



## REVIEW PAPER

# Photorespiration connects C<sub>3</sub> and C<sub>4</sub> photosynthesis

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## Abstract

C<sub>4</sub> plants evolved independently more than 60 times from C<sub>3</sub> ancestors. C<sub>4</sub> photosynthesis is a complex trait and its evolution from the ancestral C<sub>3</sub> 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<sub>2</sub> concentration in the atmosphere, the C<sub>4</sub> pathway is more efficient than C<sub>3</sub> photosynthesis because it increases the CO<sub>2</sub> concentration around the major CO<sub>2</sub> fixating enzyme Rubisco. The oxygenase reaction and, accordingly, photorespiration are largely suppressed. In the present review we describe a scenario for C<sub>4</sub> evolution that not only includes the avoidance of photorespiration as the major driving force for C<sub>4</sub> evolution but also highlights the relevance of changes in the expression of photorespiratory genes in inducing and establishing important phases on the path from C<sub>3</sub> to C<sub>4</sub>.

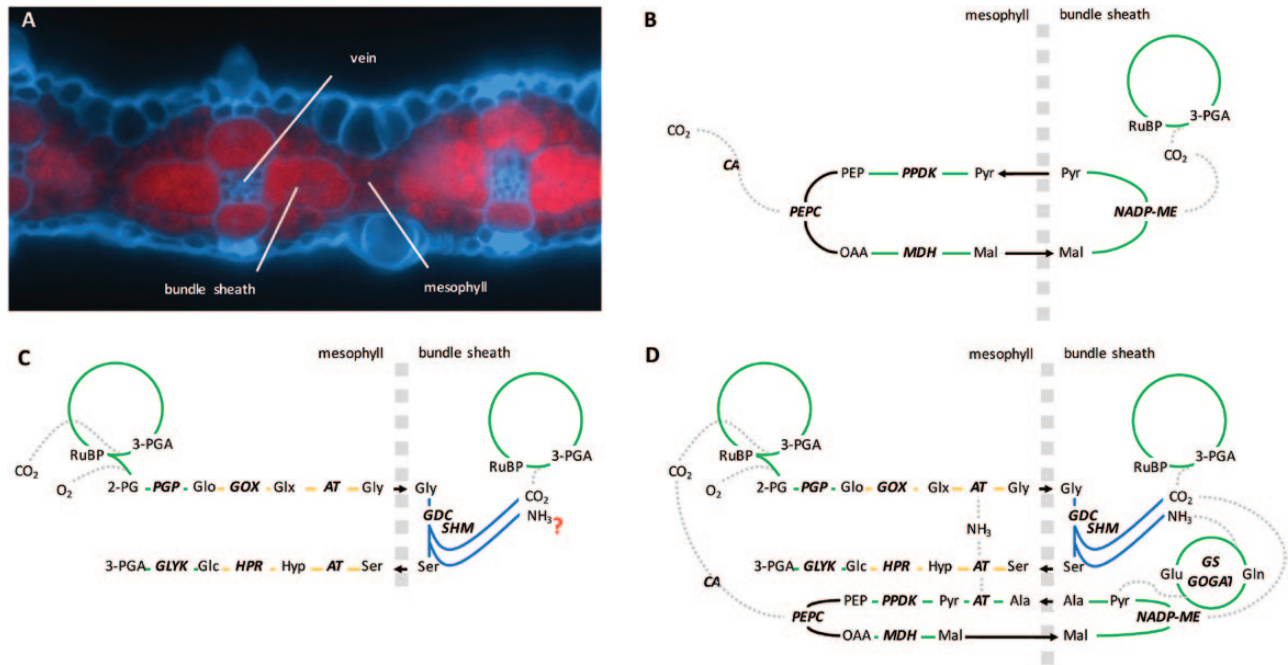
**Key words:** C<sub>4</sub> photosynthesis, CO<sub>2</sub> fixation, evolution, photorespiration

## 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<sub>2</sub> and O<sub>2</sub> 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<sub>2</sub> 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). C<sub>4</sub> photosynthesis acts as a CO<sub>2</sub> pump and inhibits the oxygenation reaction by effectively increasing the intracellular CO<sub>2</sub> to O<sub>2</sub> ratio at the site of Rubisco. C<sub>4</sub> 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 C<sub>4</sub> cycle within a single cell (Edwards *et al.*, 2004).

C<sub>4</sub> plants are characterized by high rates of photosynthesis and efficient use of water and nitrogen resources. Owing to their CO<sub>2</sub> concentration mechanism they can reduce their stomatal conductance and save water. Because Rubisco works more efficiently under higher CO<sub>2</sub> concentrations, C<sub>4</sub> plants also need less Rubisco, the most abundant enzyme in plant leaves, leading to nitrogen savings. The C<sub>4</sub> cycle itself involves the initial fixation of CO<sub>2</sub> 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.



**Fig. 1** C<sub>4</sub> photosynthesis and the photorespiratory pump. **(A)** Cross section from a leaf of *Megathyrsus maximus*. A typical C<sub>4</sub> 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 C<sub>4</sub> pathway. **(C)** Schematic representation of the photorespiratory pump. **(D)** Mechanistic interaction between the photorespiratory pump and the C<sub>4</sub> 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; Gln, 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, phosphoglycolate phosphatase; PDK, pyruvate orthophosphate dikinase; Pyr, pyruvate; RuBP, ribulose-1,5-bisphosphate; Ser, serine; SHM, serine hydroxymethyltransferase; 2-PG, 2-phosphoglycolate; 3-PGA, 3-phosphoglycerate.

These are transferred to the bundle sheath cells where CO<sub>2</sub> is set free by a decarboxylase, either the NADP-dependent malic enzyme, the NAD-dependent malic enzyme, phosphoenolpyruvate carboxylase, 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 CO<sub>2</sub> released in the bundle sheath is re-fixed by Rubisco, which is exclusively located in the bundle sheath cells in C<sub>4</sub> plants (Fig. 1B) (Hatch, 1987).

C<sub>4</sub> photosynthesis evolved independently more than 60 times within the angiosperms (Sage *et al.*, 2011). This makes C<sub>4</sub> 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, most frequently realized as the bundle sheath cell in an arrangement called the Kranz anatomy, where the bundle sheath cells surround the vascular bundles and are themselves surrounded by the mesophyll cells (Fig. 1A) (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 C<sub>4</sub>

plants are often thinner than those of C<sub>3</sub> 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).

The complex trait of C<sub>4</sub> photosynthesis requires the simultaneous presence of its anatomical and biochemical sub-traits. *Zea mays* (maize) husk leaves have increased vein spacing and lack the anatomical arrangement of Kranz anatomy. In consequence, only an incomplete version of the trait with lower carbon fixation yields develops (Pengelly *et al.*, 2011). Likewise, if any of the C<sub>4</sub> cycle enzymes are drastically reduced by mutation or molecular intervention, the pathway is not functional although the anatomical and other biochemical traits are present (Dever *et al.*, 1995; Dever *et al.*, 1997; Pengelly *et al.*, 2012). If only parts of the trait, that is the high expression of certain enzymes, are reconstituted in C<sub>3</sub> plants, the outcome is frequently detrimental to the plant (Fahnenstich *et al.*, 2007; Hausler *et al.*, 2001; Hausler *et al.*, 2002). This complexity, the requirement for all things to be present simultaneously, makes it difficult to envision how evolution may have proceeded. A step-wise model of C<sub>4</sub> evolution was proposed (Monson, 1999) and greatly refined in the following years (Sage, 2004; Sage *et al.*, 2012). Modelling of C<sub>4</sub> evolution with Bayesian approaches (Williams *et al.*, 2013) or with biochemical modelling (Heckmann *et al.*, 2013)

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 C<sub>4</sub> photosynthetic pathway. On the one hand, the reduction of photorespiration was one of the driving forces behind C<sub>4</sub> evolution. On the other hand, all of the models of C<sub>4</sub> evolution (Monson, 1999; Bauwe, 2011; Sage *et al.*, 2012; Heckmann *et al.*, 2013; Williams *et al.*, 2013) predict that the establishment of a photorespiratory CO<sub>2</sub> pump that relocates the photorespiratory CO<sub>2</sub> release to the bundle sheath cells is an important intermediate step towards the C<sub>4</sub> cycle. This photorespiratory CO<sub>2</sub> pump is also termed C<sub>2</sub> photosynthesis because the two-carbon compound glycine serves as a CO<sub>2</sub> transport metabolite (Fig. 1C; briefly, photorespiration is partitioned between two cell types with decarboxylation of glycine occurring mainly in one type, thereby enriching CO<sub>2</sub> at the site of this decarboxylation). Plants that use the photorespiratory pump (or C<sub>2</sub> photosynthesis) are often termed C<sub>3</sub>-C<sub>4</sub> intermediates owing to their physiological properties.

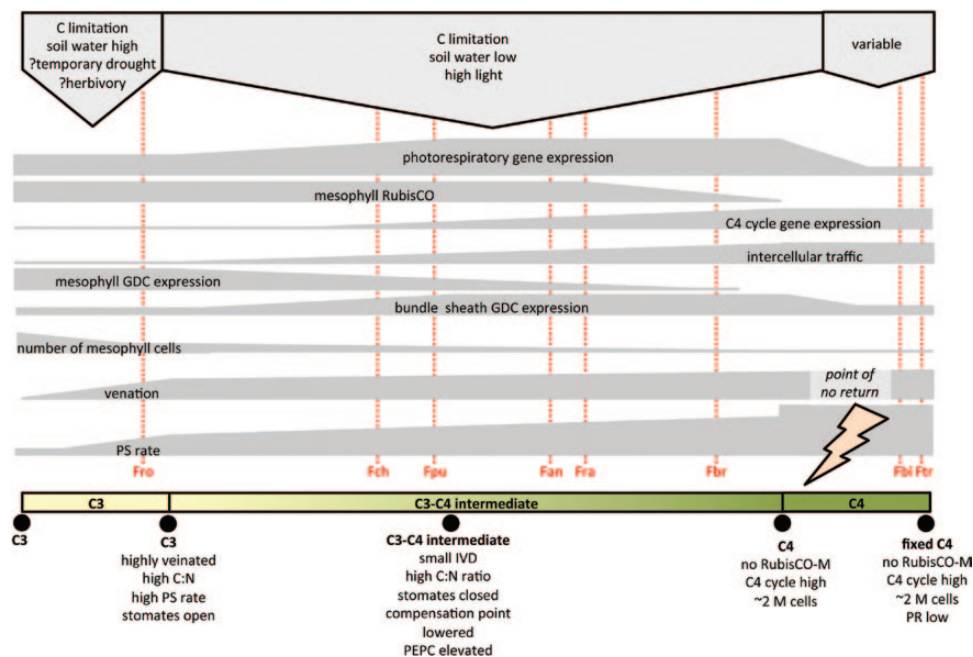
This review considers selective pressures, deduced from the properties of recent C<sub>3</sub>-C<sub>4</sub> intermediate and C<sub>4</sub> species but not from the current environments of these species (Edwards *et al.*, 2010); the changes at the molecular level; and the consequences of different phases of evolution in C<sub>3</sub>-C<sub>4</sub> intermediate and C<sub>4</sub> species as we observe them today.

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

The vast majority of C<sub>4</sub> 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).

Venation 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 venation pattern of the leaf in part determines the resistance to water flow through the plant (Sack and Holbrook, 2006). On average the venation 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 venation patterns are indirectly coupled to photosynthetic rates. Water resistance is determined more strongly by venation 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 venation 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 venation (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 venation 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.



**Fig. 2** The trajectory towards C<sub>4</sub>. Abbreviations: C, carbon; GDC, glycine decarboxylase; IVD, interveinal distance; M, mesophyll; N, nitrogen; PEPC, phosphoenolpyruvate carboxylase; PS, photosynthesis; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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  $C_4$  photosynthesis with its own set of selective pressures, one could expect that tight venation must have evolved in lineages without  $C_4$  species. And indeed such  $C_3$  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  $C_4$  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  $C_4$  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  $C_4$ .  $C_4$  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  $C_4$  species *Gynandropsis gynandra* compared to that of the closely related  $C_3$  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  $C_4$  mesophyll cells is detailed in [Fouracre \*et al.\* \(2014\)](#).

The importance of anatomical pre-conditioning for the evolution of  $C_4$  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  $C_4$  origins) and the BEP clade (including the subfamilies Bambusoideae, Ehrhartoideae, and Pooideae and containing zero  $C_4$  origins) led to the conclusion that the possibility of  $C_4$  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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> concentrations ([Agren \*et al.\*, 2012](#); [Körner, 2015](#)). Although  $C_4$  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

C<sub>4</sub> origins post-date the atmospheric decline of CO<sub>2</sub> 30 million years ago, some by over 20 million years, limited evidence indicates C<sub>4</sub> evolution prior to the decline (Prasad *et al.*, 2011; Christin and Osborne, 2014; Christin *et al.*, 2014). Both the continued evolution of the photorespiratory pump and C<sub>4</sub> photosynthesis as well the evolution prior to the CO<sub>2</sub> 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 C<sub>4</sub> trait (Fig. 2).

The photorespiratory pump is one possibility for plants to deal with limited CO<sub>2</sub> because it allows more efficient carbon assimilation (Ku *et al.*, 1983; Monson *et al.*, 1984). While the existence of so called C<sub>3</sub>–C<sub>4</sub> 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 C<sub>3</sub>–C<sub>4</sub> intermediates are characterized by a leaf anatomy that is intermediate to C<sub>3</sub> and C<sub>4</sub> species, with large, organelle-rich bundle sheath cells and close vein spacing (Edwards and Ku, 1987). Their apparent rate of photorespiration and the CO<sub>2</sub> compensation point is between the values for C<sub>3</sub> and C<sub>4</sub> plants (Edwards and Ku, 1987). The analysis of the C<sub>3</sub>–C<sub>4</sub> intermediate *Moricandia arvensis* demonstrated that these intermediate physiological parameters depend on the existence of a photorespiratory CO<sub>2</sub> pump (Rawsthorne *et al.*, 1988a, b) and confirmed earlier assumptions (Edwards and Ku, 1987; Monson *et al.*, 1984). A photorespiratory CO<sub>2</sub> pump was also found to be active in other C<sub>3</sub>–C<sub>4</sub> 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 photorespiratory 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 photorespiratory 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 photorespiratory CO<sub>2</sub> release is exclusively localized in one cell type, increasing the CO<sub>2</sub> concentration in that cell type up to 3-fold (Keerberg *et al.*, 2014). Rubisco can work much more efficiently under these CO<sub>2</sub>-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, photorespiratory CO<sub>2</sub> 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 photorespiratory 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 photorespiratory 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 C<sub>4</sub> (Fig. 2).

The evolutionary history of how the photorespiratory 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 C<sub>3</sub> *Flaveria* species analysed (Schulze *et al.*, 2013). At this point, the genus was poised to evolve the photorespiratory 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 CO<sub>2</sub> 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 C<sub>3</sub>–C<sub>4</sub> intermediates and becomes zero only in the true C<sub>4</sub> *Flaveria* species, including the pseudogenization of the GDC-P copy expressed everywhere (Schulze *et al.*, 2013). It is plausible that the photorespiratory CO<sub>2</sub> pump was not established abruptly, because the capacities to decarboxylate large amounts of glycine efficiently and to recapture the correspondingly large amounts of photorespiratory CO<sub>2</sub> 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 CO<sub>2</sub> concentration around Rubisco in the bundle sheath, it would become more engaged in CO<sub>2</sub> 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 CO<sub>2</sub> enrichment. Bundle sheath Rubisco would operate under even more favourable conditions, and so on.

Although models established the photorespiratory 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  $C_4$  remained. Models predicted the evolution of the  $C_4$  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 $C_4$ photosynthesis*

The photorespiratory pump does not only enrich  $CO_2$  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  $CO_2$  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 (Kroghmann *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  $C_4$  pathway evolved from the photorespiratory  $CO_2$  pump was linked to the question about the fate of the ammonia and analysed by a combination of computer modelling and transcriptome analysis of  $C_3$ ,  $C_4$ , and  $C_3$ – $C_4$  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  $C_4$  photosynthesis. Enzyme activity measurements and RNA-seq data had already shown low activity or expression of the key  $C_4$  gene for PEPC in  $C_3$  plants (Bräutigam *et al.*, 2011; Gowik *et al.*, 2011; Bräutigam *et al.*, 2014) and labelled  $C^{14}$  incorporation into  $C_4$  acids in  $C_3$ – $C_4$  intermediate species and even  $C_3$  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  $C_4$  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  $C_4$  cycle activity. This is due to the fact that the  $C_4$  cycle acts in concert with the photorespiratory pump in enriching  $CO_2$  in the bundle sheath while re-shuttling the ammonia to the mesophyll. Consequently, according to the model, an increase in  $C_4$  cycle activity directly translates into further biomass gains (Fig. 2).

In this model the evolution of the  $C_4$  trait is additive instead of complex, especially with respect to the biochemistry. The enzyme or transporter that limits the  $C_4$  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  $C_4$  cycle activity is likely driven by the selective pressure on the system, that is, evolution towards full  $C_4$  species proceeds only if carbon remains limiting. This evolution likely included changes to the bundle sheath walls to increase  $CO_2$  entrapment and  $O_2$  exclusion, and changes to exit pathways for  $C_4$  cycle metabolites, in addition to changes in gene expression for the  $C_4$  cycle genes. Hence once a low-activity  $C_4$  cycle takes over to replenish the ammonia imbalance resulting from the photorespiratory  $CO_2$  pump, the evolution of true  $C_4$  species becomes inevitable as long as the selective pressure—limiting carbon—persists. This model of  $C_4$  evolution shifts the question of why some branches of the phylogenetic tree of plants have never evolved  $C_4$  photosynthesis to the question of why these branches never evolved the photorespiratory pump.

#### *Fixation of the $C_4$ photosynthetic trait*

The sequence of steps establishing a highly active  $C_4$  cycle in plants with a photorespiratory pump was confirmed by the analysis of  $C_3$ – $C_4$  intermediate species from the genus *Flaveria* (Heckmann *et al.*, 2013; Mallmann *et al.*, 2014). The sequence, and the seeming inevitability, of  $C_4$  evolution once the pump is established provokes two questions: Can the  $C_4$  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  $C_4$  trait. Rubisco activity in the mesophyll may be lost gradually as PEPC activity increases but cannot be lost completely unless the  $C_4$  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  $C_4$  cycle activity increases. The photorespiratory pump will continue running until Rubisco in the mesophyll is completely shut off. This can be observed in the  $C_4$ -like species *Flaveria brownii*, which shows a reduction of mesophyll Rubisco together with other Calvin–Benson cycle and some photorespiratory 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  $C_4$  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 C<sub>4</sub> species (Bräutigam *et al.*, 2011; Gowik *et al.*, 2011; Bräutigam *et al.*, 2014). Artificial reduction of C<sub>4</sub> cycle activity to the point where it can no longer maintain sufficient CO<sub>2</sub> 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 CO<sub>2</sub> concentrations. Evolution has manoeuvred C<sub>4</sub> 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 C<sub>4</sub> species may now be limited by nutrients other than carbon.

Total Rubisco expression is also drastically reduced in C<sub>4</sub> 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 C<sub>4</sub> species (Sage, 2004). The fact that this did not happen in all C<sub>4</sub> species implies that optimization of nitrogen use was not a general selective pressure for the evolution of C<sub>4</sub> 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 C<sub>3</sub>–C<sub>4</sub> intermediate plants and no direct ancestry to C<sub>4</sub> species, meaning most of the intermediate species proceeded to C<sub>4</sub>. Assuming that all recent C<sub>4</sub> 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 C<sub>4</sub> 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 C<sub>4</sub>. This could surely be true for the extant *Flaveria* species with photorespiratory pumps because the genus *Flaveria* represents the youngest C<sub>4</sub> 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 C<sub>4</sub> 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 C<sub>4</sub> cycle, which prevents them from ever entering the slippery slope to C<sub>4</sub> photosynthesis. That might have happened as the C<sub>4</sub> 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 C<sub>4</sub> cycle under certain circumstances (Mallmann *et al.*, 2014). Third, the idea that the establishment of a low activity C<sub>4</sub> cycle automatically leads to the establishment of the full C<sub>4</sub> 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 C<sub>4</sub> photosynthesis will distinguish between these alternative hypotheses.

## Summary

The evolution of C<sub>4</sub> 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 C<sub>4</sub> cycle to replenish nitrogen after the evolution of the photorespiratory pump immediately puts the species on the slippery slope towards C<sub>4</sub> 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, C<sub>4</sub> is a dead end of evolution, albeit a very productive one.

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