Title: Strategies for improving C₄ photosynthesis

Short title: Improving C₄ photosynthesis

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Abstract

Recent activities to improve photosynthetic performance in crop plants has focused mainly on C₃ photosynthesis where there are clear identified targets such as improving Rubisco kinetics, installation of a CO₂ concentrating mechanism and alleviating limitations in chloroplast electron transport. Here we address strategies to improve photosynthetic performance in C₄ plants, which utilize a CO₂ concentrating mechanism, having evolved a complex blend of anatomy and biochemistry to achieve this. While the limitations to photosynthetic flux are not as well studied in C₄ plants, work in transgenic *Flaveria bidentis*, a transformable model C₄ dicot, and recent transcriptional analysis of leaves from diverse C₄ plants, provides several gene candidates for improvement of carbon metabolism (such as pyruvate orthophosphate dikinase, phosphoenolpyruvate carboxylase and Rubisco) and for access of CO₂ to phosphoenolpyruvate carboxylase in the mesophyll cells (such as carbonic anhydrase and CO₂ porins). Chloroplast electron transport in C₄ plants is shared between the two cell types, providing opportunities not only to alleviate limitations to flux through intersystem electron transport by targeting nuclear encoded proteins in the cytochrome (Cyt) *b₆/f* complex, but in better sharing the harvesting of light energy between mesophyll and bundle sheath chloroplasts. Gene candidates for improvement of C₄ photosynthesis could be utilized either through transgenic approaches or via mining natural allelic variation in sequenced populations of crop species.

Introduction

To meet the challenge of increasing crop yield for a growing world population, it has become apparent that photosynthetic efficiency and capacity must be increased per unit leaf area to improve yield potential [1,2]. High yields from C₄ crops have stimulated considerable interest in the C₄ photosynthetic pathway which is characterised by high photosynthetic rate and high nitrogen and water use efficiency relative to plants with the C₃ photosynthetic pathway [3]. The international C₄ rice consortium is working towards engineering rice to use C₄ photosynthesis [4]. However C₄ plants also play a key role in world agriculture. For example C₄ crops such as maize and sorghum are major contributors to both first and third world food production and the C₄ grasses sugarcane; miscanthus and

switchgrass are major plant sources of bioenergy. Strategies to manipulate and enhance C_4 photosynthesis, the focus of this review, thus have potential for major agricultural impacts.

The high photosynthetic rates of C_4 plants are achieved by a biochemical CO_2 concentrating mechanism that operates between mesophyll and bundle sheath cells of leaves (Box 1). The transition from C_3 to C_4 photosynthesis has occurred independently in more than 60 different plant taxa [5]. The C_4 pathway is not mechanistically unique and C_4 plants have been classified into several biochemical subtypes based on the enzymatics of the decarboxylation reaction and its subcellular location (Box 2, [6]). This biochemical and anatomical diversity provides many options for efficient C_4 photosynthesis and the possibility for mixtures of C_4 subtypes and diverse anatomy.

The regulation of C_4 photosynthetic flux is complicated by the compartmentation of biochemical processes and we have only a rudimentary understanding of how the C_4 and C_3 components of C_4 photosynthesis are coordinated. While the activities of many of the enzymes of C_4 photosynthesis appear to be tightly regulated, their significance in regulating photosynthetic flux has been difficult to assess. *Flaveria bidentis* (a readily transformable model C_4 dicot) has been successfully used to study this regulation using antisense and RNAi technology [7-9]. This work has been pivotal in quantitatively determining some of the rate limiting steps in the C_4 pathway by “titrating” out levels of target enzymes by gene suppression and observing the effects on physiological characteristics of the resultant transgenics. These results are reviewed in Box 3. There are however important differences between C_4 dicots and the C_4 monocots which make up the majority of agriculturally important C_4 species, which potentially limit this model system. *Setaria viridis* (green foxtail millet) has emerged as a new model grass to study C_4 photosynthesis in crops and related bioenergy species. *Setaria viridis* is an appropriate biochemical model species for *Zea mays* and Sorghum as all three belong to the NADP-ME decarboxylation type (Box 2). It is readily transformable, opening opportunities to investigate the possibility of enhancing C_4 photosynthesis [10]. Below we focus on the possible targets for C_4 photosynthetic improvement outlined in Box 1.

Enhancement of the CO_2 delivery process

High CO_2 assimilation rates require high rates of CO_2 diffusion from intercellular airspaces to the mesophyll cytosol, the site of initial CO_2 fixation. However, no information

is currently available on diffusion properties of mesophyll cell walls and plasma membranes in C_4 species and what role aquaporins, which have been implicated in facilitation CO_2 diffusion in C_3 species, may play in C_4 photosynthesis [11]. The C_4 photosynthetic pathway requires a close link between mesophyll and bundle sheath cells. This constrains mesophyll surface area exposed to intercellular airspace limiting mesophyll surface area available for CO_2 diffusion. [12]. Evidence that aquaporins may play a role in CO_2 diffusion in C_4 species comes from leaf transcriptome comparisons of two Cleome species with C_4 and C_3 photosynthesis. The study showed that the abundance of an mRNA coding for an aquaporin believed to facilitate CO_2 diffusion was 20 fold higher in the C_4 leaf transcriptome relative to the C_3 [11,13].

Carbonic anhydrase (CA) has been shown to be present in abundance at air levels of CO_2 in *F. bidentis* and *Zea mays* (Box 3 [14,15]). Reducing CA activity affects the initial slope of the CO_2 response curve of CO_2 assimilation rate (Box1, [14]) suggesting that while there may be enough CA activity to support photosynthetic flux when stomata are open, increased CA activity may be beneficial under water stress.

PEP carboxylase is the primary enzyme fixing atmospheric carbon in C_4 plants and is believed to be highly regulated by protein phosphorylation and metabolite levels. It is unlikely to be a key limiting step at ambient CO_2 but like CA may play important role under stress conditions (Box 3). The kinetic properties of PEPC such as its affinity for CO_2/HCO_3^- have not been extensively studied but variation amongst species has been documented [16-18]. It could therefore be beneficial to overexpress PEPC with a higher affinity for CO_2/HCO_3^- . It is uncertain whether altering phosphorylation regulation could improve catalytic efficiency of PEPC given the lack of phenotype observed when the PEPC protein kinase was knocked out in transgenic *F. bidentis* [19]. There may well be other opportunities for improving in vivo catalysis of PEPC and more research into its function is required.

Enhancing Rubisco function

Rubisco, the primary CO_2 fixing enzyme, is a poor catalyst of CO_2 at current atmospheric conditions and Rubisco's oxygenase activity requires the recycling of phosphoglycolate in the photorespiratory pathway resulting in an energy cost and loss of previously fixed CO_2 . Consequently attempts are being made to improve Rubisco function in C_3 species [20]. The C_4 photosynthetic CO_2 concentration mechanism has overcome some of these deficiencies by providing a high CO_2 environment for Rubisco. However increasing the

amount of Rubisco or its catalytic properties may also have the potential to improve C₄ photosynthesis. Several lines of evidence support this hypothesis.

Results with transgenic *F. bidentis* with reduced Rubisco demonstrated a close link between Rubisco content and CO₂ assimilation rates particularly at elevated CO₂ ([21], Box 1&3). As in C₃ plants, under high light and ambient CO₂, the bulk of control over photosynthetic flux lies with Rubisco [21]. Frequent independent evolution of C₄ photosynthesis has produced a diversity of C₄ Rubisco's with different kinetic properties [22] and a close link between Rubisco kinetic properties and photosynthetic nitrogen use efficiency has been demonstrated in a set of C₄ grasses [23]. Thus increasing the amount of Rubisco may be an opportunity to enhance flux particularly at low temperature (Box3).

The elevated CO₂ partial in the bundle sheath in C₄ species allows Rubisco to operate close to its maximal rate while oxygenase and the accompanying photorespiratory activity is reduced. The gas tight nature of bundle sheath cells which allows elevation of CO₂ however also leads to increased bundle sheath O₂ partial pressures above atmospheric levels in those C₄ species that have some functional photosystem II there (Box 2). Rubisco from C₄ species have superior catalytic turnover rates compared to C₃ species but this comes at a cost of a decreased affinity for CO₂ (K_c) [22]. Recent measurements of Rubisco kinetic constants in *S. viridis* [18] predict a K_c at 200 mbar O₂ of 1600 µbar which would mean Rubisco may not be operating at its V_{max} before bundle sheath CO₂ pressure reaches 8000 µbar (5*K_c). If O₂ partial pressure is elevated in the bundle sheath this increases the CO₂ pressure required for Rubisco saturation. Thus increasing catalytic turnover rate is an option that would need careful consideration. It may indeed be more advantages to focus on other kinetic properties such as the affinity of Rubisco for oxygen (K_o). Increased K_o would reduce the CO₂ partial pressure required for saturation. At elevated CO₂, specificity for CO₂ relative to O₂, which would decrease photorespiration, would appear to be less important in a high CO₂ environment of the bundle sheath; however it may be important in low light environments.

Evolutionary analysis of Rubisco from the genus of *Flaveria* has shown that two residues of the large subunit could explain the different catalytic properties of these Rubiscos [22] and this was experimentally verified in transplastomic tobacco [24]. However Rubisco kinetic properties have also been altered in rice in the directions of sorghum kinetic properties by partial replacement of rice small subunits with those of sorghum [25]. The

modification of small subunits provides the most tractable avenue for Rubisco manipulation in C_4 species.

Somewhat surprisingly, given the high CO_2 concentration in the bundle sheath cells of C_4 plants, Rubisco activase was shown to be essential for C_4 photosynthesis (Box 3) and increased activase content has been linked with increased grain yield in *Zea mays* [26,27] suggesting it may be a target for photosynthetic improvement, although photosynthetic rates in transgenic *Flaveria* were not affected until activase levels were reduced to 30% of wild type levels [28]. It may however be involved in the dynamic regulation of photosynthesis and increase the speed with which CO_2 assimilation rate responds to a change in irradiance [29]. The prospects and current challenges for improving Rubisco and its helper enzyme Rubisco activase in C_3 species have been reviewed in detail elsewhere [30,31].

Optimising substrate regeneration in C_4 and C_3 cycles.

In addition to Rubisco activity, the regeneration of PEP and RuBP together with electron transport capacity can be limiting factors for CO_2 assimilation rate at high CO_2 and high irradiance (Box1) and thus could potentially be valuable targets for increasing photosynthetic rate. For C_3 species such as tobacco, gene suppression has been used extensively to elucidate rate limiting steps in the regenerative phase of the Calvin cycle [32]. Much less information is available for C_4 plants (Box3). It is clear that some regulatory coordination exists between C_3 and C_4 cycle via the reduction of 3PGA in the mesophyll chloroplast and interconversion of 3PGA, 2PGA and PEP, as can be seen in Figure 1. However reductions of Rubisco or NADP malic enzyme in transgenic *F. bidentis* have shown that the two cycles are not tightly linked [8] allowing room for manipulation. In C_3 species overexpression of SBPase has been shown to increase photosynthetic rates under conditions where RuBP regeneration is limiting photosynthetic flux, particular at elevated CO_2 and multiple approaches are being taken to validate the importance of these discoveries in the field [33,34]. A similar approach may be of value in C_4 species, given the high CO_2 concentration in the bundle sheath and the high light environments in which C_4 crops are generally grown.

PPdK which catalyses the conversion of pyruvate to PEP with the consumption of ATP is a highly regulated enzyme and thought to be the most rate limiting step in the

regeneration of PEP [35]. It has been shown that low temperature tolerance of the bioenergy feedstock grass *Miscanthus x giganteus* was characterised by high PPdK protein content [36]. Reductions of PPdK by antisense suppression in transgenic *F. bidentis* resulted in substantial reductions in CO₂ assimilation rate with small to moderate decreases in PPdK activity [37] making it a strong candidate for overexpression. Plants with increased PPdK activity may also show improved photosynthetic rates under cooler temperatures where the enzyme is believed to become more limiting (Box3, [36]).

Transport and Diffusion

In C₄ plants CO₂ assimilation is distributed between mesophyll and bundle sheath cells and requires a massive diffusive flux of metabolites between mesophyll and bundle sheath cells and across organelle membranes [13]. It has been proposed that evolution has resulted in the optimization of metabolite flux between the cell types for exchange of C₄ acids and C₃ compounds for PEP regeneration while minimizing CO₂ leakage from the bundle sheath [38]. Abundant plasmodesmatal connections have long been proposed to be necessary for these metabolite fluxes [38] but quantitative data on the frequency of plasmodesmata and diffusion constants for metabolite movement through C₄ plasmodesmata are not available. This has restricted modelling of the potential limitations that these process may confer to C₄ photosynthesis [39] and do not allow firm conclusions on the appropriateness of these process for improving C₄ photosynthesis.

There is also little quantitative information available about the transport capacity across organelles [13]. Overexpression of the triose phosphate/phosphate translocator in the C₃ species, tobacco enhanced CO₂ assimilation at elevated CO₂ in these plants [40] and it is timely to initiate targeted gene suppression experiments to investigate whether enhancing metabolite transport at the organelle level could increase CO₂ assimilation in C₄ species.

The efficiency of C₄ photosynthesis which has been described by the term leakiness (defined as the ratio of CO₂ leak rate out of the bundle sheath to the rate of PEP carboxylation assumed to be in equilibrium with the rate of C₄ acid decarboxylation) has been a topic of research for many years [41]. The degree of leakiness of the bundle sheath compartment is a compromise between retaining CO₂, allowing O₂ to diffuse out and permitting metabolites to diffuse at rates fast enough to support the rate of CO₂ fixation. Leakiness is dependent on both the bundle sheath resistance to CO₂ diffusion and the

relative biochemical capacity of the C₃ cycle and the C₄ cycle [8]. While it has been asserted on many occasions that a suberized bundle sheath wall is essential for an efficient CO₂ concentrating mechanism, not all C₄ plants have suberized bundle sheath cell walls and modeling suggests that organelle position and liquid diffusion path length may be equally important [41]. The enzymatic steps involved in suberin biosynthesis have recently been described [42] providing the genetic resources to manipulate suberin composition of the bundle sheath cell wall.

Transgenic manipulation of Rubisco and NADP malic enzyme levels in *F. bidentis* has shown that that altering C₃ versus C₄ cycle activity can lead to increases and decreases leakiness and photosynthetic efficiency [8] and these studies suggest that increasing Rubisco content could lead to improved efficiency at high light under enzymatic limitation. Under low light, leakiness has been shown to increase with impact on the quantum efficiency [43,44]. At low light, bundle sheath pCO₂ is expected to be less and photorespiratory energy requirements may therefore also affect quantum efficiency. Improving Rubisco specificity for CO₂ could ameliorate this. This is however a difficult engineering task given that Rubisco subunits are both chloroplast and nuclear encoded. Modelling suggests that optimal partitioning of energy use between bundle sheath and mesophyll also affects leakiness [45]. High throughput screening tools are being developed to explore natural variation of photosynthetic efficiency under these conditions [46].

Enhancing chloroplast electron transport and light capture

The non-saturating nature of the light response of CO₂ assimilation rate in C₄ species indicates that chloroplast electron transport is one of the major rate limiting steps of C₄ photosynthesis at ambient CO₂ (Box 1). In C₃ species targeted transgenic reduction in the content of the Cyt *b₆/f* complex (a multi-subunit protein that uses electron flow to transport protons across thylakoid membranes) by anti-sense reduction of the Rieske iron sulphur center caused a reduction in the chloroplast electron transport rate and photosynthesis [47,48]. It has been concluded that electron flow through the Cyt *b₆/f* complex is one of the key rate-limiting steps for electron transport as at high light, photosynthetic rate is almost linearly related to the level of this complex [47]. So far there have been no such studies on the energy limitations of C₄ photosynthesis, where electron transport occurs in both mesophyll and bundle sheath chloroplasts and the ATP and NADPH demands of these cell

types are vastly different (see Box 2). The energy requirements of the 3 decarboxylation types are different and if a mixture of these pathways operates the apportioning of energy production between mesophyll and bundle sheath would also be affected. In members of the NADP-ME decarboxylation type such as *Setaria viridis*, *Zea mays* and *Sorghum bicolor* the mesophyll chloroplast performs whole chain electron transport whereas in the bundle sheath PSII activity is reduced and photophosphorylation is believed to occur mainly via cyclic electron transfer. This is because reducing equivalents are produced in the bundle sheath chloroplast from malate decarboxylation, reducing the need for whole chain electron transport (Box 2). As both cyclic and whole chain electron transport require functional Cyt b_6/f [6], increasing levels of this complex may be beneficial for both non-cyclic and cyclic photosynthesis. However, because Cyt b_6/f is a multi-subunit protein with many chloroplast encoded subunits, it is not certain whether overexpression of nuclear encoded Rieske iron sulphur centre will lead to increased Cyt b_6/f complex assembly. Similarly, it is also not clear how electron transport capacity should be proportioned between mesophyll and bundle sheath chloroplasts. Cytochrome f is mostly located in mesophyll chloroplasts, with approximately 20% , and 40% located in bundle sheath chloroplasts of NADP-ME and NAD-ME C_4 species, respectively [23].

In C_4 species of the NADP-ME subtype which lack bundle sheath PSII activity but have enhanced cyclic electron transport around PSI, PGR5 (PROTON GRADIENT REGULATION 5), and NDH-H (a subunit of the plastidial NAD(P)H dehydrogenase complex) ,have both been shown to be highly expressed in the bundle sheath relative to mesophyll chloroplasts [49,50]. As no work has been done altering levels of these proteins to examine effects on photosynthetic flux in C_4 plants, it is difficult to predict the effect of overexpression of these proteins but they could provide targets for enhancements of cyclic electron transport.

Clearly, more basic research into the portioning of electron transport capacity between the two cell types is required and production of transgenic C_4 plants with altered levels of these electron transport components would be valuable in such research.

Conclusions

C_4 photosynthesis has been studied for 50 years but until relatively recently, with the advent of transgenic technologies, there was little evidence for which key components of this complex pathway controlled photosynthetic flux. Many of the more obvious targets in

C₄ photosynthetic carbon metabolism indicated above are tractable by transgenic means but as with C₃ photosynthesis, the impact of increasing an enzyme or protein on plant performance is entirely dependent upon environmental variables. In many cases, more basic research is required on the energetics of this pathway before the more intricate aspects of C₄ photosynthesis can be addressed strategically. While improving the performance of some of the most productive plants on the planet may be ambitious, recent rapid advances in synthetic biology, combined with more “traditional” plant biochemistry approaches provide new opportunities to probe and engineer this complex pathway.

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Figures

Text Boxes and Tables

Box 1 Targets for enhancing C_4 photosynthesis

The C_4 photosynthesis pathway is a CO_2 concentrating mechanism that operates across two cell type. Atmospheric CO_2 diffuses to mesophyll cells where it is converted to HCO_3^- by carbonic anhydrase (CA) which is then fixed by phosphoenolpyruvate carboxylase (PEPC) to produce C_4 acids which diffuse to, and are decarboxylated in, the bundle sheath to supply CO_2 for ribulose biphosphate carboxylase oxygenase (Rubisco). This elevates the CO_2 partial pressure (pCO_2) in the bundle sheath allowing Rubisco to operate close to its maximal rate. This is shown in a diagrammatic representation in (A) together with suggested targets where overexpression may deliver increased photosynthetic rates.

The C_4 photosynthetic CO_2 concentrating mechanisms results in distinct characteristics of CO_2 assimilation rate in response to CO_2 partial pressure (B) and irradiance (C). The CO_2 response of CO_2 assimilation at high light is characterized by a low CO_2 compensation point

(the CO₂ partial pressure where net CO₂ assimilation rate is zero). The steep initial rise in CO₂ assimilation rate is determined by CO₂ diffusion across the cell wall, plasma membrane and cytosol and the activity of CA and PEPC. CO₂ assimilation rate saturates abruptly at low intercellular CO₂ and saturated rates of CO₂ assimilation can be determined by the amount and activity of Rubisco, the capacity for regeneration of either phosphoenol pyruvate (PEP) or ribulose biphosphate (RuBP) or the capacity of mesophyll and bundle sheath electron transport [45]. It can be seen that the light response of C₄ photosynthetic CO₂ assimilation rate does not saturate at full sunlight. At low light the rate of CO₂ assimilation is determined by the light harvesting characteristic and the requirement of energy between mesophyll and bundle sheath chloroplast. At higher light CO₂ assimilation rate is most likely limited by electron transport capacity, but can also be limited by the capacity for PEP or RuBP regeneration or Rubisco activity.

Measurements shown here were made on a Sorghum leaf at 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (B) or at atmospheric CO₂ of 380 ppm (C) and leaf temperature of 25 °C.

Box 2 The C₄ photosynthetic pathway is not biochemically unique

Hatch and Slack divided the C₄ photosynthetic mechanism into 3 decarboxylation types based on the enzyme used to decarboxylate C₄ acids. However, it is now becoming apparent from re-analysis of ¹⁴C labelling data, enzyme activities, immunological and transcriptional work and modelling [6,51-53] that mixtures of the 3 types exist and many variations exist in how these mixtures of C₄ biochemistry have been assembled during evolution. Three variations are shown in this Box. In all schemes it is assumed that the C₃ cycle uses 3 ATP and 2 NADPH per CO₂ fixed and photorespiratory energy requirements have been ignored. **Scheme A** shows an NADP-ME type C₄ pathway where, if there is no CO₂ leakage from the bundle sheath, the added energetic cost of the CO₂ concentrating mechanism is the 2ATP required to regenerate PEP in the mesophyll. Also indicated (dotted lines) is the option for aspartate to also be translocated to the bundle sheath. Notably, when malate alone is translocated, reducing equivalents are transferred from the mesophyll chloroplast to the bundle sheath chloroplast. In this mechanism, the poorly stacked thylakoids and low PSII in the bundle sheath chloroplasts is reflected in a low whole chain electron transport capacity. This is typical of the C₄ pathway in sorghum and maize. It is in this NADP-ME pathway variant that the role of cyclic photosphorylation would be most important and PGR-5

and NDH1 overexpression would be potentially be most productive. If some aspartate is translocated to the bundle sheath (dotted lines) it must be transaminated back to OAA, reduced to malate and then decarboxylated (see Furbank 2011 for review [6]). This mechanism operates in *Flaveria bidentis* and potentially in a number of other NADP-ME species. These species tend to have more grana and PSII in the bundle sheath chloroplasts. **Scheme B** is a “conventional NAD-ME type” where aspartate is translocated to the bundle sheath, alanine returned and the mitochondrion provides reducing power for OAA reduction as it is also the site of decarboxylation. **Scheme C** is the most common so called “PCK type” but is in fact a mixture NAD-ME and PCK as it is thought that mitochondrial ATP production from NADH oxidation contributes to ATP production for PEPCK to operate. The stoichiometries of ATP and NADPH required for these pathways have recently been recalculated [51,52] and are summarised in the table below as mol ATP and NADPH required per mol CO₂ fixed by PEPCK. It is likely that the PCK mechanism is also present in a number of classical NADP-ME types which transport some aspartate to the bundle sheath, although this may account for only 10-15% of flux through the CO₂ concentrating mechanism [6, 51,52,] and is not shown on the schemes in this box. The most “efficient” pathway from a light generated ATP perspective would be a PCK type mixed with 30% NAD-ME (which could generate all the ATP required for PEPCK if the NADH produced in the mitochondrion was respired with an efficiency of 2.5ATP per NADH). Light generated ATP would only be required for 30% of the PEP regeneration in the mesophyll cells, in addition to the 3 ATP and 2 NADPH required for the photosynthetic carbon reduction cycle. Interestingly, most of the commercial C₄ crops are NADP-ME types. It is also intriguing to speculate on how altering these “mixtures” of pathways in transgenic plants would alter photosynthesis and growth, particularly given the implications on the energetic “load” of mesophyll versus bundle sheath chloroplasts in providing ATP and reducing power. For example, in the mesophyll chloroplast of an NAD-ME type, 2 ATP are required but no reducing power and in a “pure” PCK-type (which appears to be a theoretical case, not appearing in nature), if all the ATP required for PEPCK was generated in the bundle sheath chloroplast, the mesophyll chloroplast would have no energetic role. With such extreme energetics, it is interesting that the mesophyll chloroplasts of these types do not display the agranal, low PSII phenotype of NADP-ME type bundle sheath chloroplasts. In reality,, the 3PGA / TrioseP shuttle between the mesophyll and bundle sheath cells (shown in Fig 1) probably plays a

major role in balancing the photochemical load between the mesophyll and bundle sheath chloroplasts in all biochemical variants of C₄ photosynthesis, as this process does not appear to be limited just to the NADP-ME types (reviewed in Edwards and Vosnesenskaya (2011), [54]).

Decarboxylation Type	Location of decarboxylase	ATP and reducing power required per CO ₂ fixed by PEPC (and cellular location)					
		Mesophyll Chloroplast		Bundle ⁵ sheath Chloroplast		Bundle sheath mitochondrion	
		ATP	NADPH	ATP	NADPH	ATP	NADH
NADP-ME ¹	Chloroplast	2	1	3	1	0	0
NADP-ME ² (Mal/Asp)	Chloroplast	2	0.5	3	1.5	0	0
NAD-ME	Mitochondrion	2	0	3	2	0	0
PCK ³	Cytosol	0	0	4	2	0	0
PCK + NADME ⁴	Cytosol + mitochondrion	0.6	0.3	3	2	0.7	0.3

¹Assuming only malate is transported to the bundle sheath

²Assuming 50% of C₄ acids transported are malate and 50% aspartate and that OAA produced from aspartate is reduced in the bundle sheath chloroplast.

³Assuming all ATP required for decarboxylation via PEP carboxykinase is generated from photophosphorylation and PEP is returned to the mesophyll cell.

⁴Assuming 70% of C₄ acids are decarboxylated by PEPCK and 30% by NADME and that the one mol NADH produced by NAD-ME is used to produce 2.5 mol ATP, used for PEP decarboxylation.

It is also assumed that there is no leakage of CO₂ occurring from the bundle sheath compartment.

⁵The role of the 3PGA/TP shuttle between mesophyll and bundle sheath cells is not considered in this table but would play a role in balancing ATP and NADPH production and consumption across the cell types.

Box 3 What we have learned from photosynthetic transgenics in *Flaveria bidentis* and photosynthetic mutants in *Zea mays* and *Amaranthus edulis*.

Flaveria bidentis (a readily transformable model C₄ dicot) has been used to study regulation of C₄ photosynthesis using antisense and RNAi technology [9]. This work has been pivotal in quantitatively determining the rate limiting steps in the C₄ pathway. **1)** Reducing the amount of Rubisco with antisense constructs to the small subunit of Rubisco in *F. bidentis* has shown a strong relationship between Rubisco content and CO₂ assimilation rate at high light indicating that Rubisco content and activity can be an important rate limiting step, particularly at low temperature[21,55]. **2)** Rubisco activase was shown to be essential for C₄ photosynthesis as plants with low activase content required very high CO₂ for growth. On the other hand activase content could be reduced to 30% of wild type levels before steady state rates were affected [28]. **3)** In C₄ species the majority of CA activity has been localised

to the cytosol of the mesophyll cell [56]. CO₂ hydration by CA is the first step of C₄ photosynthesis, converting CO₂ to HCO₃⁻, which is then fixed by phosphoenolpyruvate carboxylase (PEPC) to produce C₄ acids. *F. bidentis* with an antisense construct to CA has shown it to be essential, but, similar to activase, low levels were sufficient for steady state CO₂ assimilation rate at ambient pCO₂ and high light. [14]. This was confirmed in a CA double mutant in *Zea mays* where it was also shown to be more limiting at low pCO₂ [15]. **4)** PEP carboxylase is the primary enzyme fixing atmospheric carbon in C₄ plants and is believed to be highly regulated by protein phosphorylation and metabolite levels [19,57]. Work with mutants of *Amaranthus edulis* [58,59] indicate that PEPC levels exert strong control over photosynthetic flux at lower CO₂ concentrations but the regulatory control of photosynthesis by this enzyme is still largely unexplored. **5)** Transgenic *F. bidentis* with reduced amounts of PEPC phosphokinase suggests that phosphorylation of PEPC is not an important mechanism controlling photosynthetic flux [19]. **6)** Transgenic *F. bidentis* with reductions in the content of malate dehydrogenase showed that this enzyme could be reduced to low levels without effect on CO₂ assimilation rate because increase in the activation state of the enzyme when the content was reduced [7,9]. **7)** Malic enzyme was also shown to be present in excess [8]. **8)** *F. bidentis* with reduced PPDK were more difficult to analyse because of pleiotropic effects and it remains unclear how rate limiting this enzyme is [37]. **9)** Photorespiratory flux is considered to be small in the high CO₂ atmosphere of bundle sheath cells, however *Zea mays* knockout mutants in glycolate oxidase had a very high CO₂ requiring phenotype demonstrating an essential role for the photorespiratory pathway during C₄ photosynthesis [60]. The transgenic plants discussed have been analysed under a limited number of environmental conditions only and their impact on flux control will vary with different environmental conditions.

Figure 1

Figure 1A shows a potential link between the C₄ and C₃ cycles using the NADP-ME pathway as an example, although this pathway may occur in all C₄ photosynthetic mechanisms. 3PGA is shuttled to the mesophyll chloroplast from the bundle sheath chloroplast where it can either be reduced to triose phosphate (TP) and returned to the C₃ cycle in the bundle sheath chloroplast or converted to PEP via phosphoglycerate mutase and enolase, potentially connecting the C₃ and C₄ cycles. This connectivity between C₄ and C₃ cycle photosynthetic metabolites is illustrated by the relationship between the steady state content of 3PGA and PEP in *Zea mays* and *A. edulis* with varying intercellular CO₂ partial pressure and irradiance [61] and in wild type and transgenic *F. bidentis* with reduced activase content measured at 25 or 40 °C [62].

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Papers of particular interest, published within the period of review, have been highlighted as:

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Figure1

Figure 1