The role of proteins in C₃ plants prior to their recruitment into the C₄ pathway

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Abstract

Our most productive crops and native vegetation use a modified version of photosynthesis known as the C₄ pathway. Leaves of C₄ crops have increased nitrogen and water use efficiencies compared with C₃ species. Although the modifications to leaves of C₄ plants are complex, their faster growth led to the proposal that C₄ photosynthesis should be installed in C₃ crops in order to increase yield potential. Typically, a limited set of proteins become restricted to mesophyll or bundle sheath cells, and this allows CO₂ to be concentrated around the primary carboxylase RuBisCO. The role that these proteins play in C₃ species prior to their recruitment into the C₄ pathway is addressed here. Understanding the role of these proteins in C₃ plants is likely to be of use in predicting how the metabolism of a C₃ leaf will alter as components of the C₄ pathway are introduced as part of efforts to install characteristics of C₄ photosynthesis in leaves of C₃ crops.

Key words: C₃ photosynthesis, C₄ photosynthesis, Cleome, evolution, protein function.

C₄ photosynthesis

Plant species can be classified as using C₃ photosynthesis, C₄ photosynthesis, or Crassulacean acid metabolism depending on whether the primary product of photosynthesis contains three or four carbons (Sage, 2004). C₃ photosynthesis is considered ancestral, and the C₄ pathway is estimated to have evolved from C₃ 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 C₄ species, and this allows growth rates to be up to 50% higher than those of C₃ plants. Both the most productive native vegetation and crops use C₄ photosynthesis (Brown, 1999). C₄ 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 C₄ photosynthesis into C₃ crops (Matsuoka et al., 2001; Hibberd et al., 2008).

While a small number of species have developed a C₄ pathway that operates within single cells (Reiskind and Bowes, 1991; Voznesenskaya et al., 2001, 2002), in most C₄ 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 C₃ species (Kinsman and Pyke, 1998), but they have become more specialized in C₄ plants with changes in both cell biology and biochemistry. In M cells of most C₄ plants, after conversion of CO₂ to HCO₃⁻ by carbonic anhydrase (CA), phosphoenolpyruvate carboxylase (PEPC) acts as the initial carboxylase to produce oxaloacetate (OAA), which is then reduced to either malate or aspartate. These C₄ acids then diffuse to the BS where a C₄ acid decarboxylase releases CO₂ and so concentrates CO₂ around RuBisCO, favouring the carboxylation reaction and decreasing the oxygenation reaction. There are three C₄ acid decarboxylases known to have been recruited into C₄ photosynthesis, NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK) (Furbank, 2011). These three decarboxylases are associated with modified versions of the basic C₄ cycle (Fig. 1). To maintain the C₄ cycle, the three-carbon compound generated during decarboxylation diffuses back to the M, and the presence of...
pyruvate,orthophosphate dikinase (PPDK) in M chloro-
plasts allows regeneration of phosphoenolpyruvate (PEP),
the initial acceptor of HCO₃⁻. Extensive reviews have been
published in recent years describing the compartmentation
of metabolism (Majeran and van Wijk, 2009) and regula-
tion of gene expression (Hibberd and Covshoff, 2010)
associated with alterations to photosynthesis in C₄ leaves.

All enzymes required for the C₄ pathway are present
in C₃ plants. As a consequence, if characteristics of C₄
photosynthesis are integrated into C₃ 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 C₄ pathway prior to their
recruitment into C₄ photosynthesis are discussed. A better
understanding of the role of proteins in C₃ plants that are
recruited into the highly efficient C₄ pathway may inform
attempts to convert rice from C₃ to C₄ photosynthesis.
The function of proteins in C₃ plants that are recruited into the C₄ pathway

If proteins recruited into C₄ photosynthesis fulfill conserved roles in distantly related C₃ plants, then the limited number of studies done in specific C₃ species are useful to predict how the role of these proteins has altered. However, this has currently not been established. In addition, in C₃ species, many of the proteins recruited into the C₄ pathway are encoded by multigene families (Hibberd and Covshoff, 2010), implying that each isofrom may be carrying out specific roles. To understand how the role of these proteins has altered as they are recruited into C₄ photosynthesis, it would therefore be desirable to identify orthologues in C₃ 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 C₃ and C₄ 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 C₃ models tobacco and Arabidopsis that are relatively closely related to Flaveria and Cleome, respectively. Additionally, both Flaveria and Cleome possess C₄ and C₃ 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 C₃ and C₄ models is compared. The C₄ 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 C₄ 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 C₄ pathway, and so their role in C₃ plants is specifically addressed next. Functions appear diverse, ranging from supplying CO₂ 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 CO₂ 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 fulfills a separate role in sensing CO₂. 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 CO₂ (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 C₄ Flaveria bidentis, βCA3 has been recruited into the C₄ cycle (Tanz et al., 2009). In the closely related C₃ 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 C₄ 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 C₄ pathway (Brautigam et al., 2010). This implies that the role of βCA4 has been extended from being involved in CO₂ sensing in guard cells in Arabidopsis to a role in supplying HCO₃⁻ 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 C₃ plants. Overall, CAs have been shown to play a range of functions in different compartments of C₃ cells, and this redundancy may have in fact helped in their reductions in the BS and increase in M cells of C₄ plants (Tanz et al., 2009).

Phosphoenolpyruvate carboxylase (PEPC)

In C₃ 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.
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 C₄ F. trinervia compared with C₃ 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 at 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 C₄ compared with the C₃ species (Bräutigam 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 (Trevanian et al., 1997), and in C. gynandra there was little detectable increase in abundance of transcripts encoding MDH (Bräutigam et al., 2010). It therefore appears that little more than C₃ levels are required for the C₄ pathway to operate. To our knowledge it is not clear whether MDH used in the C₄ 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 C₃ species prior to their recruitment into the C₄ pathway.

In C₃ 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 CO₂ assimilation and higher rates of respiration (Tomaz et al., 2010). It is not clear how the multiple roles of MDH in C₃ 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 C₄ cycle
C₄ acid decarboxylases

C₄ acid decarboxylases act in the BS of C₄ plants to release CO₂ from four-carbon compounds, concentrating CO₂ around RuBisCO (Hatch, 1987). Different lineages of C₄ plants have preferentially recruited one of three distinct C₄ acid decarboxylases, and this defines the three biochemical subtypes of C₄ plants, although many plants use a mixture of the decarboxylases (see Furbank, 2011). The three enzymes are NADP-ME, NAD-ME, and PEPCK.

In C₃ 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 C₃ 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: ActPCK1 is expressed throughout the plant, whilst ActPCK2 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 C₃ 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., 2002b) 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 C₃ 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., 2002a).

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 C₄ 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 C₄ 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 insertion 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 C₄ acid decarboxylases in stems and mid-veins of C₃ plants has been proposed in which organic acids present in the xylem stream may supply CO₂ 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
pathway or up-regulation of alternative isoforms could maintain flux to shikimate. The ability of C₃ 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 C₄ pathway are commonly already present in C₃ 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 C₄ pathway (Brautigam et al., 2010). It therefore appears that during the evolution of C₄ photosynthesis, the role of NAD-GE has been refocused from one in coordinating carbon and nitrogen metabolism to supplying CO₂ 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; Imazumi et al., 1997; Parsley and Hibberd, 2006). The tissue with the highest PPDK content in C₃ plants appears to be seeds, where it has been implicated in controlling amino acid interconversions and starch biosynthesis (Aoyagi and Bassham, 1984a; 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

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<td>Stomatal movement regulation</td>
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<td>G. hirsutum</td>
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**Table 1.** Primary function of proteins involved in C₄ photosynthesis in C₃ plants
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 13C-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 C4 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 C4 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 C4 pathway. This suggests that AspAT1 has been co-opted from a relatively constitutive role in Arabidopsis to one particularly important in leaves of C4 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 C4 leaves.

Conclusions

A summary of the role of proteins recruited into C4 photosynthesis is shown in Table 1. As many of the enzymes recruited are involved in central metabolism, it would seem likely that in most C3 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 C4 pathway are identified in closely related C3 and C4 species, it is not possible to be definitive about this. The advent of deep sequencing, its use in studying C4 photosynthesis (Brautigam et al., 2010), and the inclusion of closely related C3 and C4 congenic 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 C3 plant that are closely related to each C4 species.

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