Journal of Experimental Botany, Vol. 62, No. 9, pp. 3103–3108, 2011
doi:10.1093/jxb/err080 Advance Access publication 21 April, 2011

Journal of Experimental Botany
www.jxb.oxfordjournals.org

OPINION PAPER

Evolution of the C_4 photosynthetic mechanism: are there really three C_4 acid decarboxylation types?

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Received 12 January 2011; Revised 23 February 2011; Accepted 24 February 2011

Abstract

Some of the most productive plants on the planet use a variant of photosynthesis known as the C_4 pathway. This photosynthetic mechanism uses a biochemical pump to concentrate CO_2 to levels up to 10-fold atmospheric in specialized cells of the leaf where Rubisco, the primary enzyme of C_3 photosynthesis, is located. The basic biochemical pathways underlying this process, discovered more than 40 years ago, have been extensively studied and, based on these pathways, C_4 plants have been subdivided into two broad groups according to the species of C_4 acid produced in the mesophyll cells and into three groups according to the enzyme used to decarboxylate C_4 acids in the bundle sheath to release CO_2. Recent molecular, biochemical, and physiological data indicate that these three decarboxylation types may not be rigidly genetically determined, that the possibility of flexibility between the pathways exists and that this may potentially be both developmentally and environmentally controlled. This evidence is synthesized here and the implications for C_4 engineering discussed.

Key words: C_4 acid, C_4 photosynthesis, evolution, NADME, NADPME, PEPCK, photosystem II.

Three biochemical mechanisms of C_4 photosynthesis

Plants using the C_4 pathway of photosynthesis are among the most productive and efficient biomass producers on the planet (see Byrt *et al.*, 2011, and references therein). This high efficiency in CO_2 assimilation is achieved by a biochemical CO_2-concentrating mechanism which requires both morphological and biochemical specialization of the photosynthetic apparatus (Hatch, 1987; Furbank *et al.*, 2009). In most C_4 plants, CO_2 is fixed from the intercellular spaces by PEP carboxylase in the leaf mesophyll cells to produce C_4 acids which are translocated to the bundle sheath cells where Rubisco is located. Decarboxylation in this compartment elevates the CO_2 concentration surrounding Rubisco, reducing photorespiration and allowing Rubisco to operate at close to its V_{max} (von Caemmerer and Furbank, 2003). The form of C_4 acid translocated to the bundle sheath cells varies between species and, in early work in elucidation of the pathway, C_4 plants were roughly divided into 'aspartate or malate formers' (reviewed in Kanai and Edwards, 1999). The biochemical steps potentially involved in producing the translocated C_4 acid, decarboxylating it to release CO_2 and recycling the carbon acceptor PEP are complex and varied, probably reflecting the number of times C_4 photosynthesis is thought to have independently evolved (approximately 60 times; Sage *et al.*, 2011). The traditional text book view of the biochemical pathways underpinning the three decarboxylation mechanisms of C_4 photosynthesis is shown in Fig. 1. Common to all decarboxylation types is PEP caboxylase, the primary enzyme of CO_2 fixation in C_4 plants. The product of PEP carboxylase, oxaloacetate (OAA), can either enter the mesophyll chloroplast where it is reduced to malate (by NADP-malate dehydrogenase; Fig. 1A), in the case of NADPME types (Fig. 1A; Hatch *et al.*, 1975; Kanai and Edwards, 1999) or converted in the cytosol to aspartate by asparate aminotransferase for transport to the bundle sheath cells (in the case of NAD and PEPCK types; Fig. 1B, C; Hatch *et al.*, 1975; Kanai and Edwards, 1999). In the case of the latter two decarboxylation types, aspartate is converted to OAA again in the bundle sheath cytosol by aspartate aminotransferase. The mitochondria

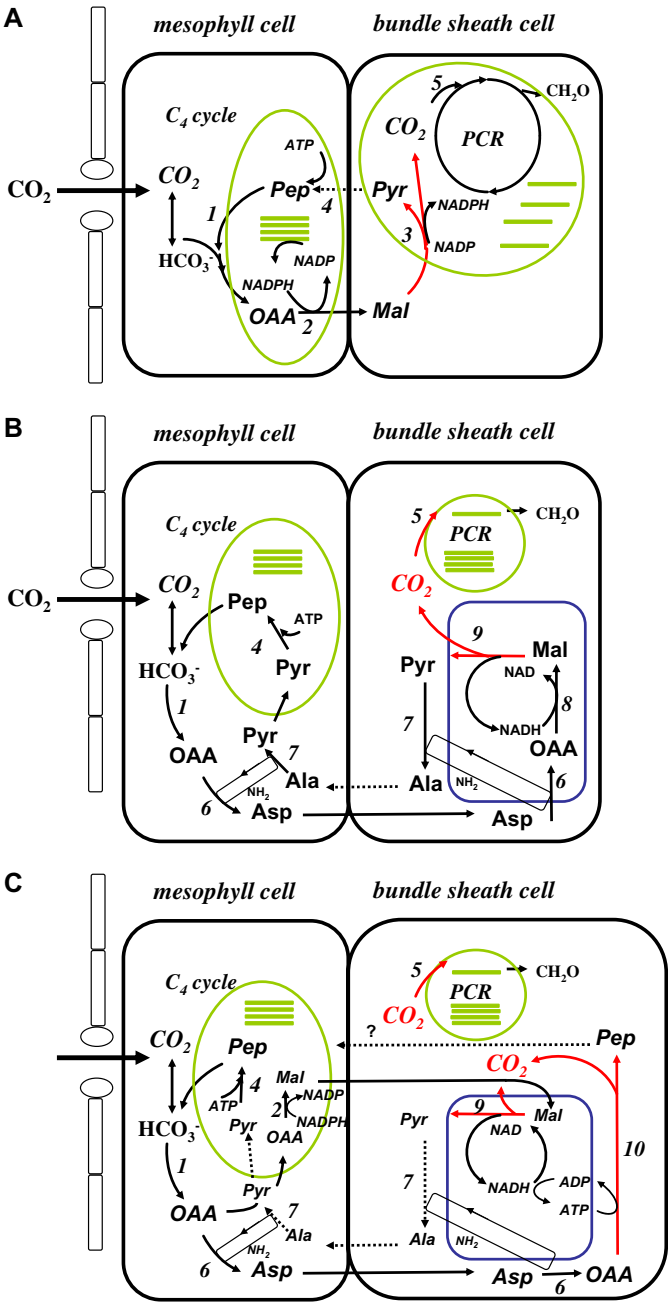

Fig. 1 Photosynthetic mechanism in NADPME- (A), NADME- (B), and PEPCK-type (C) species without consideration for flexibility between pathways and assuming that malate predominates in the NADPME-types as the translocated C_4-acid and aspartate in the other two types. Metabolite abbreviations: PEP, phosphoenolpyruvate; OAA, oxaloacetate, Asp, aspartate; Ala, alanine; Pyr, pyruvate; Mal, malate. Chloroplasts and thylakoids are coloured green, mitochondria blue, and the decarboxylation reactions are coloured red. Enzymes in the pathways are numbered as follows: (1) phosphoenolpyruvate (PEP) carboxylase; (2) NADPH- malate dehydrogenase; (3) NADP-malic enzyme; (4) pyruvate Pi-dikinase; (5) Rubisco; (6) aspartate aminotransferase; (7) alanine aminotransferase; (8) NAD-malate dehydrogenase; (9) NAD-malic enzyme; (10) phospho-enol pyruvate (PEP) carboxykinase.

are involved in the decarboxylation process in both cases, via NAD-malic enzyme, however, in PEPCK types the majority of OAA is converted to PEP by PEP-carboxykinase and NAD-malic enzyme is used to provide NADH for oxidative phosphorylation to generate ATP to fuel PEP-carboxykinase (Fig. 1C). It is also likely that the seemingly obligatory involvement of NAD-malic enzyme in the PEPCK mechanism serves to balance the amino groups between compartments via the return of alanine to the mesophyll compartment.

While there are a variety of theories concerning the nature of the selective pressure which drove evolution of the C_4 pathway, there is little information about the relative merits of one decarboxylation type over another from an energetic perspective (via radiation use efficiency) and hence 'fitness' in an ecological context (Sage *et al.*, 1999; Sage, 2004). Curiously, no PCK-type C_4 dicots have been discovered to date (Sage *et al.*, 1999; Sage, 2004). Also, the majority of C_4 crop species have arisen from the NADP-ME type mechanism (maize, sorghum, sugarcane, miscanthus, and switchgrass, for example), providing at least circumstantial evidence for the superiority of this pathway. It has been suggested that the nature of the decarboxylation mechanism may generate differences in efficiency of the CO_2-concentrating mechanism due to the subcellular localization of the decarboxylation mechanism and the physical location of the chloroplasts and mitochondria in the bundle sheath cells (von Caemmerer and Furbank, 2003). The NADP-ME mechanism for example, might provide an advantage from a perspective of 'leakiness' of the bundle sheath to CO_2 because decarboxylation occurs within the bundle sheath chloroplasts, the site of refixation by Rubisco (von Caemmerer and Furbank, 2003). Irrespective of the influence of CO_2 diffusion on the efficiency of the CO_2 pump, from a perspective of the basic energetics of C_4 photosynthesis, there should be differences in radiation use efficiency at the leaf level between the decarboxylation types (Furbank *et al.*, 1990; von Caemmerer and Furbank, 1999). This relates primarily to the mitochondrial generation of ATP in the PCK types. Previous calculations indicate that theoretical quantum yields for PEPCK-type species are around 14 quanta per CO_2 fixed while in NADME-type species this value is over 18 and potentially higher for the NADPME types (von Caemmerer and Furbank, 1999). Such differences should be apparent in quantum yield measurements, but interspecific variation in these values is far from predictable on the basis of decarboxylation types, although PCK-type species tend to fall at the lower end of quantum requirements per CO_2 fixed (Ehleringer and Pearcy, 1983). The absence of robust evidence for an energetic advantage of one type over another, either in radiation use efficiency or in growth performance (Ghannoum *et al.*, 2001), indicates that many other factors play a role in determining the 'photosynthetic efficiency' of C_4 plants, including possible variation in the efficiency of light harvesting and ATP production, spatial arrangement of organelles, presence or absence of suberized lamellae, and morphological differences related to whether the mechanism

is present in dicots or monocots (von Caemmerer and Furbank, 1999, 2003). In summary, there is little concrete evidence for advantages of one decarboxylation type over another in terms of photosynthetic efficiency or 'fitness'.

Evidence for flexibility in decarboxylation mechanisms and C_4 acid translocation

Even the rigid definitions of the three decarboxylation pathways or 'types' detailed above provide for mechanistic flexibility. It was recognized from early ^{14}C-labelling experiments that even in the NADPME-type species, some radiolabel (approximately 25%) was incorporated into aspartate, although evidence for the presence of aminotransferase activities sufficient to support appreciable photosynthetic flux out of aspartate was not forthcoming (Hatch, 1971). Chapman and Hatch (1981), however, subsequently showed that *Zea mays*, a consummate 'NADPME-type' C_4, not only produced significant proportions of radiolabel in aspartate but isolated bundle sheath cells could use aspartate and oxoglutarate to generate CO_2 to support appreciable rates of photosynthesis. More recently, this observation has been extended to include the NADPME dicot *Flaveria bidentis*, now a popular transformable model C_4 plant (Meister *et al.*, 1996). Radiolabelling demonstrated that up to half of the flux into C_4 acids appeared in aspartate and in isolated bundle sheath strands aspartate and oxoglutarate could support physiological rates of O_2 evolution (Meister *et al.*, 1996). ^{14}C-labelling data (MD Hatch, personal communication) and extractable activities for key C_4 enzymes (Kanai and Edwards, 1999) in NADPME-type species indicates that the ratio of asparate to malate translocated in this group of C_4 plants may vary a lot; an intriguing possibility is that this may be due to seasonal influences such as irradiance or other environmental conditions rather than genetically defined, species-dependent mechanisms.

Mechanistically, how does aspartate contribute to the bundle sheath CO_2 pool in NADPME-type species? Two possibilities present themselves. First, if PEP-carboxykinase were present in NADPME-type species, OAA produced by aspartate aminotransferase could be directly decarboxylated in the cytosol. ATP could come from chloroplastic sources in this model, rather than from oxidative phosphorylation, either by direct export of ATP (although the ATP porter in the chloroplast envelope generally acts to import ATP; Reinhold *et al.*, 2007) or by a shuttle of 3-phosphoglycerate and triose phosphate across the chloroplast envelope (Fig. 2; Hatch, 1987). Alternatively, the model favoured in the early literature (Fig. 2; Chapman and Hatch, 1981) is that OAA generated from aspartate is re-reduced to malate in the bundle sheath and then decarboxylated in the chloroplast by NADP-malic enzyme. In *Flaveria*, it is postulated that the reduction of OAA occurs in the bundle sheath chloroplasts, although mitochondrial NAD-MDH could also play a role (Meister *et al.*, 1996). The energetic consequences of aspartate-dependent C_4 acid

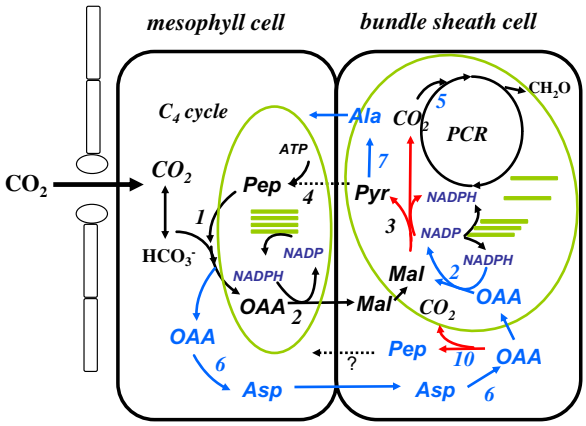

Fig. 2 Two options for a modified NADPME-type C_4 mechanism where aspartate (blue arrows) in addition to malate (black arrows) are produced in the mesophyll. Aspartate can be reconverted to OAA and reduced to malate for decarboxylation by the NADP malic enzyme or PEP carboxykinase can utilize oxaloacetate directly. The nature of the metabolite returning to the mesophyll is indicated as pyruvate, alanine or PEP, however, conversion to alanine in the bundle sheath and reconversion to pyruvate in the mesophyll (not shown) is most likely and would allow for a balance of amino residues between the cell types (Meister *et al.*, 1996). Note that utilization of aspartate as the translocated C_4 metabolite for NADPME-dependent malate decarboxylation results in no net transfer of reducing power from the mesophyll to the bundle sheath chloroplast. Numbering and abbreviations as for Fig. 1.

decarboxylation by NAD-malic enzyme in NADPME-type species will be discussed later in this article.

Direct decarboxylation of OAA by PEP-carboxykinase in NADPME-type species was not supported in the literature until more recently when compelling biochemical and molecular evidence arose to support the presence of substantial PEP-carboxykinase in the bundle sheath of both maize and sugarcane. In maize, it has been shown that appreciable active PEP-carboxykinase protein is present in bundle sheath cells of the NADPME-type species *Zea mays, Echinochloa colona*, *Digitaria sanguinalis*, *E. crus-galli*, and *Paspalum notatum*, but not in NADME-type species (Walker *et al.*, 1997). Later work indicated that the enzyme was active and capable of supporting high rates of aspartate-dependent photosynthesis in isolated maize bundle sheath cells (Wingler *et al.*, 1999). From a molecular perspective, analysis of transcripts differentially expressed in mesophyll versus bundle sheath tissue also indicated that PEP-carboxykinase was expressed at high levels in maize bundle sheath tissue (Furumoto *et al.*, 1999, 2000). Similarly, serial analysis of gene expression in sugarcane leaves indicated that PEP-carboxykinase was one of the most highly expressed bundle sheath transcripts, with relative expression levels higher than NADP-malic enzyme (Calsa and Figueira, 2007). This observation is interesting in light of the report in Walker *et al.* (1997) that PEP-carboxykinase activity could not be detected in sugarcane in their hands. Clearly there is considerable evidence for a mixed pathway of

decarboxylation of aspartate by PEP-carboxykinase in addition to the direct decarboxylation of malate in these species, if not all NADPME-type C_4 plants.

Energetic consequences of decarboxylation pathway in NADPME-type species

The synthesis of aspartate as a major translocated C_4 acid in NADPME types and its subsequent metabolism to malate in the bundle sheath chloroplast is of considerable import to cellular energy balance. If malate is the major translocated C_4 acid in an NADPME-type C_4 species, reducing power is also moved from the mesophyll chloroplast to the bundle sheath chloroplast in the form of the reduced dicarboxylic acid (Fig. 2; Chapman and Hatch, 1981). When malate is decarboxylated by the NADP-malic enzyme, NADPH is produced which can be used for the reduction of 3-phosphoglycerate in the photosynthetic carbon reduction (PCR) cycle (Fig. 2). There is a large body of evidence that, in many NADPME-type C_4 species (such as maize, sorghum, and sugarcane), PSII activity in the bundle sheath chloroplasts is reduced compared with the mesophyll chloroplasts or a C_3 chloroplast (Chapman and Hatch, 1981; see Furbank *et al.*, 2009, and references therein). While very low PSII in the bundle sheath chloroplast is often regarded as a general feature of all NADPME-type species, this is not necessarily the case. Even for maize, there is substantial variation in literature reports on the functional PSII levels in bundle sheath chloroplasts, with some reports of up to 50% of the whole chain electron transport capacity seen in thylakoids of C_3 plants (Hardt and Kok, 1978; Walker and Izawa, 1979). Functional PSII content of bundle sheath chloroplasts from a variety of NADPME types have been reported to vary from virtually zero (in sorghum and sugarcane; Meierhoff and Westhoff, 1993) to almost 'normal' C_3 levels in other species (Mayne *et al.*, 1975). Whether this variation in values is genetically or environmentally controlled is not known but it has been postulated that the PSII content of the bundle sheath in NADPME-type species may be proportional to the amount of aspartate produced as the translocated C_4 acid (Chapman and Hatch, 1981; Meister *et al.*, 1996). When aspartate is translocated to the bundle sheath and the resultant OAA is reduced to malate then decarboxylated, the NADPH balance is neutral (Fig. 2), as the NADPH produced by NADP-malic enzyme is consumed to reduce OAA within the bundle sheath. This not only has implications for the requirement for the chloroplast electron transport chain in the bundle sheath (note that the ATP/NADPH requirements in this compartment are significantly altered), but the energetic load of the mesophyll chloroplast is also affected, potentially altering the amount of 3-phosphoglycerate which returns to the mesophyll for reduction to triose phosphate (Hatch, 1987). As the reduction of 3-phosphoglycerate in the mesophyll results in the net transfer of reducing power between bundle sheath and mesophyll compartments, there could potentially be additional redox balancing occurring through the shuttle of 3-phosphoglycerate and triose phosphate (Hatch, 1987). The high level of expression of the triose phosphate porter in C_4 plants relative to their C_3 relatives would indicate that this shuttle of 3-carbon sugar phosphates is a key aspect of the C_4 mechanism (Brautigam *et al.*, 2011).

There is strong evidence for developmental control over the PSII content of the bundle sheath chloroplast and it has been demonstrated that the levels of bundle sheath PSII in maize and sorghum are regulated by the synthesis of key core components normally present in the mesophyll, but not in the bundle sheath (Meierhoff and Westhoff, 1993). While environmental regulation of PSII levels in bundle sheath chloroplasts has not been extensively examined, granal stacking in bundle sheath chloroplasts has been shown to be highly plastic and responsive to salt stress in a variety of NADPME-type species (Omoto *et al.*, 2009). While regulation of bundle sheath PSII content in response to growth irradiance has not been extensively studied, in one of the few studies reported, *Flaveria bidentis* leaf chl *a*/*b* ratio responded strongly to growth irradiance, presumably reflecting both bundle sheath and mesophyll adaptation to low-light environments by increasing the PSII content (Pengelly *et al.*, 2010). It is tempting to hypothesize that these biochemical and light-harvesting responses are linked and provide a mechanism to allow flexibility in energy balance between the mesophyll and bundle sheath chloroplasts, potentially to adapt to varying light environments. New techniques for examining functional bundle sheath PSII in intact leaf sections or even intact leaves will no doubt assist in exploring such a hypothesis (Furbank *et al.*, 2009; Hasegawa *et al.*, 2010).

Plasticity in the utilization of NADP-malic enzyme or PEP-carboxykinase could also have large ramifications for the overall light-derived energy requirements for photosynthesis in an NADPME-type C_4 plant. As discussed above, the PCK pathway of C_4 photosynthesis requires ATP for decarboxylation, which could be derived either from mitochondrial or chloroplast sources. If ATP were to be light generated within the bundle sheath for this process, and PEP returned directly to the mesophyll chloroplast, the ATP required in the mesophyll compartment would be considerably reduced. The 'work load' of the mesophyll chloroplast in a malate-producing NADPME-type is high as not only does the mesophyll consume ATP for pyruvate re-conversion to PEP, but also for a proportion of the PCR cycle glyceraldehyde 3-phosphate, plus the reducing power required for OAA reduction and 3-phosphoglycerate reduction. Once again, the plasticity of this process could be viewed as a way of balancing the energetic capacity of the two compartments under varying environmental conditions.

Energetic consequences of decarboxylation pathway in NAD- and PCK-type species

Much less biochemical work has been done on the flexibility of decarboxylation mechanisms in the 'aspartate forming'

NADME- and PCK-type species. As discussed above, there is no evidence for the operation of a 'pure' PCK-type mechanism without the operation in tandem of NAD-malic enzyme to provide ATP via mitochondrial oxidative phosphorylation (Kanai and Edwards, 1999). If photosynthetic flux was entirely via PEP-carboxykinase and ATP were to be supplied from photophosphorylation without pyruvate generation, pyruvate Pi-dikinase would not be necessary to regenerate PEP. Notably, however, if PEP returns directly to the mesophyll without regeneration of pyruvate in the alanine aminotransferase reaction, an imbalance in NH_2 flux between the mesophyll and bundle sheath compartments could result and this issue has not been resolved (Fig. 1C). There are no literature reports of PEPCK-type species without pyruvate Pi-dikinase activity in the mesophyll cells and activities in PEPCK types are generally about one-half of those found in NADPME types (Kanai and Edwards, 1999). As pyruvate Pi-dikinase activities are only just high enough in NADPME-type species to support photosynthetic flux (Furbank *et al.*, 1997), it is likely that this value could reflect the relative flux through the two decarboxylation enzymes. It is currently unknown whether relative flux through these pathways is plastic or regulated by environmental conditions.

Implications for engineering C_4 photosynthesis into C_3 species

Due to the recent interest in re-engineering C_3 crops with C_4 or C_4-like CO_2-concentrating mechanisms, the mechanistic question 'what does it take to be C_4?' is frequently asked (Edwards *et al.*, 2001; Furbank *et al.*, 2009). The prospect of introducing any of the complex pathways shown in Fig. 1 in a cell type-specific manner plus the plethora of metabolite transporters found in C_4 plants to a C_3 plant is daunting. We are encouraged, however, by frequency with which the C_4 pathway has independently evolved (Sage *et al.*, 2011) and by the presence of many of the genes encoding enzymes and transporters necessary for the pathway in related C_3 plants (Aubry *et al.*, 2011). Potential flexibility in C_4 acid decarboxylation pathways and the possible environmental plasticity of these processes might suggest that we may not have to engineer a rigid mechanism, copied from the traditional view of the C_4 mechanism in maize. It appears that the most successful agricultural crops, the NADPME types, have quite a degree of mechanistic flexibility, which could in fact be the key to their success. More work is required to elucidate the key components for an 'efficient' C_4 mechanism.

Acknowledgements

I owe a great debt of gratitude to Hal Hatch for sharing his insight and decades of experience of C_4 with me and to Susanne von Caemmerer for patiently enduring interminable discussions on the complexity of the C_4 pathway.

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