Journal of Experimental Botany, Vol. 67, No. 10 pp. 2953–2962, 2016
doi:10.1093/jxb/erw056 Advance Access publication 22 February 2016

REVIEW PAPER

Photorespiration connects C_3 and C_4 photosynthesis

Andrea Bräutigam[1,2,3] and Udo Gowik[4]*

[1] Institute of Plant Biochemistry, Universitätsstrasse 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, Universitätsstrasse 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

C_4 plants evolved independently more than 60 times from C_3 ancestors. C_4 photosynthesis is a complex trait and its evolution from the ancestral 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 C_4 pathway is more efficient than C_3 photosynthesis because it increases the CO_2 concentration around the major CO_2 fixating enzyme Rubisco. The oxygenase reaction and, accordingly, photorespiration are largely suppressed. In the present review we describe a scenario for C_4 evolution that not only includes the avoidance of photorespiration as the major driving force for 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 C_3 to C_4.

Key words: 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). 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. 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 C_4 cycle within a single cell (Edwards *et al.*, 2004).

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, C_4 plants also need less Rubisco, the most abundant enzyme in plant leaves, leading to nitrogen savings. The 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.

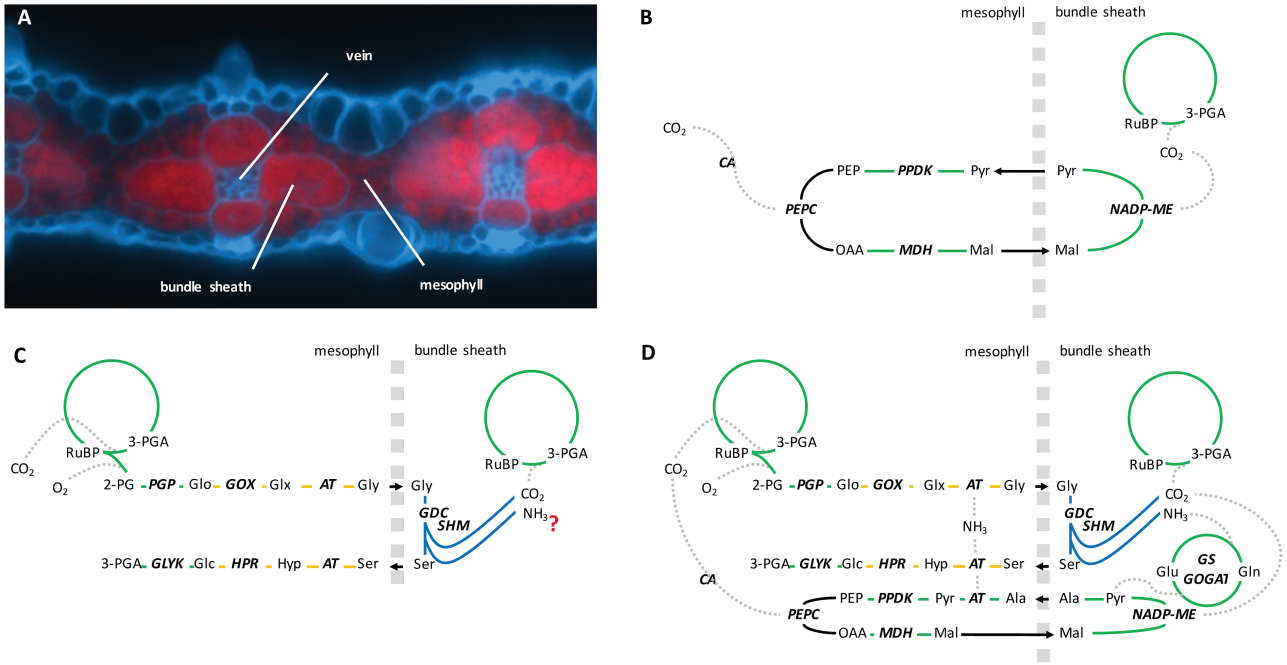

Fig. 1 C_4 photosynthesis and the photorespiratory pump. (**A**) Cross section from a leaf of *Megathyrsus maximus*. A typical C_4 leaf with bundle sheath and mesophyll cells surrounding the veins in layers. Chlorophyll fluorescence (red) was visualized by exciting fluorescence with 460–500 nm and monitoring 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_4 pathway. (**C**) Schematic representation of the photorespiratory pump. (**D**) Mechanistic interaction between the photorespiratory pump and the C_4 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; PPDK, pyruvate orthophosphate dikinase; Pyr, pyruvate; RuBP, ribulose-1,5-bisphosphate; Ser, serine; SHM, serine hydroxymethyltransferase; 2-PG, 2-phosphoglycolate; 3-PGA, 3-phospoglycerate.

These are transferred to the bundle sheath cells where CO_2 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 CO_2 released in the bundle sheath is re-fixed by Rubisco, which is exclusively located in the bundle sheath cells in C_4 plants (Fig. 1B) (Hatch, 1987).

C_4 photosynthesis evolved independently more than 60 times within the angiosperms (Sage *et al.*, 2011). This makes C_4 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_4 plants are often thinner than those of C_3 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_4 photosynthesis requires the simultaneous presence of its anatomical and biochemical subtraits. *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_4 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_3 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_4 evolution was proposed (Monson, 1999) and greatly refined in the following years (Sage, 2004; Sage *et al.*, 2012). Modelling of C_4 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_4 photosynthetic pathway. On the one hand, the reduction of photorespiration was one of the driving forces behind C_4 evolution. On the other hand, all of the models of C_4 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_2 pump that relocates the photorespiratory CO_2 release to the bundle sheath cells is an important intermediate step towards the C_4 cycle. This photorespiratory CO_2 pump is also termed C_2 photosynthesis because the two-carbon compound glycine serves as a CO_2 transport metabolite (Fig. 1C; briefly, photorespiration is partitioned between two cell types with decarboxylation of glycine occurring mainly in one type, thereby enriching CO_2 at the site of this decarboxylation). Plants that use the photorespiratory pump (or C_2 photosynthesis) are often termed C_3–C_4 intermediates owing to their physiological properties.

This review considers selective pressures, deduced from the properties of recent C_3–C_4 intermediate and C_4 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_3–C_4 intermediate and C_4 species as we observe them today.

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

The vast majority of C_4 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.

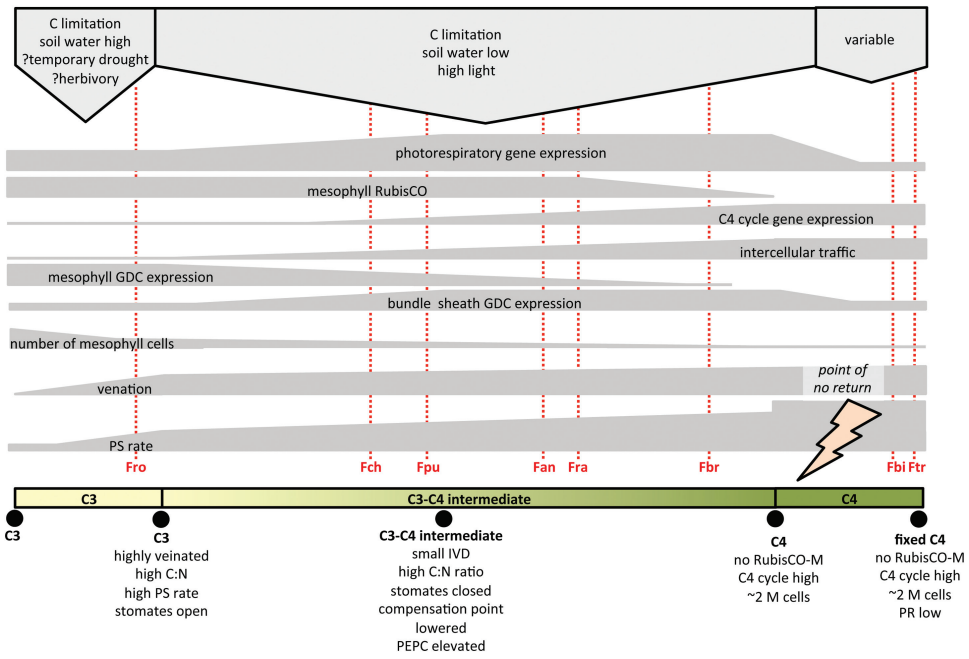

Fig. 2 The trajectory towards C_4. Abbreviations: C, carbon; GDC, glycine decarboxylase; IVD, interveinal distance; M, mesophyll; N, nitrogen; PEPC, phosphoenolpyruvate carboxylase; PS, photosynthesis; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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, Micraioideae, 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_2 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_2 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_2 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_4 origins post-date the atmospheric decline of CO_2 30 million years ago, some by over 20 million years, limited evidence indicates C_4 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_4 photosynthesis as well the evolution prior to the CO_2 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_4 trait (Fig. 2).

The photorespiratory pump is one possibility for plants to deal with limited CO_2 because it allows more efficient carbon assimilation (Ku *et al.*, 1983; Monson *et al.*, 1984). While the existence of so called C_3–C_4 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_3–C_4 intermediates are characterized by a leaf anatomy that is intermediate to C_3 and C_4 species, with large, organelle-rich bundle sheath cells and close vein spacing (Edwards and Ku, 1987). Their apparent rate of photorespiration and the CO_2 compensation point is between the values for C_3 and C_4 plants (Edwards and Ku, 1987). The analysis of the C_3–C_4 intermediate *Moricandia arvensis* demonstrated that these intermediate physiological parameters depend on the existence of a photorespiratory CO_2 pump (Rawsthorne *et al.*, 1988a, b) and confirmed earlier assumptions (Edwards and Ku, 1987; Monson *et al.*, 1984). A photorespiratory CO_2 pump was also found to be active in other C_3–C_4 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_2 release is exclusively localized in one cell type, increasing the CO_2 concentration in that cell type up to 3-fold (Keerberg *et al.*, 2014). Rubisco can work much more efficiently under these CO_2-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_2 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_4 (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_3 *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_2 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_3–C_4 intermediates and becomes zero only in the true C_4 *Flaveria* species, including the pseudogenization of the GDC-P copy expressed everywhere (Schulze *et al.*, 2013). It is plausible that the photorespiratory CO_2 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_2 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_2 concentration around Rubisco in the bundle sheath, it would become more engaged in CO_2 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_2 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 (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 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_4 species (Bräutigam *et al.*, 2011; Gowik *et al.*, 2011; Bräutigam *et al.*, 2014). Artificial reduction of C_4 cycle activity to the point where it can no longer maintain sufficient CO_2 enrichment by mutation (Dever *et al.*, 1997) or by transgenic approaches (Pengelly *et al.*, 2012) causes phenotypes reminiscent of photorespiratory mutants and, consequentially, can be alleviated by growth in elevated CO_2 concentrations. Evolution has manoeuvred C_4 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_4 species may now be limited by nutrients other than carbon.

Total Rubisco expression is also drastically reduced in C_4 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_4 species (Sage, 2004). The fact that this did not happened in all C_4 species implies that optimization of nitrogen use was not a general selective pressure for the evolution of C_4 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_3–C_4 intermediate plants and no direct ancestry to C_4 species, meaning most of the intermediate species proceeded to C_4. Assuming that all recent C_4 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_4 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_4. This could surely be true for the extant *Flaveria* species with photorespiratory pumps because the genus *Flaveria* represents the youngest C_4 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_4 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_4 cycle, which prevents them from ever entering the slippery slope to C_4 photosynthesis. That might have happened as the C_4 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_4 cycle under certain circumstances (Mallmann *et al.*, 2014). Third, the idea that the establishment of a low activity C_4 cycle automatically leads to the establishment of the full C_4 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_4 photosynthesis will distinguish between these alternative hypotheses.

Summary

The evolution of C_4 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_4 cycle to replenish nitrogen after the evolution of the photorespiratory pump immediately puts the species on the slippery slope towards C_4 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_4 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

Agren GI, Wetterstedt JAM, Billberger MFK. 2012. Nutrient limitation on terrestrial plant growth - modeling the interaction between nitrogen and phosphorus. New Phytologist **194,** 953–960.

Bauwe H. 1984. Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose-1, 5-bisphosphate carboxylase/oxygenase in leaves of C_3, C_4, and C_3–C_4 intermediate species of *Flaveria* (Asteraceae). Biochemie und Physiologie der Pflanzen **179,** 253–268.

Bauwe H, Kolukisaoglu U. 2003. Genetic manipulation of glycine decarboxylation. Journal of Experimental Botany **54,** 1523–1535.

Bauwe H, Hagemann M, Fernie AR. 2010. Photorespiration: players, partners and origin. Trends in Plant Science **15,** 330–336.

Bauwe H. 2011. Photorespiration: The bridge to C_4 photosynthesis. In: Raghavendra AS, Sage RF, eds. C_4 photosynthesis and related CO_2 concentrating mechanisms . Springer, Netherlands: Dordrecht, 81–108.

Botha CEJ. 1992. Plasmodesmatal distribution, structure and frequency in relation to assimilation in C_3 And C_4 grasses in Southern Africa. Planta **187,** 348–358.

Bräutigam A, Kajala K, Wullenweber J, Sommer M, Gagneul D, Weber KL, Carr KM, Gowik U, Mass J, Lercher MJ, Westhoff P, Hibberd JM, Weber APM. 2011. An mRNA blueprint for C_4 photosynthesis derived from comparative transcriptomics of closely related C_3 and C_4 species. Plant Physiology **155,** 142–156.

Bräutigam A, Schliesky S, Külahoglu C, Osborne CP, Weber APM. 2014. Towards an integrative model of C_4 photosynthetic subtypes: insights from comparative transcriptome analysis of NAD-ME, NADP-ME, and PEP-CK C_4 species. Journal of Experimental Botany **65,** 3579–3593.

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

Cheng S-H, Moore BD, Wu J, Edwards GE, Ku MSB. 1989. Photosynthetic plasticity in *Flaveria brownii*: growth irradiance and the expression of C_4 photosynthesis. Plant Physiology **89,** 1129–1135.

Christin PA, Osborne CP, Sage RF, Arakaki M, Edwards EJ. 2011a. C_4 eudicots are not younger than C_4 monocots. Journal of Experimental Botany **62,** 3171–3181.

Christin PA, Sage TL, Edwards EJ, Ogburn RM, Khoshravesh R, Sage RF. 2011b. Complex evolutionary transitions and the significance of C_3–C_4 intermediate forms of photosynthesis in Molluginaceae. Evolution **65,** 643–660.

Christin PA, Osborne CP, Chatelet DS, Columbus JT, Besnard G, Hodkinson TR, Garrison LM, Vorontsova MS, Edwards EJ. 2013. Anatomical enablers and the evolution of C_4 photosynthesis in grasses. Proceedings of the National Academy of Sciences of the United States of America **110,** 1381–1386.

Christin PA, Osborne CP. 2014. The evolutionary ecology of C_4 plants. New Phytologist **204,** 765–781.

Christin PA, Spriggs E, Osborne CP, Stromberg CA, Salamin N, Edwards EJ. 2014. Molecular dating, evolutionary rates, and the age of the grasses. Systematic Biology **63,** 153–165.

Dal'Molin CGD, Quek LE, Palfreyman RW, Brumbley SM, Nielsen LK. 2010. C4GEM, a genome-scale metabolic model to study C_4 plant metabolism. Plant Physiology **154,** 1871–1885.

Dengler NG, Nelson T. 1999. Leaf structure and development in C_4 plants. In: Rowan FS, Russell KM, eds. C_4 plant biology . Academic Press: San Diego, 133–172.

Dever LV, Blackwell RD, Fullwood NJ, Lacuesta M, Leegood RC, Onek LA, Pearson M, Lea PJ. 1995. The isolation and characterization of mutants of the C_4 photosynthetic pathway. Journal of Experimental Botany **46,** 1363–1376.

Dever LV, Bailey KJ, Leegood RC, Lea PJ. 1997. Control of photosynthesis in *Amaranthus edulis* mutants with reduced amounts of PEP carboxylase. Australian Journal of Plant Physiology **24,** 469–476.

Döring F, Streubel M, Bräutigam A, Gowik U. 2016. Most photorespiratory genes are preferentially expressed in the bundle sheath cells of the C_4 grass *Sorghum bicolor*. Journal of Experimental Botany accepted for publication MS ID#: JEXBOT/2015/166421.

Edwards EJ, Osborne CP, Stromberg CA, Smith SA, Consortium CG, Bond WJ, Christin PA, Cousins AB, Duvall MR, Fox DL, Freckleton RP, Ghannoum O, Hartwell J, Huang Y, Janis CM, Keeley JE, Kellogg EA, Knapp AK, Leakey AD, Nelson DM, Saarela JM, Sage RF, Sala OE, Salamin N, Still CJ, Tipple B. 2010. The origins of C_4 grasslands: integrating evolutionary and ecosystem science. Science **328,** 587–591.

Edwards GE, Ku MS. 1987. Biochemistry of C_3–C_4 intermediates. In: Hatch MD, Boardman NK, eds. The biochemistry of plants . Academic Press: New York, 275–325.

Edwards GE, Franceschi VR, Voznesenskaya EV. 2004. Single-cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annual Review of Plant Biology **55,** 173–196.

Engelmann S, Wiludda C, Burscheidt J, Gowik U, Schlue U, Koczor M, Streubel M, Cossu R, Bauwe H, Westhoff P. 2008. The gene for the P-subunit of glycine decarboxylase from the C_4 species *Flaveria trinervia*: analysis of transcriptional control in transgenic *Flaveria bidentis* (C_4) and Arabidopsis (C_3). Plant Physiology **146,** 1773–1785.

Evert RF, Eschrich W, Heyser W. 1977. Distribution and structure of plasmodesmata in mesophyll and bundle-sheath cells of *Zea Mays* L. Planta **136,** 77–89.

Evert RF, Russin WA, Bosabalidis AM. 1996. Anatomical and ultrastructural changes associated with sink-to-source transition in developing maize leaves. International Journal Of Plant Sciences **157,** 247–261.

Fahnenstich H, Saigo M, Niessen M, Zanor MI, Andreo CS, Fernie AR, Drincovich MF, Flugge UI, Maurino VG. 2007. Alteration of organic acid metabolism in Arabidopsis overexpressing the maize C_4NADP-malic enzyme causes accelerated senescence during extended darkness. Plant Physiology **145,** 640–652.

Farquhar GD, Caemmerer SV, Berry JA. 1980. A biochemical-model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta **149,** 78–90.

Fouracre JP, Ando S, Langdale JA. 2014. Cracking the Kranz enigma with systems biology. Journal of Experimental Botany **65,** 3327–3339.

Furbank RT. 2011. Evolution of the C_4 photosynthetic mechanism: are there really three C_4 acid decarboxylation types? Journal of Experimental Botany **62,** 3103–3108.

Gowik U, Bräutigam A, Weber KL, Weber APM, Westhoff P. 2011. Evolution of C_4 photosynthesis in the genus *Flaveria*: how many and which genes does it take to make C_4? The Plant Cell **23,** 2087–2105.

Griffiths H, Weller G, Toy LF, Dennis RJ. 2013. You're so vein: bundle sheath physiology, phylogeny and evolution in C_3 and C_4 plants. Plant, Cell & Environment **36,** 249–261.

Hatch MD. 1987. C_4 Photosynthesis - a unique blend of modified biochemistry, anatomy and ultrastructure. Biochimica et Biophysica Acta **895,** 81–106.

Hausler RE, Rademacher T, Li J, Lipka V, Fischer KL, Schubert S, Kreuzaler F, Hirsch HJ. 2001. Single and double overexpression of C_4-cycle genes had differential effects on the pattern of endogenous enzymes, attenuation of photorespiration and on contents of UV protectants in transgenic potato and tobacco plants. Journal of Experimental Botany **52,** 1785–1803.

Hausler RE, Hirsch HJ, Kreuzaler F, Peterhansel C. 2002. Overexpression of C_4-cycle enzymes in transgenic C_3 plants: a biotechnological approach to improve C_3-photosynthesis. Journal of Experimental Botany **53,** 591–607.

Heckmann D, Schulze S, Denton A, Gowik U, Westhoff P, Weber APM, Lercher MJ. 2013. Predicting C_4 photosynthesis evolution: modular, individually adaptive steps on a Mount Fuji fitness landscape. Cell **153,** 1579–1588.

Holaday AS, Brown RH, Bartlett JM, Sandlin EA, Jackson RC. 1988. Enzymic and photosynthetic characteristics of reciprocal F(1) hybrids of *Flaveria pringlei* (C_3) and *Flaveria brownii* (C_4-like species). Plant Physiology **87,** 484–490.

Hylton C, Rawsthorne S, Smith A, Jones DA, Woolhouse H. 1988. Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C_3–C_4 intermediate species. Planta **175,** 452–459.

John CR, Smith-Unna RD, Woodfield H, Covshoff S, Hibberd JM. 2014. Evolutionary convergence of cell-specific gene expression in independent lineages of C_4 grasses. Plant Physiology **165,** 62–75.

Keerberg O, Parnik T, Ivanova H, Bassuner B, Bauwe H. 2014. C_2 photosynthesis generates about 3-fold elevated leaf CO_2 levels in the C_3–C_4 intermediate species *Flaveria pubescens*. Journal of Experimental Botany **65,** 3649–3656.

Kennedy RA, Laetsch WM. 1974. Plant species intermediate for C_3, C_4 photosynthesis. Science **184,** 1087–1089.

Körner C. 2015. Paradigm shift in plant growth control. Current Opinion in Plant Biology **25,** 107–114.

Krogmann DW, Jagendorf AT, Avron M. 1959. Uncouplers of spinach chloroplast photosynthetic phosphorylation. Plant Physiology **34,** 272–277.

Ku MSB, Monson RK, Littlejohn RO, Nakamoto H, Fisher DB, Edwards GE. 1983. Photosynthetic characteristics of C_3–C_4 intermediate flaveria species: 1. Leaf anatomy, photosynthetic responses to O_2 and CO_2, and activities of key enzymes in the C_3 and C_4 pathways. Plant Physiology **71,** 944–948.

Kulahoglu C, Denton AK, Sommer M, Mass J, Schliesky S, Wrobel TJ, Berckmans B, Gongora-Castillo E, Buell CR, Simon R, De Veylder L, Bräutigam A, Weber AP. 2014. Comparative transcriptome atlases reveal altered gene expression modules between two Cleomaceae C_3 and C_4 plant species. The Plant Cell **26,** 3243–3260.

Li P, Ponnala L, Gandotra N, Wang L, Si Y, Tausta SL, Kebrom TH, Provart N, Patel R, Myers CR, Reidel EJ, Turgeon R, Liu P, Sun Q, Nelson T, Brutnell TP. 2010. The developmental dynamics of the maize leaf transcriptome. Nature Genetics **42,** 1060–1067.

Lundgren MR, Osborne CP, Christin P-A. 2014. Deconstructing Kranz anatomy to understand C_4 evolution. Journal of Experimental Botany **65,** 3357–3369.

Majeran W, Friso G, Ponnala L, Connolly B, Huang MS, Reidel E, Zhang CK, Asakura Y, Bhuiyan NH, Sun Q, Turgeon R, van Wijk KJ. 2010. Structural and metabolic transitions of C_4 leaf development and differentiation defined by microscopy and quantitative proteomics in maize. The Plant Cell **22,** 3509–3542.

Mallmann J, Heckmann D, Braeutigam A, Lercher MJ, Weber APM, Westhoff P, Gowik U. 2014. The role of photorespiration during the evolution of C_4 photosynthesis in the genus Flaveria. Elife **3,** e02478.

Monson RK, Edwards GE, Ku MSB. 1984. C_3–C_4 intermediate photosynthesis in plants. Bioscience **34,** 563–574.

Monson RK. 1989. The relative contributions of reduced photorespiration, and improved water-and nitrogen-use efficiencies, to the advantages of C_3–C_4 intermediate photosynthesis in Flaveria. Oecologia **80,** 215–221.

Monson RK. 1999. The origins of C_4 genes and evolutionary pattern in the C_4 metabolic phenotype. In: Sage RF, Monson RK, eds. C_4 plant biology . Academic Press: San Diego, 377–410.

Morgan CL, Turner SR, Rawsthorne S. 1993. Coordination of the cell-specific distribution of the four subunits of glycine decarboxylase and of serine hydroxymethyltransferase in leaves of C_3–C_4 intermediate species from different genera. Planta **190,** 468–473.

Muhaidat R, Sage TL, Frohlich MW, Dengler NG, Sage RF. 2011. Characterization of C_3–C_4 intermediate species in the genus *Heliotropium* L. (Boraginaceae): anatomy, ultrastructure and enzyme activity. Plant, Cell & Environment **34,** 1723–1736.

Nardini A, Peda G, La Rocca N. 2012. Trade-offs between leaf hydraulic capacity and drought vulnerability: morpho-anatomical bases, carbon costs and ecological consequences. New Phytologist **196,** 788–798.

Niinemets U, Portsmuth A, Tena D, Tobias M, Matesanz S, Valladares F. 2007. Do we underestimate the importance of leaf size in plant economics? Disproportional scaling of support costs within the spectrum of leaf physiognomy. Annals Of Botany **100,** 283–303.

Pengelly JJL, Kwasny S, Bala S, Evans JR, Voznesenskaya EV, Koteyeva NK, Edwards GE, Furbank RT, von Caemmerer S. 2011. Functional analysis of corn husk photosynthesis. Plant Physiology **156,** 503–513.

Pengelly JJL, Tan J, Furbank RT, von Caemmerer S. 2012. Antisense reduction of NADP-malic enzyme in *Flaveria bidentis* reduces flow of CO_2 through the C_4 cycle. Plant Physiology **160,** 1070–1080.

Pick TR, Bräutigam A, Schlüter U, Denton AK, Colmsee C, Scholz U, Fahnenstich H, Pieruschka R, Rascher U, Sonnewald U, Weber AP. 2011. Systems analysis of a maize leaf developmental gradient redefines the current C_4 model and provides candidates for regulation. The Plant Cell **23,** 4208–4220.

Pinto H, Tissue DT, Ghannoum O. 2011. *Panicum milioides* (C_3–C_4) does not have improved water or nitrogen economies relative to C_3 and C_4 congeners exposed to industrial-age climate change. Journal of Experimental Botany **62,** 3223–3234.

Prasad V, Stromberg CA, Leache AD, Samant B, Patnaik R, Tang L, Mohabey DM, Ge S, Sahni A. 2011. Late Cretaceous origin of the rice tribe provides evidence for early diversification in Poaceae. Nature Communications **2,** 480.

Raines CA. 2011. Increasing photosynthetic carbon assimilation in C_3 plants to improve crop yield: current and future strategies. Plant Phys*iology* **155,** 36–42.

Rajendrudu G, Prasad JS, Das VS. 1986. C_3–C_4 Intermediate species in *Alternanthera* (Amaranthaceae): leaf anatomy, CO_2 compensation point, net CO_2 exchange and activities of photosynthetic enzymes. Plant Physiology **80,** 409–414.

Rawsthorne S, Hylton CM, Smith AM, Woolhouse HW. 1988a. Photorespiratory metabolism and immunogold localization of photorespiratory enzymes in leaves of C_3 and C_3–C_4 intermediate species of Moricandia. Planta **173,** 298–308.

Rawsthorne S, Hylton CM, Smith AM, Woolhouse HW. 1988b. Distribution of photorespiratory enzymes between bundle-sheath and mesophyll cells in leaves of the C_3–C_4 intermediate species *Moricandia arvensis* (L.) DC. Planta **176,** 527–532.

Rawsthorne S. 1992. C_3–C_4 intermediate photosynthesis: linking physiology to gene expression. The Plant Journal **2,** 267–274.

Roth-Nebelsick A, Uhl D, Mosbrugger V, Kerp H. 2001. Evolution and function of leaf venation architecture: a review. Annals of Botany **87,** 553–566.

Sack L, Tyree MT, Holbrook NM. 2005. Leaf hydraulic architecture correlates with regeneration irradiance in tropical rainforest trees. New Phytologist **167,** 403–413.

Sack L, Holbrook NM. 2006. Leaf hydraulics. Annual Review of Plant Biology **57,** 361–381.

Sack L, Scoffoni C, McKown AD, Frole K, Rawls M, Havran JC, Tran H, Tran T. 2012. Developmentally based scaling of leaf venation architecture explains global ecological patterns. Nature Communications **3,** 837.

Sack L, Scoffoni C. 2013. Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. New Phytologist **198,** 983–1000.

Sage RF. 2004. The evolution of C_4 photosynthesis. New Phytologist **161,** 341–370.

Sage RF, Christin P-A, Edwards EJ. 2011. The C_4 plant lineages of planet Earth. Journal of Experimental Botany **62,** 3155–3169.

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C_4 photosynthesis. Annual Review of Plant Biology **63,** 19–47.

Sage TL, Busch FA, Johnson DC, Friesen PC, Stinson CR, Stata M, Sultmanis S, Rahman BA, Rawsthorne S, Sage RF. 2013. Initial events during the evolution of C_4 photosynthesis in C_3 species of *Flaveria*. Plant Physiology **163,** 1266–1276.

Scarpella E, Francis P, Berleth T. 2004. Stage-specific markers define early steps of procambium development in Arabidopsis leaves and correlate termination of vein formation with mesophyll differentiation. Development **131,** 3445–3455.

Scarpella E, Meijer AH. 2004. Pattern formation in the vascular system of monocot and dicot plant species. New Phytologist **164,** 209–242.

Scheres B, Xu L. 2006. Polar auxin transport and patterning: grow with the flow. Genes & Development **20,** 922–926.

Schulze S, Mallmann J, Burscheidt J, Koczor M, Streubel M, Bauwe H, Gowik U, Westhoff P. 2013. Evolution of C_4 photosynthesis in the genus *Flaveria*: establishment of a photorespiratory CO_2 pump. The Plant Cell **25,** 2522–2535.

Slewinski TL, Baker RF, Stubert A, Braun DM. 2012. Tie-dyed2 encodes a callose synthase that functions in vein development and affects symplastic trafficking within the phloem of maize leaves. Plant Physiology **160,** 1540–1550.

Slewinski TL. 2013. Using evolution as a guide to engineer Kranz-type C_4 photosynthesis. Frontiers in Plant Science **4,** 212.

Sowinski P, Szczepanik J, Minchin PEH. 2008. On the mechanism of C_4 photosynthesis intermediate exchange between Kranz mesophyll and bundle sheath cells in grasses. Journal of Experimental Botany **59,** 1137–1147.

van der Ploeg RR, Bohm W, Kirkham MB. 1999. On the origin of the theory of mineral nutrition of plants and the law of the minimum. Soil Science Society of America Journal **63,** 1055–1062.

Vogan PJ, Sage RF. 2011. Water-use efficiency and nitrogen-use efficiency of C_3–C_4 intermediate species of *Flaveria* Juss. (Asteraceae). Plant, Cell & Environment **34,** 1415–1430.

von Caemmerer S. 1989. A model of photosynthetic CO_2 assimilation and carbon-isotope discrimination in leaves of certain C_3–C_4 intermediates. Planta **178,** 463–474.

von Caemmerer S. 2000. Biochemical models of leaf photosynthesis . CSIRO Publishing: Collingwood.

Wang P, Kelly S, Fouracre JP, Langdale JA. 2013. Genome-wide transcript analysis of early maize leaf development reveals gene cohorts associated with the differentiation of C_4 Kranz anatomy. The Plant Journa*l* **75,** 656–670.

Wang Y, Bräutigam A, Weber AP, Zhu XG. 2014. Three distinct biochemical subtypes of C_4 photosynthesis? A modelling analysis. Journal of Experimental Botany **65,** 3567–3578.

Wessinger ME, Edwards GE, Ku MSB. 1989. Quantity and kinetic properties of ribulose 1,5-bisphosphate carboxylase in C_3, C_4, and C_3–C_4 intermediate species of *Flaveria* (Asteraceae). Plant and Cell Physiology **30,** 665–671.

Westhoff P, Gowik U. 2004. Evolution of C_4 phosphoenolpyruvate carboxylase. Genes and proteins: a case study with the genus *Flaveria*. Annals of Botany **93,** 13–23.

Williams BP, Johnston IG, Covshoff S, Hibberd JM. 2013. Phenotypic landscape inference reveals multiple evolutionary paths to C_4 photosynthesis. Elife **2,** e00961.