The Road to C₄ Photosynthesis: Evolution of a Complex Trait via Intermediary States

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C₄ photosynthesis enables high photosynthetic energy conversion efficiency as well as high nitrogen and water use efficiencies. Given the multitude of biochemical, structural and molecular changes in comparison with C₃ photosynthesis, it appears unlikely that such a complex trait would evolve in a single step. C₄ photosynthesis is therefore believed to have evolved from the ancestral C₃ state via intermediary stages. Consequently, the identification and detailed characterization of plant species representing transitory states between C₃ and C₄ is important for the reconstruction of the sequence of evolutionary events, especially since C₄ evolution occurred in very different phylogenetic backgrounds. There is also significant interest in engineering of C₄ or at least C₄-like elements into C₃ crop plants. A detailed and mechanistic understanding of C₃–C₄ intermediates is likely to provide guidance for the experimental design of such approaches. Here we provide an overview on the most relevant results obtained on C₃–C₄ intermediates to date. Recent knowledge gains in this field will be described in more detail. We thereby concentrate especially on biochemical and physiological work. Finally, we will provide a perspective and outlook on the continued importance of research on C₃–C₄ intermediates.

Keywords: C₄ photosynthesis • C₃–C₄ intermediate • Evolution • Photorespiration.

Abbreviations: CBC, Calvin–Benson cycle; BS, bundle sheath; GDC, glycine decarboxylase complex; M, mesophyll; NUE, nitrogen use efficiency; PGA, 3-phosphoglycerate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; WUE, water use efficiency; PEPC, phosphoenolpyruvate carboxylase; 2PG, 2-phosphoglyceric acid.

A Historical Perspective on the Discovery of C₃–C₄ Intermediate Species

The main principle of C₄ photosynthesis was reported 50 years ago by Hatch and Slack (1966): after short-term exposure of illuminated sugarcane leaf segments to ¹⁴CO₂ for 1 s, the vast majority of the label (93%) was detected in C₄ dicarboxylic acids (malate, aspartate and oxaloacetate), and not in 3-phosphoglyceric acid (3PGA), as would be the case in C₃ plants. After 20 s, approximately 30% of the ¹⁴C-label was detectable in 3PGA, whereas the proportion of label in C₄ acids declined, which indicated that the radiolabeled carbon was transferred via a C₄ compound to the classical C₃ Calvin–Benson cycle (CBC). Later work revealed that C₄ photosynthesis involves a spatial separation of the two consecutive carboxylation reactions: After primary fixation of carbon by phosphoenolpyruvate carboxylase (PEPC), the resulting C₄ compound is transported to the site of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) reaction, where CO₂ is released again by decarboxylation of the C₄ acid (Hatch 1987; Fig. 1D). By concentrating CO₂ at the site of RuBisCO via the C₄ biochemical carbon pump, the oxygenation reaction of RuBisCO, which leads to the formation of the toxic metabolic intermediate 2-phosphoglyceric acid (2PG), is suppressed. Detoxifying 2PG requires the photosynthetic cycle, which demands energy and leads to the loss of previously fixed CO₂. The high CO₂ concentration around RuBisCO achieved through C₄ photosynthesis suppresses the photorespiratory cycle, a situation that enables higher photosynthetic efficiency in C₄ plants and hence frequently also higher productivity than in their ancestral C₃ relatives, especially in sunny, hot and dry climates.

Within the angiosperms, C₄ photosynthesis evolved independently >60 times (Sage et al. 2012). In the majority of C₄ plants, the C₄ carbon-concentrating mechanism and the CBC are distributed over two distinct leaf cell types, the mesophyll (M) and the bundle sheath (BS) cells, with a few exceptions that employ a single-celled C₄ mechanism (Voznesenskaya et al. 2001). Reaping the benefits of C₄ photosynthesis therefore depends on numerous leaf anatomical and biochemical re-adjustments. Traditionally, C₄ plants are distinguished from C₃ plants by the following characteristic features: (i) short-term labeling with ¹³C leads to initial incorporation of the label into the C₄ compounds oxaloacetate, malate and, frequently also, aspartate; (ii) a very low CO₂ compensation point; (iii) reduced discrimination against the heavy carbon isotope ¹³C; (iv) anatomical changes associated with narrow vein spacing and increased numbers of chloroplasts in enlarged BS cells; (v) high abundance and cell-specific expression of C₄ shuttle enzymes; and (vi) reduced abundance and altered cell-specific expression of CBC and photorespiratory enzymes (reviewed in Sage et al. 2012).

Despite the frequent convergent evolution of C₄ species from C₃ ancestors, it is unlikely that such a complex trait would have evolved in just one single giant step. The existence
Fig. 1 Evolution from C₃ to C₄ photosynthesis. (A) C₃ photosynthesis: the Calvin–Benson cycle and photorespiration work independently in both cell types. (B) Basic or early intermediate photosynthesis: the activity of the GDC has shifted towards the bundle sheath, activating CO₂ transport from the mesophyll to the bundle sheath by a photorespiratory glycine pump. In addition to CO₂, the GDC reaction also releases ammonium and consequently motivates N balancing mechanisms between the cells. This can be realized by transport of different metabolites; the model of Mallmann et al. (2014) suggests an alanine–pyruvate shuttle as one of the most likely solutions (the oxygenase reaction of Rubisco (continued))
of evolutionary intermediate forms was therefore expected and, indeed, in 1974, Kennedy and Laetsch presented *Mallugo verticillata* as the first candidate for a C₃–C₄ intermediate photosynthesis type (Kennedy and Laetsch 1974). The general leaf anatomy of *M. verticillata* appeared to be typical C₃, but the BS cells had numerous, well-developed chloroplasts similar to the typical picture in C₄. Photorespiratory rates of *M. verticillata* were in between the values of C₃ and C₄ species. Exposure to ¹⁴CO₂ resulted in almost equal labeling of C₃ and C₄ compounds as primary photosynthetic products.

At the time of initial discovery, the mechanisms enabling a mixture of C₃ and C₄-related features in *M. verticillata* were far from understood, and the assignment of this species as intermediary between C₃ and C₄ photosynthesis was merely a hypothesis. The interest in the discovery of possible C₃–C₄ intermediates was, however, stimulated, in particular because it was very clear from the beginning that knowledge gained from the study of intermediates would enable an understanding of the mechanisms underlying the evolution of C₄ photosynthesis. In the years following the description of *M. verticillata*, many more potential C₃–C₄ intermediates have been identified and studied. A survey of CO₂ compensation points in several hundred eudicot and monocot species identified the Brassicaceae *Moricandia arvensis* and the grass specie *Panicum milioides* (= *Steinchisma hians*) as further C₃–C₄ intermediates (Krenzer et al. 1975). A comprehensive review of C₃–C₄ intermediates published in 1987 (Edwards and Ku 1987) already listed 22 potential C₃–C₄ species, most of them belonging to orders which also included C₄ species. Investigation of this increased variety of intermediates permitted the definition of common distinctive features: (i) a reduced CO₂ compensation point; (ii) a reduced sensitivity of photosynthesis to O₂; and (iii) BS cells with an increased number of centripetally arranged chloroplasts and mitochondria, as compared with C₃ species. However, the δ¹³C values of C₃–C₄ intermediates were found to be close to those of C₃ species, which clearly distinguishes the intermediates from *bona fide* C₄ plants (Edwards and Ku 1987). On the basis of the observed reduction in the apparent rate of photorespiration and high rates of light-dependent ¹⁴C labeling of glycine, Monson, Edwards and Ku (1984) proposed that glycine is transported from the M to the BS cells, where CO₂ released from the glycine decarboxylase reaction would be refixed by the CBC, thereby establishing a glycine-based mechanism for pumping carbon into BS cells (Monson et al. 1984, Edwards and Ku 1987). This model implies that the majority of glycine-decarboxylating activity in leaves would be located in BS cells, and not in M cells, as it is typical for C₃ species.

Experimental support for the proposed confinement of the glycine decarboxylase reaction to the BS cells was later provided in the intermediate *M. arvensis* by immunolocalization as well as enzyme activity assays in M- and BS-enriched extracts (Rawsthorne et al. 1988a, Rawsthorne et al. 1988b). These results led to the first experimentally confirmed C₃–C₄ photosynthesis model (Rawsthorne et al. 1988b). Absence of a functioning glycine decarboxylase complex (GDC) in the M cells causes glycine accumulation in the M, which drives diffusion of glycine to the BS, where its oxidative decarboxylation by the mitochondrial GDC system produces CO₂ (Fig. 1B). The close association of mitochondria and chloroplasts at the inner wall of BS cells enhances the potential for re-assimilation of photorespiratory CO₂, while at the same time increasing the concentration of CO₂ at the site of RuBisCO in BS cells. The products of the decarboxylation reaction, in particular serine, then need to move back to the M cells for re-establishment of C and N balance between the cells (Rawsthorne et al. 1988b).

In addition to this feature, that is common to all C₃–C₄ intermediate species studied to date, gradual differences were observed for the presence of other C₄-like features in various intermediates (Ku et al. 1983, Monson et al. 1986). This includes especially the abundance and cell specificity of PEPC and other typical C₄ enzymes, the ratio between primary ¹³C label recovered in C₃ and C₄ compounds, and a reduction in vein spacing. Analysis of these features in different C₃–C₄ intermediates led to the definition of the sequence of key events on the road from C₃ to C₄: (i) confinement of the GDC to the BS; (ii) increase of PEPC and C₄ cycle activity; (iii) complete shift of RuBiSCO activity to the BS cells; and (iv) finally the optimization of the system (Monson and Rawsthorne 2000, Sage 2004, Gowik
showed that C3–C4 intermediacy evolved more than once in \( \text{C}_3 \) species (Lyu et al. 2015). Detailed phylogenetic analyses revealed that C3–C4 intermediacy evolved more than once in the genus \( \text{Flaveria} \) and that the C3–C4 intermediates represent starting points for the evolution of C4-like and true C4 photosynthesis (McKown et al. 2005, Lyu et al. 2015). This made \( \text{Flaveria} \) a preferred research object for the investigation of physiological, biochemical and phenotypic adaptation during evolution from C3 to C4 photosynthesis (Ku et al. 1983, Monson et al. 1986, McKown and Dengler 2007, Mallmann et al. 2014, Lyu et al. 2015). Hence the current model of the evolution of C4 photosynthesis is based to a large extent on work in \( \text{C}_3 \), C3–C4 and C4 \( \text{Flaveria} \) species (Monson and Rawsthorne 2000, Sage 2004).

**Computational Modeling of the Evolution of C4 Photosynthesis from C3 Ancestors**

Recently, new insights into the evolution of C4 photosynthesis came from the application of different modeling approaches. This work capitalized on a mechanistic biochemical model of photosynthetic CO2 assimilation that was previously developed by von Caemmerer (1989, 2000). Using the well-characterized kinetic constants of RuBisCO and various gas exchange characteristics, this model accurately predicts the amount of CO2 assimilated per unit leaf area and time, given a defined CO2 concentration and amount of RuBisCO. The conceptual advance by Heckmann and colleagues (2013) was that they used this mechanistic model to simulate a fitness landscape for the transition from C3 to C4, defining the overall fitness gain as proportional to the amount of CO2 that can be fixed using a given quantity of RuBisCO per leaf area. In other words, Heckmann et al. (2013) assumed that higher assimilation rates with a given amount of RuBisCO, light and water equals higher fitness since more biomass can be gained with identical input of resources. The simulation included the following parameters: \( \xi \), the fraction of photorespiratory glycine moved for decarboxylation from M to BS cells; \( \beta \), the fraction of RuBisCO active sites in \( \text{M} \) and \( \text{BS} \); \( V_{\text{pmax}} \), the activity of the \( \text{C}_4 \) cycle expressed as maximal velocity of the committing enzymatic reaction, PEPC; \( K_{\text{ccar}} \), the maximal turnover rate of RuBisCO; \( g_o \), the BS gas conductance; and \( K_p \), the Michaelis–Menten constant of PEPC for its substrate phosphoenolpyruvate (PEP). These six parameters were varied systematically in computational simulations, and the resulting CO2 assimilation rates calculated using the mechanistic model, which resulted in a six-dimensional fitness landscape. Two main conclusions could be drawn from the model: first, the computational model predicts a modular sequence of events that is consistent with the succession of events predicted by the experimental studies (Monson and Rawsthorne 2000, Sage 2004, Leegood 2013). In agreement with previous phylogenetic analyses, it further supports the hypothesis that the identified C3–C4 intermediates indeed represent transitory stages on the path to C4. Importantly, the computational simulation predicts that in anatomically pre-conditioned plants the first step towards C4 photosynthesis is indeed the relocation of GDC activity from \( \text{M} \) to BS cells. Secondly, the path through the biochemical fitness landscape is smooth, without local minima or maxima, because there always exists at least one possible parameter change towards C4 that is associated with immediate fitness gain under the given environmental conditions.

The evolution of C4 photosynthesis is therefore predicted to be repeatable in different species and provides a convincing explanation for the phylogenetic diversity of C4 species. The robustness of C4 evolution in different phylogenetic backgrounds was tested in a Bayesian modeling approach by Williams and colleagues (2013). Their study includes 37 species from 22 genera, probably representing 18 distinct evolutionary origins of C3–C4 intermediacy. Their model confirmed the predicted sequence of key events on the evolutionary path to C4 but it also showed that considerable flexibility exists for the shaping and succession of adjusting steps concerning biochemistry as well as parallel changes in cellular structure and leaf anatomy. The flexibility of the various biochemical, cellular biological and anatomical parameters probably contributes to the facilitation of convergent evolution of the complex trait. In agreement with the phylogenetic and biochemical models, the Bayesian approach also places the described C3–C4 intermediates on the evolutionary trajectory from C3 to C4 (Williams et al. 2013).

Taken together, the computational studies indicate that C4 evolution is characterized by essential key events starting with the shift of GDC activity to the BS followed by a flexible succession of adjustment steps. The most recent findings on mechanistic realization of the key events on the path to C4 are summarized in the next paragraphs.

**Pre-Conditioning for C4 Photosynthesis**

Despite the predictable modular trajectory of C4 evolution presented above, C4 photosynthesis is still only present in approximately 3% of the angiosperms (Osborne and Sack 2012, R.F. Sage et al. 2011). Apparently several pre-conditions must be met before traveling the road to C4 evolution can be initiated. Environmental conditions have, of course, a major impact on these events. As evident from the computational models discussed above, C4 evolution would be promoted under conditions that cause high photorespiratory rates, in particular under low atmospheric CO2 and limiting water supply, in warm and...
open environments (Osborne and Sack 2012). C4 evolution further depends on certain anatomic and genetic pre-conditions. Within the angiosperms, C4 origins therefore cluster in some branches while they are absent in others (R.F. Sage et al. 2011). This is particularly obvious in the grasses, where all C4 species identified to date belong to the Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae (PACMAD) clade and none has been discovered yet in the Bambusoideae, Ehrhartioideae and Pooidae (BEP) clade, to which many of our most important crop species belong, such as wheat and rice. Analysis of leaf anotomical traits, such as vein spacing and size of BS cells, in 157 grass species in combination with phylogenetic and statistical analysis clearly associated C4 evolvability with the presence of anatomic enablers, in particular relatively large BS cells and low numbers of M cells between veins (Christin et al. 2013). These lead to increased proportions of vascular and BS tissue in relation to total leaf cross-sectional area (Christin et al. 2013). On the biochemical level, shifting GDC activity from M to the BS cells has been identified as a decisive initial event on the road to C4, but the successful implementation of a photorespiratory glycine shuttle obviously depends on a proper anatomic set-up of the leaf, given the need for transport of photorespiratory glycolate to a specific cell type in the leaf. That is, a low M to BS cell ratio is expected to promote the installation of a glycine-based photorespiratory carbon pump (Sage et al. 2014).

The genetic make-up of a particular plant species also either constrains or promotes C4 evolvability (Williams et al. 2012). C4 biochemistry depends on enzyme activities that are present in all C3 species, but during evolution of C4 become recruited into a new metabolic context, primarily by changes in enzyme abundance and cell specificity of enzyme expression. The presence of multiple copies of genes encoding these enzymes (and other factors required for C4) is therefore likely to be advantageous, so that one copy of the gene will continuously support the C3 housekeeping function whereas the second copy can be recruited to C4 metabolism without jeopardizing vital functions associated with the C3 ortholog. The prominent role of gene duplications as a genetic enabler of C4 evolution has recently been demonstrated as a prerequisite for the shift of GDC activity from the M to the BS in Flaveria (see below; Wiludda et al. 2012, Schulze et al. 2013).

Installation of a Photorespiratory Pump by Shift of GDC Activity to BS Cells: An Early and Essential Step on the Road to C4

According to our current knowledge, the number of C4 lineages and species on Earth by far outnumbers that of C3-C4 intermediates. R.F. Sage et al. (2011) estimate that we know about 7,500 C3 species, but they list only 43 intermediates. Because intermediates are slightly more difficult to detect (requiring gas exchange analysis), it is possible that the proportions presented above do not accurately mirror the true picture, but the currently available numbers and the proximity of the majority of C3-C4 intermediates to C4 species indicate that after the essential initial step is done, the likelihood of full C4 evolution increases significantly. This view is also supported by the computational models of C4 evolution discussed above (McKown et al. 2005, Heckmann et al. 2012, Williams et al. 2013, Mallmann et al. 2014). A detailed mechanistic understanding of the molecular events that enable the shift of GDC activity from M to BS cells is therefore of particular importance for understanding of the entire process, but also for evolutionary-inspired engineering approaches (Denton et al. 2013).

Given the complexity of the GDC system, modification of GDC activity by random mutations has a relatively high probability. The GDC multi-enzyme system consists of four different subunits, and manipulation of any of them has the potential to change the activity of the entire complex (Engel et al. 2007). Further targets of random mutations could be any entity that exhibits regulatory power over any of the subunits (Heckmann et al. 2013).

In the C3 Flaveria species F. pringlei and F. robusta, two copies of the GLDP subunit gene exist, GLDPA and GLDPB. GLDPA is already expressed in a BS-specific manner in C4 Flaveria species (Wiludda et al. 2012), while GLDPB is expressed in all leaf cells (Schulze et al. 2013). In the C3-C4 intermediate Flaveria species, the expression of the ubiquitously expressed GLDPB gene is down-regulated as a consequence of an altered promoter activity, which causes a shift of GDC activity towards BS cells because now the GLDPA gene is almost exclusively responsible for expressing the GLDP subunit of the GDC system. This disappearance of GLDPB expression from M cells proceeds gradually from C3 to C4 rather than abruptly, a factor that is perhaps crucial for the adjustment of the intercellular metabolism (Schulze et al. 2013). Later on in the evolutionary trajectory towards C4 in Flaveria, GLDPA gene expression is lost by pseudogenization, which leaves only the BS-specific GLDPA gene active. Surprisingly, GLDPB expression is not completely lost from M cells in fully evolved C4. This is due to low expression levels of GLDPA in M cells, which is the result of less stringent BS-specific expression of the C4 GLDPA promoter (Wiludda et al. 2012, Schulze et al. 2013). Apparently, low levels of active GDC in M cells are also essential for maintenance of basic cellular functions in the C4 species (Schulze et al. 2013). The presence of a BS-specific GLDPA gene already in C3 Flaveria species most probably is a powerful enabler for C4 evolution in this genus. For comparison, the Arabidopsis genome harbors two GLDPA genes with apparently redundant functions, which allows compensation when just one copy is lost (Engel et al. 2007). The evolutionary scenario in Flaveria is probably lineage specific and it will hence be required to unravel the mechanisms underlying cell-specific GDC expression in independent cases of C4 evolution in other phylogenetic groups.

The Photorespiratory Glycine Shuttle is Mechanistically Linked to C4 Evolution

The implementation of the photorespiratory glycine shuttle (in some publications also called the C2 shuttle or C2
photosynthesis) between M and BS cells of the early C_3–C_4 intermediates causes a metabolic imbalance between the two cell types. In the initial description of the process, Rawsthorne et al. (1988b) already pointed out that one of the downstream products of the glycine decarboxylation reaction would need to move back to the M for replacement of the carbon lost from the CBC by oxygenation. The GDC reaction in conjunction with serine hydroxymethyltransferase (SHMT; Voll et al. 2006) uses two molecules of glycine for the production of one molecule of serine. In addition, CO_2 and ammonium are released. If we now assume that serine is the product of the GDC/SHMT reaction that is transported back to the M cells, continuous operation of the photosynthetic glycine shuttle would therefore also require re-balancing of N metabolism between the cells because for two units of N in the form of glycine that are imported into BS cells only one unit in the form of serine is returned. This potential N imbalance was already mentioned in the original report by Rawsthorne et al. (1988b), but until recently did not receive much attention. Mallmann et al. (2014) applied a metabolic modeling approach to explore potential routes for re-adjustment of the N balance and to identify candidate metabolites and enzymes involved in such an N shuttle.

When the model was optimized for maximization of leaf biomass production and minimization of the sum of all fluxes, the model suggests three alternative shuttle mechanisms: (i) glutamate/2-oxoglutarate; (ii) alanine/pyruvate (Fig. 1B); and (iii) aspartate/malate. Introduction of even low PEPC activity in the M cells already leads to the prediction of a partial C_4 shuttle mechanism in the C_3–C_4 intermediate plants, including the activity of alanine aminotransferase, glutamate-glyoxylate aminotransferase, pyruvate:phosphate dikinase, NADP-malic enzyme and malate dehydrogenase. The predictions of the metabolic model were then experimentally tested using nine enzyme and malate dehydrogenase. The predictions of the metabolic model were then experimentally tested using nine

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**Physiology of C_3–C_4 Intermediates**

In contrast to the now well-developed biochemical C_3–C_4 model, information on the physiology of C_3–C_4 intermediates is still limited. A first model of gas exchange in C_3–C_4 intermediates was presented by von Caemmerer (1989). It demonstrated the quantitative feasibility of this pathway and provided a mathematical explanation for C_3-like characteristic features such as the reduction of the CO_2 compensation point by operation of the glycine shuttle (Fig. 1B).

Measurements of CO_2 compensation points by infrared gas exchange analysis are, however, influenced by multiple factors and they do not permit the consideration of leaf internal differences (Keerberg et al. 2014). Dynamic analysis of ^14^CO_2 release from labeled photosynthates generated by long-term exposure of leaves to ^14^CO_2 recently enabled an estimation of the different fluxes within the leaf. Comparison of Flaveria pubescens, a C_3–C_4 intermediate species, with its C_3 relative *F. crongquistii* indicates that the photorespiratory pump elevates the mean intraplasmoidal CO_2 concentration during steady-state photosynthesis by about 3-fold (Keerberg et al. 2014). In an ecological context, this would translate into a fitness advantage under C-limiting conditions.

In contrast to the situation in C_3 and C_4 plants, the CO_2 compensation point of C_3–C_4 intermediates is strongly influenced by environmental conditions, especially CO_2, temperature and light (Brown and Morgan 1980, Holaday et al. 1982, Hunt et al. 1987). Under low light conditions, the CO_2 compensation point of C_3–C_4 intermediates is almost C_3-like but then it declines under increasing illumination. Hence the efficiency of the glycine pump is dependent on high rates of ribulose 1,5-bisphosphate regeneration and glycine production (Sage et al. 2013). Compared with C_3 relatives, C_3–C_4 intermediates from the genera *Flaveria*, *Heliotropium* and *Alternanthera* showed superior photosynthetic performance, especially under high temperature and low CO_2 concentrations (Vogan and Sage 2012). This correlates with the predicted evolution of C_3–C_4 intermediates and C_4 ancestors in open habitats under conditions of low CO_2 and high temperatures (Osborne and Sack 2012).

Fully evolved C_4 plants also possess higher water (WUE) and nitrogen use efficiency (NUE) than C_3 plants. Due to their efficient carbon concentration mechanism, C_4 plants display high
assimilatory rates even when stomatal conductance is low. The C₄ carbon pump allows for a reduced investment into RuBisCO protein and hence N, which translates into increased rates of assimilation of carbon per unit leaf nitrogen. For C₃–C₄ intermediates, the picture is not consistent. In Heliotropium species, the reduction in the CO₂ compensation point was associated with enhanced WUE (Vogan et al. 2007). Assessment of WUE and NUE in Flaveria species with different degree of C₄-ness could, however, not confirm a positive effect on WUE and NUE of the early intermediates (Kocacinar and Sage 2008, Vogan and Sage 2011, Way et al. 2014). Only at the later stages of C₄ evolution, when a C₄ pump is already in operation, did the water economy improve significantly (Kocacinar and Sage 2008, Way et al. 2014). Similar results were obtained for the monocotyledonous C₃–C₄ intermediate Panicum milioides (Pinto et al. 2011).

Advantages of the intermediate photosynthesis type seem to occur mainly under conditions of high temperature and the low CO₂ conditions present in recent geological times (Vogan and Sage 2012). Under specific environmental conditions, plants could, however, also benefit from intermediate photosynthesis under present CO₂ conditions. In Flaveria, C₃–C₄ intermediate species tended to have shorter vessels than C₃ relatives, which could contribute to xylem safety under very dry conditions (Kocacinar and Sage 2008). Carbon economy of intermediates could also be advantageous when low stomatal conductance limits CO₂ concentrations within the leaf. A general influence of the photorespiratory pump on whole-plant water economy, on the other hand, could not be established to date.

**Outlook on Impact of New Technologies on Studies of C₄ Evolution**

In recent years, the study of C₃–C₄ intermediates, mainly from the Flaveria genus, contributed substantially to the development of advanced models explaining the biochemical aspects of the evolution of C₄ photosynthesis. These models suggest that after the initial installation of a photorespiratory glycine pump, implemented by altered cell specificity of GDC expression, evolution can continue towards C₄ on a smooth path through a Mount Fuji-like fitness landscape (Heckmann et al. 2013, Mallmann et al. 2014). Changes in metabolic capacities on this path seem to be gradual rather than discrete steps, and consist of a multitude of metabolic adjustments (Heckmann et al. 2013). The succession and shaping of these adjustments seem to be very flexible and robust in different genetic backgrounds (Williams et al. 2013). So far the detailed studies in the genus Flaveria confirmed the events predicted by the models, and it will be interesting to compare them with scenarios from other C₃ lineages. Over the course of the next few years, the study of C₃–C₄ intermediates will be particularly exciting in the following fields.

**Application of transcriptome and genome sequencing**

With the advent of next-generation sequencing, the cost of transcript and genome sequencing continues to drop dramatically while throughput is constantly increasing (Weber 2015). This provides exciting new opportunities for the investigation of C₄ evolution. As demonstrated for the case of GLDP in Flaveria, detailed genetic information is crucial for elucidating the mechanisms underlying some of the key events of C₄ evolution. Investigation of phylogenetically distant systems will increase the power to understand potential plasticity within the evolutionary trajectory towards C₄. New species with C₃–C₄ intermediate photosynthesis are continually being discovered. In a detailed review by Sage et al. (2012), 21 different intermediate lineages have been described. The majority of those have close C₃ relatives, but for only nine of them no progression towards C₄ could be demonstrated so far, and among them are also at least two lineages from the Brassicales, for example Moricandia species and Diplotaxis tenuifolia. There are reports that in the genus Alote ropis, C₃ and C₄ photosynthesis are even found within the same species (Lundgren et al. 2015). Construction of advanced high-resolution phylogenies will aid in the design of new experimental systems for identification of additional C₄-specific parameters. So far, only genes involved in biochemistry of the trait are well described, but additional players such as regulatory elements and factors influencing organelle, cell and leaf structure are still mostly unknown. Besides the biochemically relevant genes, these could also have great influence on the advancement or retardation or even abortion of C₄ evolution.

**Dynamic flux analysis**

The work of Keerberg et al. (2014) clearly showed that dynamic metabolic flux analysis is essential to dissect fully the events in the different leaf compartments. Such knowledge will be required for the identification of events that exert the greatest instant effect on photosynthesis of the leaf. Dynamic flux experiments with ¹³C helped recently to advance our knowledge on photosynthetic events in Arabidopsis leaves (Szecowka et al. 2013, Ma et al. 2014). Application of similar methods to C₃–C₄ intermediates will promote a more detailed and mechanistic understanding of the evolutionary drivers beside the biochemical networks. Knowledge of the influence of environmental factors on C₃–C₄ photosynthesis is also very limited. Particularly interesting will be studies on the interplay of light, CO₂ and water of the C₃–C₄ photosynthetic dynamic.

**Quantitative genetics analysis of hybrids between C₃ and C₃–C₄**

For a number of C₃–C₄ intermediates genetic crosses to related C₃ species that produce a fertile hybrid offspring have been reported. Successful crossings have been performed between Flaveria species with C₃ and C₄-like photosynthesis (Holaday et al. 1988). Moricandia arvensis (C₁–C₃) can be hybridized with the crop species B. napus and B. oleracea (Apel et al. 1984, McVetty et al. 1989. Bang et al. 2009). Also M. moricandioides and M. arvensis can be hybridized, which enables genetic approaches towards identifying the genes controlling C₃–C₄ intermediate photosynthesis. In combination with genome sequencing and deep genotyping by next-generation
sequencing, the analysis of segregating populations of these hybrids provides a powerful tool to identify genes that encode enablers of $C_4$ evolution. This knowledge can then be translated into breeding programs that aim at increasing photosynthetic energy conversion efficiency.

**Summary**

The recent application of different modeling approaches allowed a refinement of the current model of $C_4$ evolution via $C_3-C_4$ intermediate stages. The models predict that the evolution from $C_3$ to $C_4$ is characterized by a succession of common key events starting with the implementation of the photorespiratory glycine pump and the subsequent need for additional balancing steps between the M and BS. A multitude of additive adjustment steps seems to mediate stages on the trajectory to $C_4$ independently by phylogenetic as well as biochemical modeling. The modeling work is also in agreement with the detailed experimental work done (Heckmann et al. 2013, Williams et al. 2013, Mallmann et al. 2014) Plant species with features between $C_3$ and $C_4$ have been confirmed as true intermediate stages on the trajectory to $C_4$ independently by phylogenetic as well as biochemical modeling. The modeling work is also in agreement with the detailed experimental work done (Heckmann et al. 2013, Williams et al. 2013, Mallmann et al. 2014) especially in *Flaveria* species on very different stages of $C_4$ evolution. In the future it will be interesting to see if lack of progression to $C_4$ in some lineages is likely to be connected to chance and the absence of selective pressure or if specific anatomical and metabolic changes can be identified which prevented progression towards $C_4$.

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