Photorespiration connects \( \text{C}_3 \) and \( \text{C}_4 \) photosynthesis

Andrea Bräutigam\(^1,2,3\) and Udo Gowik\(^4\)*

\(^1\) Institute of Plant Biochemistry, Universitätstrasse 1, Heinrich-Heine-University, 40225 Düsseldorf, Germany
\(^2\) Cluster of Excellence on Plant Sciences (CEPLAS) “From Complex Traits towards Synthetic Modules”, 40225 Düsseldorf, Germany
\(^3\) Present address: Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Corrensstraße 3, 06466 Stadt Seeland, Germany
\(^4\) Institute of Plant Molecular and Developmental Biology, Universitätstrasse 1, Heinrich-Heine-University, 40225 Düsseldorf, Germany

* Correspondence: gowik@uni-duesseldorf.de

Received 2 December 2015; Accepted 28 January 2016

Editor: Martin Hagemann, University Rostock

Abstract

\( \text{C}_4 \) plants evolved independently more than 60 times from \( \text{C}_3 \) ancestors. \( \text{C}_4 \) photosynthesis is a complex trait and its evolution from the ancestral \( \text{C}_3 \) photosynthetic pathway involved the modification of the leaf anatomy and the leaf physiology accompanied by changes in the expression of thousands of genes. Under high temperature, high light, and the current CO\(_2\) concentration in the atmosphere, the \( \text{C}_4 \) pathway is more efficient than \( \text{C}_3 \) photosynthesis because it increases the CO\(_2\) concentration around the major CO\(_2\) fixing enzyme Rubisco. The oxygenase reaction and, accordingly, photorespiration are largely suppressed. In the present review we describe a scenario for \( \text{C}_4 \) evolution that not only includes the avoidance of photorespiration as the major driving force for \( \text{C}_4 \) evolution but also highlights the relevance of changes in the expression of photorespiratory genes in inducing and establishing important phases on the path from \( \text{C}_3 \) to \( \text{C}_4 \).

Key words: \( \text{C}_4 \) photosynthesis, CO\(_2\) fixation, evolution, photorespiration

Introduction

The vast majority of organic carbon on earth is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The enzyme functions as an oxygenase as well as a carboxylase using both CO\(_2\) and O\(_2\) depending on their concentrations, with carboxylation generating 3-phosphoglyceric acid (3-PGA) and oxygenation additionally generating 2-phosphoglycolate (2-PG). Photorespiration, the pathway used to regenerate 2-PG, takes place in the chloroplasts, peroxisomes, and mitochondria. It consumes ATP and NADPH and leads to a net loss of CO\(_2\) for the plant. This reduces the efficiency of carbon fixation in plants by up to 30% under hot and dry conditions (Bauwe et al., 2010; Raines, 2011). \( \text{C}_4 \) photosynthesis acts as a CO\(_2\) pump and inhibits the oxygenation reaction by effectively increasing the intracellular CO\(_2\) to O\(_2\) ratio at the site of Rubisco. \( \text{C}_4 \) photosynthesis usually involves two different cell types, the mesophyll and the bundle sheath cells (Fig. 1A), whereas only few species are known that realize a \( \text{C}_4 \) cycle within a single cell (Edwards et al., 2004).

\( \text{C}_4 \) plants are characterized by high rates of photosynthesis and efficient use of water and nitrogen resources. Owing to their CO\(_2\) concentration mechanism they can reduce their stomatal conductance and save water. Because Rubisco works more efficiently under higher CO\(_2\) concentrations, \( \text{C}_4 \) plants also need less Rubisco, the most abundant enzyme in plant leaves, leading to nitrogen savings. The \( \text{C}_4 \) cycle itself involves the initial fixation of CO\(_2\) in the form of bicarbonate in the mesophyll cells by phosphoenolpyruvate carboxylase (PEPC), resulting in the four-carbon compound oxaloacetate that is converted to the transport metabolites malate or aspartate.
These are transferred to the bundle sheath cells where CO₂ is set free by a decarboxylase, either the NADP-dependent malic enzyme, the NAD-dependent malic enzyme, phosphoenolpyruvate carboxykinase, or a combination of two of these enzymes (Furbank, 2011; Pick et al., 2011; Wang et al., 2014). The resulting pyruvate is transferred back to the mesophyll where phosphoenolpyruvate is regenerated by pyruvate orthophosphate dikinase. The CO₂ released in the bundle sheath is re-fixed by Rubisco, which is exclusively located in the bundle sheath cells in C₄ plants (Hatch, 1987).

C₄ photosynthesis evolved independently more than 60 times within the angiosperms (Sage et al., 2011). This makes C₄ photosynthesis one of the most remarkable cases of convergent evolution of a complex trait (Westhoff and Gowik, 2004). It requires two compartments, one for initial carbon fixation by PEPC, most frequently realized as a mesophyll cell, and one for carbon fixation by Rubisco in an arrangement called the Kranz anatomy, where the bundle sheath cells surround the vascular bundles and are themselves surrounded by the mesophyll cells (Hatch, 1987). The different cell types are adapted to the trait. Bundle sheath cells are enlarged and photosynthetically competent, surrounded by a less permeable cell wall that may or may not be suberized (Botha, 1992; Evert et al., 1996). They are connected to the mesophyll by many plasmodesmata (Evert et al., 1977; Botha, 1992; Sowinski et al., 2008; Majeran et al., 2010). Leaves of C₄ plants are often thinner than those of C₃ plants and exhibit a higher vein density to ensure that every mesophyll cell is in direct contact with a bundle sheath cell (Fig. 1A) (Dengler and Nelson, 1999). Both mesophyll and bundle sheath cells undergo gene expression changes for adaptation (Bräutigam et al., 2011; Bräutigam et al., 2014; Gowik et al., 2011).

Fig. 1 C₄ photosynthesis and the photorespiratory pump. (A) Cross section from a leaf of *Megathyrsus maximus*. A typical C₄ leaf with bundle sheath and mesophyll cells surrounding the veins in layers. Chlorophyll fluorescence (red) was visualized by exciting fluorescence with 460–500 nm and monitoring the emission above 593 nm. The autofluorescence of lignified cell walls (blue) was excited at 335–383 nm and monitored at 420–470 nm. (B) Schematic representation of the C₄ pathway. (C) Schematic representation of the photorespiratory pump. (D) Mechanistic interaction between the photorespiratory pump and the C₄ pathway. In (B), (C), and (D), enzyme localizations are colour coded: green chloroplast, orange peroxisomes, blue mitochondria. Abbreviations: Ala, alanine; Asp, aspartate; AT, aminotransferase; CA, carbonic anhydrase; GDC, glycine decarboxylase; Glc, glycerate; Gin, glutamine; Glo, glycolate; Glu, glutamate; Glx, glyoxylate; Gly, glycine; GOX, glycolate oxidase; HPR, Hyp reductase; Hyp, hydroxypyruvate; Mal, malate; MDH, malate dehydrogenase; NADP-ME, NADP-dependent malic enzyme; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PGP, phosphoglycerate phosphate; PPDK, pyruvate orthophosphate dikinase; Pyr, pyruvate; RuBP, ribulose-1,5-bisphosphate; Ser, serine; SHM, serine hydroxymethyltransferase; 2-PG, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.
confirmed the succession of steps proposed earlier, but indicated that the evolutionary path is smooth (Heckmann et al., 2013).

Photorespiration is strongly associated with the evolution of the C₄ photosynthetic pathway. On the one hand, the reduction of photorespiration was one of the driving forces behind C₄ evolution. On the other hand, all of the models of C₄ evolution (Monson, 1999; Bauwe, 2011; Sage et al., 2012; Heckmann et al., 2013; Williams et al., 2013) predict that the establishment of a photorespiratory CO₂ pump that relocates the photorespiratory CO₂ release to the bundle sheath cells is an important intermediate step towards the C₄ cycle. This photorespiratory CO₂ pump is also termed C₂ photosynthesis because the two-carbon compound glycine serves as a CO₂ transport metabolite (Fig. 1C; briefly, photorespiration is partitioned between two cell types with decarboxylation of glycine occurring mainly in one type, thereby enriching CO₂ at the site of this decarboxylation). Plants that use the photorespiratory pump (or C₂ photosynthesis) are often termed C₃–C₄ intermediates owing to their physiological properties.

This review considers selective pressures, deduced from the properties of recent C₃–C₄ intermediate and C₄ species but not from the current environments of these species (Edwards et al., 2010); the changes at the molecular level; and the consequences of different phases of evolution in C₃–C₄ intermediate and C₄ species as we observe them today.

**Setting the stage—increased leaf venation creates a carbon-needy plant**

The vast majority of C₄ species exhibit Kranz anatomy in their leaves, that is, they have high vein density with only two mesophyll cells spacing two veins and their bundle sheaths (Fig. 1A). The step-wise model considers changes in venation patterns as one of the early steps (Sage, 2004), which was confirmed in a Bayesian model (Williams et al., 2013) (Fig. 2). Veneration itself is a variable trait both within a species and between species (Lundgren et al., 2014). It is under high selective pressure (Roth-Nebelsick et al., 2001) because the veneration pattern of the leaf in part determines the resistance to water flow through the plant (Sack and Holbrook, 2006). On average the veneration contributes about a third to total water resistance, but can reach up to 98% (summarized in Sack and Holbrook, 2006). The water potential is of key importance because it determines stomatal opening via the water status of the cells, which in turn determines photosynthetic rates (Sack and Holbrook, 2006; Brodribb et al., 2007). Hence the veneration patterns are indirectly coupled to photosynthetic rates. Water resistance is determined more strongly by veneration pattern in species that establish under high light conditions (~70%) than in species that establish in low light conditions (52%; Sack et al., 2005). Based on these results, it is expected that species with high veneration density establish in high light, high air temperature, and low air humidity conditions. At the same time, enough soil water must be available to secure the benefits of increased veneration (Fig. 2). Given that more veins with their reinforced walls require a higher investment, photosynthetic gains need to outstrip the investment to realize a competitive advantage. Higher veneration density also lowers the leaf water potential at which leaf water conductance is halved, indicating higher tolerance to (temporary) drought (Nardini et al., 2012). Under these conditions, having more veins might be beneficial to counteract the loss of water conductivity due to xylem collapse or the effect of cavitation (Griffiths et al., 2013). Griffiths et al. (2013) also proposed that there might be an evolutionary advantage to enlarged bundle sheath cells because they could acquire functions in cavitation repair and maintaining hydraulic conductance.
Finally, higher venation density may reduce loss through grazing by altering palatability. Based on these analyses, an alternative environment in which species with high venation patterns establish may be a high light, high temperature environment with generally high, but fluctuating and at times limited, water availability. Competing plants will wilt in such an environment and thus no longer compete. Modelling analyses may show in future which of the two scenarios is true under a given set of conditions. If venation has evolved independently from C₄ photosynthesis with its own set of selective pressures, one could expect that tight venation must have evolved in lineages without C₄ species. And indeed such C₃ species exist, as one could expect that tight venation must have evolved in lineages without C₄ species. And indeed such C₃ species exist, as shown by Christin et al. (2013).

There are two consequences that follow from a tighter venation pattern in otherwise similar leaves: (i) space for photosynthetically active mesophyll is reduced in favour of vein tissue (Fig. 1A), and (ii) veins with reinforced cell walls result in a higher C:N ratio because the walls require virtually only C to be built (Niinemets et al., 2007; Sack and Scoffoni, 2013). Both of these consequences lead to the evolutionary pressure to increase photosynthetic capacity. Not only is leaf size constrained by a variety of factors (Niinemets et al., 2007; Sack et al., 2012), simply increasing leaf size to add more mesophyll cells is likely ecologically unfavourable (Niinemets et al., 2007). To achieve a higher number of photosynthesizing cells on the same leaf lamina, the bundle sheath cells were likely under evolutionary pressure to enhance their competence to photosynthesize, leading to enlarged bundle sheath cells with an increased number of chloroplasts. Because photorespiration occurs in all cells containing Rubisco, this consequently also requires an increase in the number of mitochondria. With regard to the complex trait of C₄ photosynthesis, at this point during evolution the tight venation was in place with a high likelihood of photosynthetically competent, organelle-containing bundle sheath cells. This type of anatomy is also termed as proto Kranz anatomy (Sage et al., 2012). None of the other trait components were likely in place at this point. In fact, the poorly permeable walls of bundle sheath cells typical for C₄ species would have been counterproductive for active photosynthesis in the cell type. Increased venation, although not necessarily to the point of Kranz anatomy, was likely a necessary but insufficient condition for enabling progress towards C₄. C₄ photosynthesis, as well as the photorespiratory pump, require additional anatomical features, such as close contact between mesophyll and bundle sheath cells and large enough bundle sheath cells to house enough chloroplasts for the Calvin–Benson–Bassham cycle (Lundgren et al., 2014).

The molecular mechanisms that lead to the changes in venation density are largely unknown. Initiation of veins is governed by directed auxin transport followed by the temporal succession of marker gene expression for vein development (Scarpella and Meijer, 2004; Scheres and Xu, 2006). Once mesophyll cells differentiate, vein formation is terminated (Scarpella et al., 2004), prompting the hypothesis that delayed mesophyll differentiation enables more vein formation in dicots. Indeed, Kulahoglu et al. (2014) observed that the differentiation of mesophyll cells is delayed in the leaves of the C₄ species Gynandropsis gynandra compared to that of the closely related C₃ species Tarenaya hassleriana. The molecular identity of factors controlling these changes remains unknown to date. Once vein identity is established, cell identities in the leaf need to be established. Transcriptome analysis of developing maize foliar and husk leaves as well as the examination of maize mutants implicate a role of the SCARECROW/SHORTROOT regulatory network in establishing Kranz anatomy (Slewinski et al., 2012; Slewinski, 2013; Wang et al., 2013). A model describing how the SCARECROW/SHORTROOT pathway might be involved in Kranz patterning and the specification of bundle sheath and C₄ mesophyll cells is detailed in Fouracre et al. (2014).

The importance of anatomical pre-conditioning for the evolution of C₄ and likely also the evolution of the photorespiratory pump is shown in a study by Christin et al. (2013). Leaf anatomy analyses of 157 grass species from the PACMAD clade (including the subfamilies Aristidoideae, Arundinoideae, Chloridoideae, Danthonioideae, Micrairoideae, and Panicoideae and exhibiting 22–24 independent C₄ origins) and the BEP clade (including the subfamilies Bambusoideae, Ehhrartioideae, and Pooidae and containing zero C₄ origins) led to the conclusion that the possibility of C₄ evolution strongly increases when the proportion of bundle sheath tissue exceeds 15%. This was achieved by increased bundle sheath cell size and decreased vein spacing.

The result of increased venation is plants that are highly competitive in high temperature, low air humidity, and high soil moisture environments. However, they are critically dependent on high photosynthetic rates to maintain their high investment in carbon-intense vein architecture (Fig. 2).

The photorespiratory CO₂ pump as the initial solution to limited soil water availability

Plant populations with high investment into the venation system to maintain high photosynthetic rates may encounter limited water availability. This encounter may be temporal with changing climate over time within their current niche or spatial at the edges of the niche. A solution to limited soil water availability and thus limited carbon may be the reversal to lower density venation to save carbon. Alternatively, carbon concentration mechanisms could be the answer to maintaining the present venation density, assuming CO₂ is the limiting resource for growth and reproduction. Plant growth is limited by the scarcest resource according to the Liebig law of the minimum as summarized in van der Ploeg et al. (1999). In most niches, plants are not limited by carbon assimilation, but by nitrogen or phosphorus availability in the soil even under today’s low CO₂ concentrations (Agren et al., 2012; Körner, 2015). Although C₄ photosynthesis itself leads to high nitrogen use efficiency (Sage, 2004), the intermediate stages by no means have higher nitrogen use efficiency (Monson, 1989; Pinto et al., 2011; Vogan and Sage, 2011). Evolution of photosynthetic types that increase the carbon assimilation efficiency must have occurred under conditions in which carbon and not nitrogen or phosphorus (or indeed any other nutrient) was the limiting factor. Although most
C₄ origins post-date the atmospheric decline of CO₂ 30 million years ago, some by over 20 million years, limited evidence indicates C₄ evolution prior to the decline (Prasad et al., 2011; Christin and Osborne, 2014; Christin et al., 2014). Both the continued evolution of the photosynthetic pump and C₄ photosynthesis as well the evolution prior to the CO₂ decline indicate that local changes of the environmental conditions, like a local decline in water availability, are critical for carbon limitation and hence for the evolution of the C₄ trait (Fig. 2).

The photosynthetic pump is one possibility for plants to deal with limited CO₂ because it allows more efficient carbon assimilation (Ku et al., 1983; Monson et al., 1984). While the existence of so called C₃–C₄ intermediate plant species was known for a long time, the detailed biochemical mechanisms underlying this type of photosynthesis remained unclear (Edwards and Ku, 1987). Most C₃–C₄ intermediates are characterized by a leaf anatomy that is intermediate to C₃ and C₄ species, with large, organelle-rich bundle sheath cells and close vein spacing (Edwards and Ku, 1987). Their apparent rate of photosynthesis and the CO₂ compensation point is between the values for C₃ and C₄ plants (Edwards and Ku, 1987). The analysis of the C₃-C₄ intermediate Moricandia arvensis demonstrated that these intermediate physiological parameters depend on the existence of a photosynthetic CO₂ pump (Rawsthorne et al., 1998a, b) and confirmed earlier assumptions (Edwards and Ku, 1987; Monson et al., 1984). A photosynthetic CO₂ pump was also found to be active in other C₃–C₄ intermediate species from the genera Flaveria, Panicum, Mollugo, Alternanthera, and others (Kennedy and Laetsch, 1974; Rajendrudu et al., 1986; Hylton et al., 1988; Morgan et al., 1993; Sage et al., 2012). The pump essentially requires mesophyll with limited glycine decarboxylation activity, which forces photosynthetic glycine to the bundle sheath for decarboxylation and high photosynthetic rates to achieve carbon concentration in the bundle sheath (Fig. 1C) (Rawsthorne et al., 1988a). The increased photosynthetic rate in plants with dense venation is a pre-condition for the photosynthetic pump. In M. arvensis the pump is realized by restricting the P subunit of the glycine decarboxylase complex (GDC) to the bundle sheath cells (Rawsthorne et al., 1988a, b). In other species, the P subunit as well as other GDC subunits and serine hydroxymethyltransferase, which is involved in glycine decarboxylation, are similarly absent in the mesophyll cells (Morgan et al., 1993). It was shown later that the cell-specific activity of the GDC is regulated on the transcriptional level (Engelmann et al., 2008; Schulze et al., 2013).

By moving the decarboxylation step to the mitochondria of the bundle sheath, the photosynthetic CO₂ release is exclusively localized in one cell type, increasing the CO₂ concentration in that cell type up to 3-fold (Keerberg et al., 2014). Rubisco can work more efficiently under these CO₂-enriched conditions and the unfavourable oxygenation reaction is largely suppressed (Bauwe and Kolukisaoglu, 2003; Rawsthorne, 1992; von Caemmerer, 1989). In addition, by restricting GDC to the bundle sheath, photosynthetic CO₂ is released in the interior compartment of the leaf, increasing the chance of refixation before it is lost from the plant. This qualitative model of the photosynthetic pump was largely confirmed by physiological data and the quantitative model by von Caemmerer (1989). Using the von Caemmerer/Farquhar model of photosynthesis (Farquhar et al., 1980; von Caemmerer, 2000) and starting with a species with tight venation and assuming unlimited light availability, Heckmann et al. (2013) demonstrated that the photosynthetic pump provides a small fitness gain in terms of higher carbon assimilation rates, and predicted it to be the first change occurring in the evolution of C₄ (Fig. 2).

The evolutionary history of how the photosynthetic pump was established in the genus Flaveria was recently investigated in molecular detail (Schulze et al., 2013). A gene duplication released the glycine decarboxylase P protein from adaptive conflict. Both copies were sub-functionalized by duplication, degeneration, and complementation with regard to the expression domains (Monson, 1999). One GDC-P copy was found to be bundle sheath–specific whereas another GDC-P gene was expressed in all photosynthetic leaf cells in the C₃ Flaveria species analysed (Schulze et al., 2013). At this point, the genus was poised to evolve the photosynthetic pump. Gradual loss of the whole leaf-expressed copy left only the bundle sheath–specific copy. Under the assumption that the transport capacity of the mesophyll–bundle sheath cell interface was sufficient, enrichment of CO₂ at the site of the bundle sheath occurred.

The detailed analyses in Flaveria showed that GDC-P was not abruptly lost from the mesophyll cells but that GDC-P mesophyll expression is reduced gradually in C₃–C₄ intermediates and becomes zero only in the true C₄ Flaveria species, including the pseudogenization of the GDC-P copy expressed everywhere (Schulze et al., 2013). It is plausible that the photosynthetic CO₂ pump was not established abruptly, because the capacities to decarboxylate large amounts of glycine efficiently and to recapture the correspondingly large amounts of photosynthetic CO₂ were likely not present in the bundle sheaths at this stage. Also, the bundle sheath cells of recent proto Kranz species are still relatively poor in chloroplasts and mitochondria (Muhaidat et al., 2011; Sage et al., 2013). The abrupt loss of all glycine decarboxylation activity in the mesophyll would most probably have been fatal.

The gradual reduction of glycine decarboxylation in the mesophyll cells implies a series of self-reinforcing steps (Bauwe, 2011; Muhaidat et al., 2011; Sage et al., 2012). By creating a higher CO₂ concentration around Rubisco in the bundle sheath, it would become more engaged in CO₂ fixation than the mesophyll enzyme. This creates a selection pressure to enhance the number of bundle sheath chloroplasts and the amount of Rubisco in the bundle sheath. More glycine decarboxylation activity could be shifted to the bundle sheath cells and the number of bundle sheath mitochondria would increase and lead to further CO₂ enrichment. Bundle sheath Rubisco would operate under even more favourable conditions, and so on.

Although models established the photosynthetic pump as the first change in biochemistry (Heckmann et al., 2013) and molecular analysis demonstrated the succession of events at the gene level (Schulze et al., 2013), the question whether the
photorespiratory pump might be a dead end or an intermediate inevitably leading to C₄ remained. Models predicted the evolution of the C₄ cycle as the next step (Heckmann et al., 2013; Williams et al., 2013) but did not provide explanations about the mechanism.

**From the photorespiratory pump to C₄ photosynthesis**

The photorespiratory pump does not only enrich CO₂ in the bundle sheath cells. Two molecules of glycine are moved into the bundle sheath and only one molecule of serine is moved back in the most straightforward version of the pathway. Hence, not only the CO₂ but also the ammonia accumulates in the bundle sheath (Fig. 1C). This leads to a massive nitrogen imbalance between mesophyll and bundle sheath cells when the photorespiratory pump runs with high activity. Ammonia is toxic and known to effectively uncouple electrochemical gradients (Krogmann et al., 1959), thus it has to be refixed in the bundle sheath cells and shuttled back to the mesophyll in the form of amino acids. This ammonia problem was recognized at the time the scheme was proposed (Rawsthorne et al., 1988b).

The question of how the C₄ pathway evolved from the photorespiratory CO₂ pump was linked to the question about the fate of the ammonia and analysed by a combination of computer modelling and transcriptome analysis of C₃, C₄, and C₃–C₄ intermediate species of the genus Flaveria (Mallmann et al., 2014). Using a flux balance analysis model modified from C4GEM (Dal'Molin et al., 2010) the possible return routes for the ammonia were determined. Biomass neutral possibilities with increasing metabolic complexity were (i) a glutamate 2-oxoglutarate shuttle, (ii) an alanine pyruvate shuttle, and (iii) an aspartate malate shuttle. The second and third possibility contained reactions required for C₄ photosynthesis. Enzyme activity measurements and RNA-seq data had already shown low activity or expression of the key C₄ gene for PEPC in C₃ plants (Bräutigam et al., 2011; Gowik et al., 2011; Bräutigam et al., 2014; and labelled C¹⁴ incorporation into C₄ acids in C₃–C₄ intermediate species and even C₃ species had been demonstrated (Monson et al., 1984). Hence, the model was queried for the optimal result if PEPC was active. PEPC activity immediately leads to a C₄ cycle that interacts with the photorespiratory pump at the point of the ammonia return (Fig. 1D) (Mallmann et al., 2014). Ammonia is shuttled to the mesophyll cells in the form of alanine, while malate is transferred to the bundle sheath in return, where it is decarboxylated and the resulting pyruvate used for alanine synthesis. Assuming carbon limitation of growth, fitness increases linearly with C₄ cycle activity. This is due to the fact that the C₄ cycle acts in concert with the photorespiratory pump in enriching CO₂ in the bundle sheath while re-shuttling the ammonia to the mesophyll. Consequently, according to the model, an increase in C₄ cycle activity directly translates into further biomass gains (Fig. 2).

In this model the evolution of the C₄ trait is additive instead of complex, especially with respect to the biochemistry. The enzyme or transporter that limits the C₄ cycle will come under high selective pressure because its increase will immediately translate into biomass and hence fitness gain. When it increases in expression, selective pressure will immediately shift to the next enzyme or transporter (or cellular interface) that is limiting (Mallmann et al., 2014).

The increase in C₄ cycle activity is likely driven by the selective pressure on the system, that is, evolution towards full C₄ species proceeds only if carbon remains limiting. This evolution likely included changes to the bundle sheath walls to increase CO₂ entrapment and O₂ exclusion, and changes to exit pathways for C₄ cycle metabolites, in addition to changes in gene expression for the C₄ cycle genes. Hence once a low-activity C₄ cycle takes over to replenish the ammonia imbalance resulting from the photorespiratory CO₂ pump, the evolution of true C₄ species becomes inevitable as long as the selective pressure—limiting carbon—persists. This model of C₄ evolution shifts the question of why some branches of the phylogenetic tree of plants have never evolved C₄ photosynthesis to the question of why these branches never evolved the photorespiratory pump.

**Fixation of the C₄ photosynthetic trait**

The sequence of steps establishing a highly active C₄ cycle in plants with a photorespiratory pump was confirmed by the analysis of C₃–C₄ intermediate species from the genus Flaveria (Heckmann et al., 2013; Mallmann et al., 2014). The sequence, and the seeming inevitability, of C₄ evolution once the pump is established provokes two questions: Can the C₄ trait revert and why are there intermediate stages today despite millions of years of evolution.

We posit that complete loss of Rubisco in the mesophyll and the subsequent reduction in photorespiratory gene expression fix the C₄ trait. Rubisco activity in the mesophyll may be lost gradually as PEPC activity increases but cannot be lost completely unless the C₄ cycle as a whole is adapted to carry the full load. The model of Heckmann et al. (2013) predicts the gradual loss of Rubisco as C₄ cycle activity increases. The photorespiratory pump will continue running until Rubisco in the mesophyll is completely shut off. This can be observed in the C₄-like species Flaveria brownii, which shows a reduction of mesophyll Rubisco together with other Calvin-Benson cycle and some photosynthetic genes, with the exception of the enzymes directly involved in glycine decarboxylation (Bauwe, 1984; Holaday et al., 1988; Mallmann et al., 2014). As long as mesophyll Rubisco is active, high photorespiratory gene expression is required (Fig. 2).

Only after the complete loss of mesophyll Rubisco activity can the final adjustment phase of C₄ evolution proceed. The loss of mesophyll Rubisco activity relaxes the selective pressure for high expression of photorespiratory genes because high activity and therefore high expression is no longer required. Because there is no more Rubisco in the mesophyll, expression of most photorespiratory genes in this tissue becomes obsolete and will be lost—most likely by drift—as can be observed in the highly optimized grass species maize, Sorghum bicolor, or Setaria italica (Li et al., 2010; Majeran et al., 2010; John et al., 2014; Döring et al., 2016). In consequence, high expression of photorespiratory genes can
no longer be detected in C₄ species (Bräutigam et al., 2011; Gowik et al., 2011; Bräutigam et al., 2014). Artificial reduction of C₄ cycle activity to the point where it can no longer maintain sufficient CO₂ enrichment by mutation (Dever et al., 1997) or by transgenic approaches (Pengelly et al., 2012) causes phenotypes reminiscent of photorespiratory mutants and, consequently, can be alleviated by growth in elevated CO₂ concentrations. Evolution has manoeuvred C₄ plants into a corner: escape requires simultaneous gain of Rubisco expression in the mesophyll and elevated expression of the photorespiratory genes, and is thus unlikely (Fig. 2). Because the trait is fixed, carbon limitation is no longer required to maintain it, hence C₄ species may now be limited by nutrients other than carbon.

Total Rubisco expression is also drastically reduced in C₄ species (Bräutigam et al., 2011; Gowik et al., 2011; Bräutigam et al., 2014), as is Rubisco protein content (Bauwe, 1984; Wessinger et al., 1989) along with the Calvin–Benson cycle enzymes, excluding those required for reduction of 3-PGA to triosephosphate (Bräutigam et al., 2011; Gowik et al., 2011; Bräutigam et al., 2014). In some species, even a reduction in expression of protein synthesis-related genes has been observed (Bräutigam et al., 2011; Gowik et al., 2011). This reduction in expression and likely protein abundance of highly abundant leaf proteins lead to better nitrogen use efficiency in some C₄ species (Sage, 2004). The fact that this did not happened in all C₄ species implies that optimization of nitrogen use was not a general selective pressure for the evolution of C₄ photosynthesis, and it can thus be considered a secondary effect.

Intermediate species are comparably rare; there are only seven known groups with independent origins of C₃–C₄ intermediate plants and no direct ancestry to C₄ species, meaning most of the intermediate species proceeded to C₄. Assuming that all recent C₄ lineages evolved via intermediates (Bauwe, 2011; Sage et al., 2012; Heckmann et al., 2013; Williams et al., 2013), the photorespiratory pump independently evolved 73 times and over 90% of these intermediate plant–containing lineages also contain species with C₄ photosynthesis. This raises the question of why the recent intermediate species are still persistent and, for some like the intermediate Mollugo group, for such a long time (Christin et al., 2011b).

There are several hypotheses that may explain this observation. First, the current status may be a snapshot and the species remain on their way towards C₄. This could surely be true for the extant Flaveria species with photorespiratory pumps because the genus Flaveria represents the youngest C₄ origin known to date (Christin et al., 2011a, Heckmann et al., 2013). It appears unlikely for the 15 million-year-old Mollugo verticillata (Christin et al., 2011b). Second, for some reason plants developed the photorespiratory pump but never used the C₄ pathway for adjusting the nitrogen imbalance. One could envision that these plants lack the basal activity of one or more enzymes or transporters of the C₄ cycle, which prevents them from ever entering the slippery slope to C₄ photosynthesis. That might have happened as the C₄ cycle genes have to be duplicated to be released from adaptive conflict but they were not. These plants must have developed an alternative way to cope with the nitrogen imbalance. For example, amino acids carrying two amino groups, like glutamine or asparagine, could be considered as transport metabolites, which might be superior to using the C₄ cycle under certain circumstances (Mallmann et al., 2014). Third, the idea that the establishment of a low activity C₄ cycle automatically leads to the establishment of the full C₄ physiology assumes continuous selective pressure. When carbon was no longer limiting for some reason or when the environment was variable (Cheng et al., 1989), that plant would have been trapped at its current stage. Future research on groups with only a photorespiratory pump but no C₄ photosynthesis will distinguish between these alternative hypotheses.

Summary

The evolution of C₄ plants occurred in phases that can be delineated by the selective pressures that drive the changes. Initially, the dense venation pattern is selected for high light, high temperature environments, in which soil water availability prevents stomatal closing if water conductance is high enough. The second phase of evolution is driven by carbon limitation, which may occur whenever stomatal aperture is limited, such as in salt stress or in drought stress conditions or in niches exceptionally rich in other nutrients. The use of the C₄ cycle to replenish nitrogen after the evolution of the photorespiratory pump immediately puts the species on the slippery slope towards C₄ and species are predicted to slide as long as the selective pressure is present. In theory, species may slide backwards if the selective pressure drops. This is only possible until further optimizations, like the loss of mesophyll activity of photorespiratory enzymes, occur. In this sense, C₄ is a dead end of evolution, albeit a very productive one.

Acknowledgements

This work was supported Deutsche Forschungsgemeinschaft through the Research Group FOR1186, the 3to4 EU program, and the Excellence Cluster EXC 1028 (From Complex Traits towards Synthetic Modules).

References


Brodribb TJ, Feild TS, Jordan GJ. 2007. Leaf maximum photosynthetic rate and venation are linked by hydraulics. Plant Physiology 144, 1890–1898.


Pinto H, Tissue DT, Ghannoum O. 2011. Panicum milioides (C₄−C₃) does not have improved water or nitrogen economies relative to C₃ and C₄ congeners exposed to industrial-age climate change. Journal of Experimental Botany 62, 3223–3234.


