Arabidopsis* has a major impact, it is not clear whether central metabolism would be significantly perturbed if the amount of PPCk was increased in concert with PEPC. In *C. gynandra* and *C. spinosa*, transcripts encoding two isoforms of PPCk have been identified by deep sequencing, and mRNA derived from *CgPPCk1* is more abundant in the C_4 compared with the C_3 species (Brautigam et al., 2010). This implies that PPCk1 function has been modified from regulating the TCA cycle and photorespiration in *Arabidopsis* to regulating photosynthesis in *C. gynandra*.

Malate dehydrogenase (MDH)

MDH interconverts OAA and malate using NADH or NADPH. Isoforms of NAD-MDH are found in the cytosol, mitochondria, glyoxysomes, and peroxisomes (Gietl, 1990, 1992; Gietl et al., 1990), while NADH- and NADPH-dependent isoforms are found in chloroplasts (Berkemeyer et al., 1998). In *F. bidentis* 90% of NADP-MDH can be removed without an effect on photosynthesis (Trevanion et al., 1997), and in *C. gynandra* there was little detectable increase in abundance of transcripts encoding MDH (Brautigam et al., 2010). It therefore appears that little more than C_3 levels are required for the C_4 pathway to operate. To our knowledge it is not clear whether MDH used in the C_4 pathway is recruited directly from those already present in chloroplasts and mitochondria of the NADP-ME and NAD-ME subtypes, respectively. It is therefore relevant to consider the role of each of these isoforms in C_3 species prior to their recruitment into the C_4 pathway.

In C_3 plants, MDH plays important roles in early seedling growth as well as in mature leaves. For example, during early seedling growth of *Arabidopsis* double insertional mutants in the two *MDH* genes encoding peroxisomal NAD-MDH proteins, β-oxidation of fatty acids is compromised because less NAD was regenerated (Pracharoenwattana et al., 2007). In mature leaves, peroxisomal NAD-MDH is important in maintaining optimal rates of photorespiration (Cousins et al., 2007), and in chloroplasts NADP-MDH controls the resupply of NADH to the photosynthetic electron transport chain in a process known as the 'malate valve' (Scheibe, 2004). The abundance of transcripts encoding NADP-MDH, and the maximum catalytic activity of the enzyme increases in response to both low temperature and high light (Hameister et al., 2007). Chloroplastic NADP-MDH is activated by light, and through the conversion of OAA to malate also produces NADP. This NADP can then be reduced to NADPH by ferredoxin NADP-dependent reductase associated with photosystem I. Maintaining the supply of NADP is important to sustain photosynthetic electron transport. If light-driven electron transport slows down, the amount of NADP builds up in the chloroplast stroma and this inactivates NADP-MDH through specific cysteine residues that are present in the chloroplastic isoform of the protein (Ocheretina et al., 2000). It has also been proposed that plastidic NAD-MDH is responsible for redox homeostasis in non-photosynthetic plastids or in chloroplasts at night (Scheibe et al., 1990; Berkemeyer et al., 1998). An *Arabidopsis* double mutant of the mitochondrial NAD-MDH has reduced photorespiration due to the change in malate homeostasis, but growth retardation due to lower rates of CO_2 assimilation and higher rates of respiration (Tomaz et al., 2010). It is not clear how the multiple roles of MDH in C_3 plants, including involvement in the TCA cycle, photorespiration, response to low temperature, and partitioning of carbon in leaves, will impact on attempts to place components of the C_4 cycle

into rice. However, it would seem sensible for the processes in which MDH is known to operate in C_3 species to be assessed carefully in the lines of rice that are generated.

C_4 acid decarboxylases

C_4 acid decarboxylases act in the BS of C_4 plants to release CO_2 from four-carbon compounds, concentrating CO_2 around RuBisCO (Hatch, 1987). Different lineages of C_4 plants have preferentially recruited one of three distinct C_4 acid decarboxylases, and this defines the three biochemical subtypes of C_4 plants, although many plants use a mixture of the decarboxylases (see Furbank, 2011). The three enzymes are NADP-ME, NAD-ME, and PEPCK.

In C_3 plants the enzymes have differing roles depending on tissue type and the stage of development. PEPCK, for example, has a clearly defined function in germinating seeds of C_3 plants, where it allows the mobilization of sugars from lipids and some amino acids by gluconeogenesis (Leegood and ap Rees, 1978; Rylott *et al.*, 2003; Penfield *et al.*, 2004; Malone *et al.*, 2007). In trichomes, however, PEPCK is thought to have a role in defence, providing PEP to the shikimate pathway for the biosynthesis of aromatic compounds (Leegood *et al.*, 1999). In *Arabidopsis*, cucumber, and grape, immunolocalization has demonstrated that PEPCK is present in phloem companion cells (Walker *et al.*, 1999; Malone *et al.*, 2007), where it may function in metabolism of nitrogenous compounds and pH regulation (Walker *et al.*, 1999; Delgado-Alvarado *et al.*, 2007; Malone *et al.*, 2007). PEPCK may also have an anaplerotic role in phloem, replenishing TCA intermediates, or acting to generate PEP from amino acids in the phloem, either for gluconeogenesis or for the shikimate pathway (Walker *et al.*, 1999; Brown *et al.*, 2010). In *Arabidopsis*, two genes encode PEPCK: *AtPCK1* is expressed throughout the plant, whilst *AtPCK2* transcripts are only detectable in roots and flowers (Rylott *et al.*, 2003; Malone *et al.*, 2007).

Both cytosolic and chloroplastic NADP-MEs are found in C_3 plants (Edwards and Andreo, 1992; Drincovich *et al.*, 2001), with differing roles suggested for each (Gerrard-Wheeler *et al.*, 2005). The reader is referred to Maier et al. (2011) for a detailed assessment of the enzymology and role of these proteins. The present analyses are therefore restricted to a limited number of points. Roles for cytosolic NADP-ME include provision of reducing power for anabolic processes, for example in assisting the oxidative pentose phosphate pathway (Gerrard-Wheeler *et al.*, 2005); and lignin biosynthesis by providing NADPH (Walter *et al.*, 1994; Schaaf *et al.*, 1995; Gerrard-Wheeler *et al.*, 2005). Cytosolic NADP-ME can act to regulate malate concentration, as well as control cytosolic pH (Martinoia and Rentsch, 1994; Lai *et al.*, 2002*b*) and turgor pressure in guard cells (Outlaw *et al.*, 1981; Maurino *et al.*, 1997; Laporte *et al.*, 2002). Chloroplastic NADP-ME is less well studied in C_3 species, although a possible role in lipid biosynthesis has been suggested (Gerrard-Wheeler *et al.*, 2005). Additionally both forms of NADP-ME may be involved in plant defence (Schaaf *et al.*, 1995; Casati *et al.*, 1999; Lai *et al.*, 2002*a*).

In *Arabidopsis* there are four genes encoding NADP-MEs (*NADP-ME1–NADP-ME4*). Transcript analysis has demonstrated that *NADP-ME2* and *NADP-ME4* are expressed in multiple organs throughout the plant, whereas transcripts for *NADP-ME1* are only detected in roots and *NADP-ME3* predominantly in flowers (Gerrard-Wheeler *et al.*, 2005). AtNADP-ME2 is responsible for the majority of NADP-ME activity in leaves and mid-veins, and is thought to be cytosolic, whereas NADP-ME4 is chloroplastic (Gerrard-Wheeler *et al.*, 2005, 2008; Brown *et al.*, 2010) and structurally resembles maize NADP-MEs in terms of its oligomerization pattern *in vitro* (Detarsio *et al.*, 2003; Saigo *et al.*, 2004; Gerrard-Wheeler *et al.*, 2005). NADP-ME4 has been proposed to act in fatty acid synthesis, which is supported by the *NADP-ME4* promoter directing high expression of *uidA* during embryogenesis and germination (Gerrard-Wheeler *et al.*, 2005). Because *C. gynandra* has not recruited NADP-ME as its C_4 acid decarboxylase (Marshall *et al.*, 2007) these studies of *Arabidopsis* do not provide significant insight into how its function has altered as it is recruited into the C_4 pathway.

The functional *Arabidopsis* NAD-ME enzyme in leaves is a heterodimer formed from the gene products of the two *AtNAD-ME* genes, although the single gene products also have the capacity to form functional homodimers (Tronconi *et al.*, 2008). NAD-ME has also been shown to have a heterodimeric structure in other plant species such as potato (Grover and Wedding, 1982; Willeford and Wedding, 1987) and *Amaranthus* (Long and Berry, 1996). NAD-ME has long been thought to have a role in determining flux through the TCA cycle by providing pyruvate for oxidation (Grover *et al.*, 1981). However, studies with an antisense NAD-ME potato line found no detectable changes in flux through the TCA cycle, but instead alterations in glycolytic metabolism (Jenner *et al.*, 2001). In *Arabidopsis* leaves, studies with *NAD-ME* insertional mutants have revealed a role in coordinating carbon and nitrogen metabolism (Tronconi *et al.*, 2008). In an *Arabidopsis* double mutant, completely lacking NAD-ME activity, excess malate in the mitochondria at the end of the night was found to be directed into synthesis of amino acids via the TCA intermediates OAA and 2-oxoglutarate (Tronconi *et al.*, 2008).

A role for the C_4 acid decarboxylases in stems and mid-veins of C_3 plants has been proposed in which organic acids present in the xylem stream may supply CO_2 to photosynthesis and, in combination with PPDK, supply PEP to the shikimate pathway (Hibberd and Quick, 2002; Brown *et al.*, 2010). A significant flux of carbon into the shikimate pathway is probably required in veins during periods of high lignin production. When photosynthesis was removed from cells around veins in *Arabidopsis*, reduced amounts of shikimate were measured, in addition to alterations in transcripts encoding proteins involved in pathways that generate PEP (Janacek *et al.*, 2009). Insertional mutants in either NAD-ME or NADP-MEs showed little impact on shikimate content (Gerrard-Wheeler *et al.*, 2005; Tronconi *et al.*, 2008), but redundancy in the

Table 1. Primary function of proteins involved in C_4 photosynthesis in C_3 plants

Protein	Species	Location	Function	References
βCA	*Arabidopsis*	Leaves, seedlings	Carbon transfer to RubisCO, guard cell aperture, seedling survivorship	Price *et al.* (1994); Ferreira *et al* (2008); Hu *et al.* (2010)
	Gossypium hirsutum	Seeds	Lipid biosynthesis	Hoang *et al.* (2002)
	Lotus japonicus, *Glycine max*	Roots	Providing carbon to PPC during nodule development	Kavroulakis *et al.* (2000); Flemetakis *et al.* (2003)
PEPC	*Arabidopsis*	Leaves	Anaplerotic, amino acid synthesis, nitrogen metabolism	Miyao and Fukayama (2003)
	Amaranthus edulis	Leaves	Stomatal movement regulation	Cousins *et al.* (2007)
	Gossypium hirsutum	Stems	Fibre elongation	Li *et al.* (2010)
	Oryza	Roots	Ammonium fixation in anaerobia	Masumoto *et al.* (2010)
PPCk	*Arabidopsis*	Rosette, roots	Modifying PEPC activity to nutritional status	Gregory *et al.* (2009)
NAD-MDH	*Arabidopsis*	Leaves	Redox homeostasis at night	Berkemeyer *et al.* (1998)
NADP-MDH	*Arabidopsis*	Seeds, leaves	TCA cycle and photorespiration, supplying OH-pyruvate reductase, β-oxidation in seed development	Cousins *et al.* (2007); Pracharoenwattana *et al.* (2010)
	Spinacia oleracea	Leaves	Malate valve. Stabilizing stromal ATP/NADPH ratio	Scheibe (2004)
PEPCK	*Arabidopsis*	Seeds, trichomes, non-green tissues	Seed germination, defence in trichomes. Gluconeogenesis	Leegood *et al.* (1999); Rylott *et al.* (2003)
	Arabidopsis, *Pisum*, *Vitis vinifera*	Leaves,	Nitrogen metabolism in phloem companion cells. Anaplerotic in phloem	Walker *et al.* (1999); Brown *et al.* (2010)
NAD-ME	*Solanum tuberosum*, *Arabidopsis*	Leaves	Anaplerotic. Providing pyruvate for oxidation. Coordinate carbon and nitrogen metabolism	Grover *et al.* (1981); Jenner *et al.* (2001); Tronconi *et al.* (2008)
NADP-ME	*Arabidopsis*	Leaves	Assisting the oxidative pentose phosphate pathway	Gerrard-Wheeler *et al.* (2005)
	Pisum sp.			Walter *et al.* (1994)
	Flaveria	Fruits	Malate concentration and cytosolic pH, defence	Lai *et al.* (2002*b*)
	Tobacco, *Triticum aestivum*, *Vicia faba*	Leaves	Turgor pressure in guard cells. Providing NADPH to lignin biosynthesis	Outlaw *et al.* (1981); Maurino *et al.* (1997); Laporte *et al.* (2002)
	Ricinus communis	Seeds	Lipid biosynthesis	Eastmond *et al.* (1997)
PPDK	*Oryza*, *Arabidopsis*		Providing PEP to the shikimate pathway. Nitrogen remobilization during senescence	Hibberd and Quick (2002); Taylor *et al.* (2010)
	Oryza	Roots	Root anoxia	Moons *et al.* (1998)
AsAT	*Arabidopsis*	Leaves	Central metabolism, nitrogen fixation, and transport	Schulz and Coruzzi (1995)
AlAT	*Hordeum*	Roots	Hypoxia response	Miyashita *et al.* (2007)

pathway or up-regulation of alternative isoforms could maintain flux to shikimate. The ability of C_3 plants to decarboxylate organic acids from the xylem stream is phylogenetically widespread among dicotyledons (Hibberd and Quick, 2002; Brown *et al.*, 2010), and suggests that some of the prerequisites for evolution of the C_4 pathway are commonly already present in C_3 plants. The promoter regions were shown to be sufficient for the accumulation of decarboxylases in veinal cells (Brown *et al.*, 2010).

In *C. gynandra*, genes orthologous to *AtNADME1* and *AtNADME2* have been recruited into the C_4 pathway (Brautigam *et al.*, 2010). It therefore appears that during the evolution of C_4 photosynthesis, the role of NAD-ME has been refocused from one in coordinating carbon and nitrogen metabolism to supplying CO_2 to RuBisCO.

Pyruvate,orthophosphate dikinase (PPDK)

PPDK catalyzes the reversible conversion of pyruvate and PEP. In rice, *Flaveria*, and *Arabidopsis*, two promoters give rise to two forms of transcript that encode chloroplastic and cytosolic isoforms of PPDK (Rosche and Westhoff, 1995; Imaizumi *et al.*, 1997; Parsley and Hibberd, 2006). The tissue with the highest PPDK content in C_3 plants appears to be seeds, where it has been implicated in controlling amino acid interconversions and starch biosynthesis (Aoyagi and Bassham, 1984*a*, *b*; Aoyagi and Chua, 1988; Kang *et al.*, 2005). Activities of PPDK are also substantial in mid-veins of leaves where it has been proposed to provide PEP to the shikimate pathway for lignin biosynthesis (Hibberd and Quick, 2002). In *Arabidopsis*, transcripts encoding the cytosolic PPDK are abundant in cotyledons during early seedling growth (Parsley and Hibberd, 2006), but they also increase substantially during both dark-induced and natural senescence of *Arabidopsis* leaves (Lin and Wu, 2004; Parsley and Hibberd, 2006; Taylor *et al.*, 2010), and in rice they increase in roots during anoxia (Moons *et al.*, 1998). The coordinate increase in abundance of transcripts encoding PPDK and other proteins that would allow production of transport amino acids in leaves

during dark-induced senescence led to the proposal that PPDK functions in nitrogen remobilization from leaves (Lin and Wu, 2004). In naturally senescing leaves both the cytosolic and chloroplastic isoforms of PPDK are up-regulated and, while cytosolic PPDK accumulates preferentially in veins, chloroplastic PPDK also accumulates in M cells (Taylor *et al.*, 2010). Analysis of microarrays and incorporation patterns after feeding ^{13}C-labelled pyruvate led to the proposal that PPDK functions in a pathway that generates the transport amino acid glutamine that is loaded into the phloem (Taylor *et al.*, 2010). In *Arabidopsis*, overexpression of *PPDK* during senescence can significantly accelerate nitrogen remobilization from leaves, and thereby increase rosette growth rate and the weight and nitrogen content of seeds (Taylor *et al.*, 2010). As the C_4 cycle relies on chloroplastic PPDK in M cells, it appears that it has been recruited from a role in natural senescence of leaves, and so its accumulation needs to increase in M cells of young leaves and be repressed in BS cells. It is not clear whether PPDK still plays an important role in senescing leaves of C_4 plants.

Transaminases

Five aspartate aminotransferases (AspATs) are encoded in the *Arabidopsis* genome (Miesak and Coruzzi, 2002). In *Arabidopsis*, AspAT2 and AspAT4 are cytosolic, AspAT1 is mitochondrial, AspAT3 is peroxisomal, and AspAT5 is chloroplastic (Schultz and Coruzzi, 1995). While transcripts encoding mitochondrial AspAT1 are abundant in all tissues of *Arabidopsis*, the cytosolic AspAT2 is thought to function primarily in aspartate synthesis and metabolism in leaves and siliques (Miesak and Coruzzi, 2002). Of the four alanine aminotransferases in *Arabidopsis*, AlaAT1 and AlaAT2 are proposed to function in the breakdown of alanine to pyruvate during recovery from hypoxia (Miyashita *et al.*, 2007). In *C. gynandra*, transcripts encoding the orthologue to the mitochondrial AtAspAT1 are significantly more abundant than those in *C. spinosa*, indicating its recruitment into the C_4 pathway. This suggests that AspAT1 has been co-opted from a relatively constitutive role in *Arabidopsis* to one particularly important in leaves of C_4 *C. gynandra*. Transcripts encoding AlaAT1 are abundant in *C. gynandra* compared with *C. spinosa* (Brautigam *et al.*, 2010), indicating that it has been recruited from a function in recovering from hypoxia to a more constitutive role in C_4 leaves.

Conclusions

A summary of the role of proteins recruited into C_4 photosynthesis is shown in Table 1. As many of the enzymes recruited are involved in central metabolism, it would seem likely that in most C_3 plants they fulfil similar functions. However, this may not be the case for those proteins that are encoded by large multigene families. Until orthologous genes encoding each enzyme recruited into the C_4 pathway are identified in closely related C_3 and C_4 species, it is not possible to be definitive about this. The advent of deep sequencing, its use in studying C_4 photosynthesis (Brautigam *et al.*, 2010), and the inclusion of closely related C_3 and C_4 congeneric pairs in the 1000 plant transcriptomes programme (http://www.onekp.com/project.html) may help to give us answers to at least the second part of this puzzle. The subsequent challenge will be to devise assays for the function of these isozymes in lineages of C_3 plant that are closely related to each C_4 species.

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