The Path from C_3 to C_4 Photosynthesis[1]

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C_4 PHOTOSYNTHESIS

The C_4 photosynthetic carbon cycle is an elaborated addition to the C_3 photosynthetic pathway. It evolved as an adaptation to high light intensities, high temperatures, and dryness. Therefore, C_4 plants dominate grassland floras and biomass production in the warmer climates of the tropical and subtropical regions (Edwards et al., 2010).

In all plants CO_2 is fixed by the enzyme Rubisco. It catalyzes the carboxylation of ribulose-1,5-bisphosphate, leading to two molecules of 3-phosphoglycerate. Instead of CO_2, Rubisco can also add oxygen to ribulose-1,5-bisphosphate, resulting in one molecule each of 3-phosphoglycerate and 2-phosphoglycolate. Phosphoglycolate has no known metabolic purpose and in higher concentrations it is toxic for the plant (Anderson, 1971). It therefore has to be processed in a metabolic pathway called photorespiration. Photorespiration is not only energy demanding, but furthermore leads to a net loss of CO_2. Thus the efficiency of photosynthesis can be decreased by 40% under unfavorable conditions including high temperatures and dryness (Ehleringer et al., 1991). The unfavorable oxygenase reaction of Rubisco can be explained as a relict of the evolutionary history of this enzyme, which evolved more than 3 billion years ago when atmospheric CO_2 concentrations were high and oxygen concentrations low. Apparently, later on, it was impossible to alter the enzyme's properties or to exchange Rubisco by another carboxylase. Nevertheless, plants developed different ways to cope with this problem. Perhaps the most successful solution was C_4 photosynthesis.

The establishment of C_4 photosynthesis includes several biochemical and anatomical modifications that allow plants with this photosynthetic pathway to concentrate CO_2 at the site of Rubisco. Thereby its oxygenase reaction and the following photorespiratory pathway are largely repressed in C_4 plants. In most C_4 plants the CO_2 concentration mechanism is achieved by a division of labor between two distinct, specialized leaf cell types, the mesophyll and the bundle sheath cells, although in some species C_4 photosynthesis functions within individual cells (Edwards et al., 2004). Since Rubisco can operate under high CO_2 concentrations in the bundle sheath cells, it works more efficiently than in C_3 plants. Consequently C_4 plants need less of this enzyme, which is by far the most abundant protein in leaves of C_3 plants. This leads to a better nitrogen-use efficiency of C_4 compared to C_3 plants, since the rate of photosynthesis per unit nitrogen in the leaf is increased (Oaks, 1994). Additionally C_4 plants exhibit better water-use efficiency than C_3 plants. Because of the CO_2 concentration mechanism they can acquire enough CO_2 even when keeping their stomata more closed. Thus water loss by transpiration is reduced (Long, 1999).

In the mesophyll cells of C_4 plants CO_2 is converted to bicarbonate by carbonic anhydrase and initially fixed by phospho*enol*pyruvate (PEP) carboxylase (PEPC) using PEP as CO_2 acceptor. The resulting oxaloacetate is composed of four carbon atoms, which is the basis for the name of this metabolic pathway. Oxaloacetate is rapidly converted to the more stable C_4 acids malate or Asp that diffuse to the bundle sheath cells. Here, CO_2 is released by one of three different decarboxylating enzymes, which define the three basic biochemical subtypes of C_4 photosynthesis, NADP-dependent malic enzyme (NADP-ME), NAD-dependent ME (NAD-ME), and PEP carboxykinase (PEPCK). The released CO_2 is refixed by Rubisco, which exclusively operates in the bundle sheath cells in C_4 plants. The three-carbon compound resulting from CO_2 release diffuses back to the mesophyll cells where the primary CO_2 acceptor PEP is regenerated by pyruvate orthophosphate dikinase by the consumption of, at the end, two molecules of ATP (Hatch, 1987).

Figure 1 shows a scheme of the NADP-ME subtype of C_4 photosynthesis. Here malate is the dominant transport metabolite while Asp can be used in parallel. The synthesis of malate occurs in the mesophyll chloroplasts, the decarboxylation by NADP-ME in the bundle sheath chloroplasts.

The two other biochemical subtypes differ from the NADP-ME type by the transport metabolites used and the subcellular localization of the decarboxylation reaction. In NAD-ME plants Asp, which is synthesized in the mesophyll cytosol, is used as transport metabolite. After deamination and reduction, the resulting malate is decarboxylated by NAD-ME in the bundle sheath mitochondria. Plants of the PEPCK type use Asp as well as malate as transport metabolites. Asp is synthesized in the cytosol of mesophyll cells and

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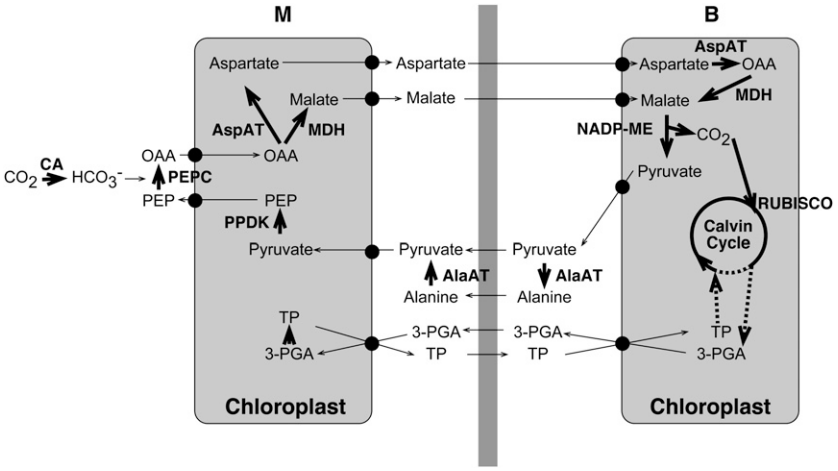

Figure 1. NADP-ME type of C_4 photosynthesis. 3-PGA, 3-Phosphoglyceric acid; AspAT, Asp aminotransferase; AlaAT, Ala aminotransferase; CA, carbonic anhydrase; MDH, malate dehydrogenase; OAA, oxaloacetate; PPDK, pyruvate orthophosphate dikinase; TP, triosephosphate.

decarboxylated in the cytosol of bundle sheath cells by the combined action of Asp amino transferase and PEPCK. As in NADP-ME-type C_4 species, malate is synthesized in the mesophyll chloroplasts but decarboxylated by NAD-ME in the mitochondria of bundle sheath cells. This reaction produces NADH that is used in the mitochondria to produce the ATP needed to drive the PEPCK reaction (Hatch, 1987). If Asp is used as transport metabolite, usually the three-carbon decarboxylation product, pyruvate, is partially transported back to the mesophyll cells in the form of Ala to maintain the ammonia balance between the two cell types (Hatch, 1987).

Compared to C_3 plants the bundle sheath cells of C_4 plants have expanded physiological functions. This is reflected by the enlargement and a higher organelle content of these cells in most C_4 species. For the efficient function of the C_4 pathway a close contact between mesophyll and bundle sheath cells is indispensable and they are tightly interconnected to each other by high numbers of plasmodesmata (Dengler and Nelson, 1999). To ensure a direct contact between bundle sheath and mesophyll cells, C_4 plants possess a characteristic leaf anatomy. The bundle sheath cells enclose the vascular bundles and are themselves surrounded by the mesophyll cells. The high vein density in the leaves of C_4 plants leads to a nearly one-to-one ratio of the volumes of mesophyll and bundle sheath tissues. The internal anatomy of a C_4 leaf is often composed of a repeating pattern of vein-bundle sheath-mesophyll-mesophyll-bundle sheath-vein. Because of its wreath-like structure this type of leaf anatomy was termed Kranz anatomy by the German botanist G. Haberlandt (1904). Kranz anatomy is found with more or less considerable variations in nearly all monocotyledonous and dicotyledonous lineages that use the two-cell mode of C_4 photosynthesis.

While the above differences are directly related to the CO_2 concentration mechanism, there are many further modifications known that evolved to integrate the C_4 pathway optimally into the plant's metabolism. For instance, C_4 species of the NADP-ME subtype are depleted in PSII in their bundle sheath cells to lower oxygen production in these cells. Accordingly, the production of reduction equivalents in the bundle sheath cells is reduced and the reduction phase of the Calvin-Benson cycle, i.e. the conversion of 3-phosphoglycerate to triose phosphate, has been at least partially shifted to the mesophyll cells (Fig. 1). There is another adaptation in C_4 plants that affects the light reactions of photosynthesis. Compared to C_3 photosynthesis the C_4 pathway consumes one (PEPCK type) or two (NADP-ME and NAD-ME type) additional molecules of ATP per fixed CO_2 without the need of additional reduction equivalents. This increase in ATP-to-NADPH ratio is compensated for in some C_4 plants by enhancing cyclic electron flow around PSI, which provides additional ATP without concomitantly producing NADPH. Large-scale trancriptomic and proteomic approaches also revealed that other metabolic pathways such as amino acid synthesis, nitrogen or sulfur assimilation, and lipid metabolism are compartmentalized between mesophyll and bundle sheath cells in at least some C_4 plants (Majeran and van Wijk, 2009).

POLYPHYLETIC EVOLUTION OF C_4 PHOTOSYNTHESIS

C_3 angiosperms evolved more than 50 times independently into C_4 plants (Muhaidat et al., 2007). Most of the C_4 species occur in the grasses (approximately 4,600) and sedges (approximately 1,600). Only a total of about 1,600 C_4 species are found in the dicots where they are spread over 16 families with 75% of them clustering in the four families Chenopodiaceae, Amaranthaceae, Euphorbiaceae, and Asteraceae (Muhaidat et al., 2007). C_4 grasses probably evolved in the early Oligocene about 30 million years ago, while C_4 dicots appeared later, less than 20 million years ago (Sage, 2004).

The polyphyletic origin of C_4 photosynthesis indicates that only relatively small evolutionary changes

were required for the establishment of this photosynthetic pathway. It can be assumed that C_4 evolution must have been easy in genetic terms. This raises the question of whether we can use the information about the genetic architecture and evolution of this pathway and introduce modules of C_4-ness into present C_3 plant and thereby transform them into C_3-C_4 intermediate or even C_4-like plants (Sheehy et al., 2007).

THE PATH TO C_4 PHOTOSYNTHESIS

The currently most widely accepted model of C_4 evolution proposes a stepwise sequence of changes leading from C_3 to C_4 plants (Fig. 2). Each of these changes on its own is leading to a distinct evolutionary benefit for the resulting species independent of whether it will progress toward the full expression of the C_4 syndrome. This scenario explains why the evolution of this complex trait could occur so many times independently. The model is mainly based on comparative analyses of extant C_3, C_4, and especially C_3-C_4 intermediate species, and a detailed elaboration can be found in Sage (2004). Here, we only present a short summary and elucidate how the evolutionary changes might have been realized through modifications at the molecular/genetic level.

It is thought that the existence of many redundant genes in the genomes of the relevant organisms and species was a general prerequisite for C_4 evolution (Monson, 2003). These gene redundancies have been acquired by duplications of whole genomes, genome segments, or only single genes. Multiple copies of a gene allow evolutionary modifications of one copy without losing the original function of the gene itself. Thus redundant gene copies prevent deleterious consequences of evolutionary changes that alter or switch off the specific function of a certain gene. In further steps, leaves have been altered toward Kranz anatomy, a photorespiratory CO_2 pump was established, and finally a C_4 cycle was created. All these steps were accompanied by massive changes in gene regulation. Also the kinetic properties of enzymes, involved in metabolic pathways that were affected by these evolutionary changes, were adjusted to the new requirements (Fig. 2).

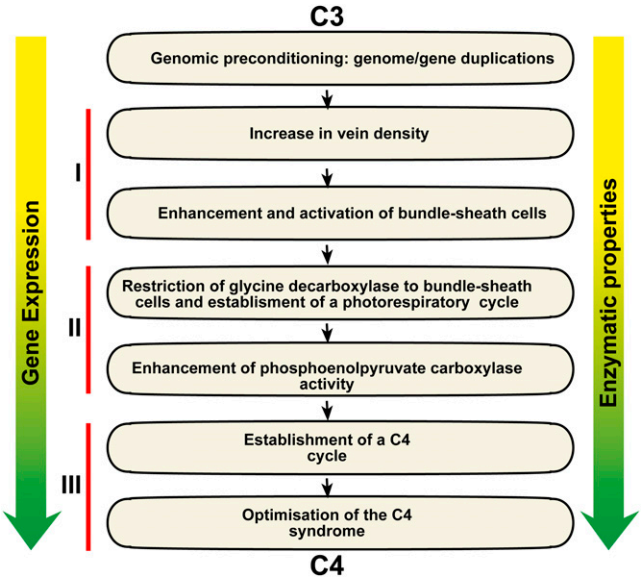

Figure 2. Stepwise evolution of C_4 photosynthesis.

I. Development of Kranz Anatomy

The first step toward C_4 evolution was the development of the Kranz anatomy. To establish a mechanism that efficiently concentrates CO_2 in bundle sheath cells the mean distance of a mesophyll cell to the next bundle sheath cell must be as short as possible. Ideally each mesophyll cell should be directly adjacent to at least one bundle sheath cell. Therefore, in planar leaves the vein density had to be enhanced. A higher vein density increased also the mechanical integrity of the leaves, which could be beneficial in windy habitats, or improved the water supply of leaves in dry and hot biotopes (Sage, 2004). In succulent terete or semiterete leaves, evolution of C_4 occurred in some dicots with development of a single Kranz unit surrounding the vascular and water storage tissue (Edwards et al., 2004).

A comparative analysis of the leaf development in both monocot and dicot C_3 and C_4 species revealed that the close vein spacing in leaves of C_4 plants is due to changes in the initiation frequency and pattering of the minor and not the major veins (Ueno et al., 2006; McKown and Dengler, 2009).

In Arabidopsis (*Arabidopsis thaliana*) the formation of veins from ground tissue is triggered by polar auxin flow mediated by auxin efflux carriers. Cell files along the auxin transport route convert to procambial cells and later on develop into vascular bundles (Scarpella et al., 2006). Either modifications of auxin production and allocation and/or modifications of the competency of ground tissue cell to become procambial cells are responsible for the greater vein density observed in C_4 compared to C_3 leaves (McKown and Dengler, 2009). Since the molecular events causing the initiation of veins are not even completely understood in C_3 model plants, it is presently challenging to predict the changes that led to the C_4 typical leaf anatomy.

The activation of bundle sheath cells—the enlargement of these cells and the increase in the number of organelles in this tissue might be a secondary effect of the higher vein density. Typically, the bundle sheath cells of C_3 plants possess only a few chloroplasts, and the photosynthetic activity is low. With higher vein densities also the ratio of bundle sheath to mesophyll cells increases. Since only the mesophyll cells show high photosynthetic activity, this would imply that the overall photosynthetic activity of a leaf with a given size decreases. The evolutionary pressure to maintain

the overall photosynthetic activity could have led to an increase of the number of chloroplasts in the bundle sheath cells. Due to the necessity to metabolize the photorespiratory Gly in bundle sheath cells the increase of chloroplast numbers would also require an increase in the numbers of mitochondria and peroxisomes in these cells.

II. The Photorespiratory CO_2 Pump: C_3-C_4 Intermediate Photosynthesis

Extant C_3-C_4 intermediate species possess a photorespiratory Gly shuttle that pumps CO_2 into the bundle sheath cells (Bauwe, 2010). This is achieved by restricting the Gly decarboxylation reaction to the bundle sheath mitochondria, thus all Gly produced by photorespiration in the mesophyll has to be transferred to the bundle sheath cells for further processing. The Gly shuttle affects photosynthetic CO_2 fixation in two ways. All photorespiratory CO_2 is set free inside the leaf far apart from the outer surface. Therefore it has to diffuse through several cell layers, before it could escape from the leaf. This enhances the plant's chances of refixing the photorespired CO_2 and minimizes the loss of carbon due to photorespiration. In some C_3-C_4 intermediate species this refixation capacity is supported by the spatial distribution of the organelles within the bundle sheath cell, since the mitochondria concentrate adjacent to the vascular bundles (Rawsthorne et al., 1998). Additionally, the Gly shuttle enhances the CO_2 concentration within the bundle sheath cells. As a consequence, the carboxylation activity of Rubisco in the bundle sheath cells increases, while its oxygenase reaction is outcompeted (Bauwe, 2010).

It is assumed that the establishment of such a photorespiratory CO_2 pump is an important intermediate step on the way toward C_4 photosynthesis. A photorespiratory CO_2 pump can easily be accomplished at the molecular level. The expression of only one gene, encoding a subunit of the Gly decarboxylase multienzyme complex, had to be restricted to the bundle sheath cells. This might have been achieved through relatively subtle changes in the cis-regulatory elements that control the expression of these genes (compare with Akyildiz et al., 2007). In cases where several isogenes with different leaf expression specificities existed already in the respective C_3 ancestral species this process might also have included the pseudogenization of those isogenes that are not bundle sheath specific.

In the C_3-C_4 intermediate species *Moricandia arvensis*, for example, only the P subunit of Gly decarboxylase is restricted to the bundle sheath. Since the enzyme is inactive without this subunit, Gly cannot be decarboxylated in the mesophyll (Rawsthorne et al., 1988). For other C_3-C_4 intermediates from the genera *Flaveria* and *Panicum*, it was found that also the other subunit genes were expressed specifically or at least preferentially in the bundle sheath cells (Morgan et al., 1993). It follows that once Kranz anatomy and enlarged bundle sheath cells with increased amounts of organelles were established, a photorespiratory CO_2 pump could be easily achieved in genetic terms. The photorespiratory CO_2 pump and the resulting elevated CO_2 content in the bundle sheath cells might have led to a further increase in organelle numbers in these cells (Sage, 2004).

The next step toward true C_4 photosynthesis might have been an increase in the levels of carbonic anhydrase and PEPC in the cytosol of the mesophyll cells. This would have aided in recapturing the photorespiratory CO_2 that escaped from the bundle sheath into the mesophyll cells. Also this evolutionary step is reflected by C_3-C_4 intermediate species of the genus *Flaveria*, which contain significantly higher levels in PEPC transcript and protein amounts as compared to C_3 *Flaveria* species but do not exhibit C_4 cycle activity yet (Ku et al., 1991; Engelmann et al., 2003).

To establish a limited C_4 cycle activity the remaining C_4 cycle enzymes must have been elevated at this point. It is known that even in C_3 plants the activity of the decarboxylating enzymes NADP-ME and NAD-ME is massively increased in vascular tissues (Hibberd and Quick, 2002). Thus the expression of the related genes must have been shifted to the bundle sheath cells. To complete the C_4 cycle the expression of chloroplastic pyruvate orthophosphate dikinase must have been enhanced to allow an efficient PEP regeneration. Plants in this phase of C_4 evolution exhibit high activities of C_4 cycle enzymes, but still high Rubisco activity in the mesophyll cells. Consequently, CO_2 is only partially fixed through the C_4 pathway.

III. Establishment of the C_4 Cycle

The key step in establishing true C_4 photosynthesis and to integrate the C_4 pathway and the Calvin-Benson cycle was the spatial separation of the two carboxylation reactions. PEPC was restricted to the mesophyll and Rubisco to the bundle sheath cells. This step became necessary when the C_4 cycle activity increased to such a level that CO_2 fixation by PEPC reached the same magnitude as by Rubisco and hence the C_4 and the Calvin-Benson cycle competed for CO_2 and ATP (Monson, 1999). Now the vast majority of the photoassimilated CO_2 passed initially through the C_4 cycle before it was fixed by Rubisco. The evolving C_4 pathway was further optimized by compartmentalizing other enzymes of both the C_4 and Calvin-Benson cycles, by adapting the light reaction of photosynthesis and by strongly increasing carbonic anhydrase activity in the cytosol of mesophyll cells.

The C_4 photosynthetic pathway is characterized by the extensive shuffling of metabolites between the organelles and the cytosol within mesophyll and bundle sheath cells, respectively. The evolution of this pathway, therefore, required also the establishment of the necessary transport capacity. In plants of the NADP-ME type, for example, for every molecule of

CO_2 fixed, one molecule of pyruvate and oxaloacetate each have to be transported into the mesophyll chloroplasts and in a countermove PEP and malate have to be translocated to the cytosol. In bundle sheath cells, on the other hand, malate has to enter and pyruvate has to leave the chloroplast matching the rate of CO_2 assimilation.

Large-scale transcriptome and proteome analyses indicate that also other pathways related to sulfur, nitrogen, and carbon metabolism were modified with respect to either overall activity or to mesophyll/bundle sheath compartmentation (Friso et al., 2010; Bräutigam et al., 2011). This was most likely necessary due to differences in the supply of energy and reduction equivalents in the different tissues and to optimize the overall integration of the various metabolic pathways.

Changes in Gene Expression

The evolution of C_4 photosynthesis was accompanied by massive changes in gene expression. Recently, the transcriptomes of mature leaves of the C_4 plant *Cleome gynandra* and the closely related C_3 species *Cleome spinosa* were compared quantitatively by a RNA-Seq-based digital gene expression approach (Bräutigam et al., 2011). About 2.8% of the transcripts detected differed significantly in their abundance between the two species. As to be expected the expression levels of genes involved in the C_4 cycle, the photorespiratory pathway, and the photosynthetic light reactions changed. However, several other pathways showed explicit alterations in their corresponding transcript levels, too. For instance, the C_4 *Cleome* showed reduced steady-state levels in transcripts associated with one carbon compound metabolism, the shikimate pathway, and amino acid metabolism (Bräutigam et al., 2011). Most interestingly, genes encoding components of the cytosolic and plastidic protein synthesis machinery are down-regulated in the C_4 species. Higher steady-state transcript levels in the C_4 leaf are observed for genes involved in starch metabolism, cofactor synthesis, and nitrogen metabolism (Bräutigam et al., 2011).

Besides quantitative alterations C_4 evolution required changes in the spatial gene expression patterns. Sawers et al. (2007) reported that in maize (*Zea mays*) about 18% of the genes are differentially expressed between mesophyll and bundle sheath cells. According to the Rice Atlas database (http://plantgenomics.biology.yale.edu/riceatlas/; Jiao et al., 2009), only less than 2.5% of the rice (*Oryza sativa*) genes (729 out of 32,119 genes) are differentially expressed ($P < 0.05$) between the mesophyll and bundle sheath cells of this C_3 grass. This comparison indicates that the establishment of C_4 photosynthesis involved a dramatic redesign and restructuring of leaf functions.

Most of the evolutionary alterations, leading to the quantitative and qualitative changes in gene expression, are not yet understood at the molecular level and only a few have been analyzed in great detail. These cases demonstrate that nature appeared to have been quite flexible in achieving the desired goal, i.e. different genes were altered in different ways to adapt them for their function in the C_4 pathway (Hibberd and Covshoff, 2010).

Cell-specific gene expression can be achieved by transcriptional control. For instance, the mesophyll-specific expression of the photosynthetic PEPC gene, *ppcA*, of the C_4 plant *Flaveria trinervia* depends on a cis-regulatory element, the MESOPHYLL EXPRESSION MODULE1, which is located about 1,900 bp upstream of the transcriptional start site (Gowik et al., 2004). A very similar element was also found in the promoters of the orthologous *ppcA* genes from C_3 *Flaverias*; however, these elements lack the ability to direct mesophyll specificity. Accordingly, slight modifications within a cis-regulatory element were sufficient to convert a gene with no apparent expression specificity into a mesophyll-specific gene (Akyildiz et al., 2007).

In contrast, the bundle sheath-specific expression of one of the genes encoding the small subunit of Rubisco in the C_4 plant *Flaveria bidentis*, FbRbcS1, was reported to be regulated mainly at the posttranscriptional level (Patel et al., 2006). Most likely, the FbRcS1 transcripts are differentially stable in mesophyll and bundle sheath cells. This is controlled by stability determinants that are located in the 5′ and 3′ untranslated regions of the mRNA (Patel et al., 2006).

The massive changes in gene expression during the transition from C_3 to C_4 photosynthesis combined with the fact that C_4 evolution must have been easy in genetic terms implies that preexisting gene regulatory networks in C_3 plants were probably the foundation for multiple evolutionary changes toward C_4 photosynthesis (compare with Matsuoka, 1995). In C_3 plants gene regulatory networks exist that assure a coordinated response of genes involved in photosynthesis and related metabolic pathways (Mentzen and Wurtele, 2008). Promoters driving mesophyll- or bundle sheath-specific gene expression in C_4 species partly maintain their cell preference of expression in C_3 species (Matsuoka et al., 1993; Engelmann et al., 2008), suggesting that the gene regulatory networks controlling the development and differentiation of mesophyll and bundle sheath cells of C_4 plants are not fundamentally different from those of C_3 species. Consequently, networks for regulating developmental and metabolic processes operated already in C_3 ancestral angiosperms and could serve as a platform for the establishment of C_4 leaf anatomy and metabolism.

Unfortunately, our understanding of gene regulatory networks controlling the development and anatomy of a typical leaf of a C_3 angiosperm is rather rudimentary. With the exceptions discussed above we know little about the molecular nature of cis-and trans-regulatory factors that regulate gene expression in the mesophyll and bundle sheath cells of both C_3 and C_4 plants.

The GOLDEN2-LIKE (GLK) transcription factors GLK1 and GLK2 are the only exceptions. This pair of transcription factors occurs in all land plants. In Arabidopsis the GLK proteins are largely redundant and control the expression of more than 100 genes that are mainly connected with photosynthesis. In the mesophyll and bundle sheath of the C_4 species maize, however, the two GLK genes are expressed differentially with GOLDEN2 specifically affecting only chloroplast development in the bundle sheath cells (Waters and Langdale, 2009). Thus the GLK proteins appear to be an important component of the gene regulatory network of mesophyll/bundle sheath differentiation in the C_4 plant maize.

Optimization of Enzyme Properties

All C_4 cycle enzymes evolved from nonphotosynthetic isoforms. To ensure high fluxes through the C_4 pathway, the concentration of substrates and effector metabolites is elevated as compared to the original metabolic environment in the ancestral C_3 species. Accordingly, the evolution of the C_4 isoforms involved changes in their kinetic and regulatory properties.

The C_4 isoform of PEPC is perhaps the best-documented example for these evolutionary processes (for review, see Gowik and Westhoff, 2010). C_4 PEPCs bind PEP with a lower affinity than the nonphotosynthetic PEPCs, while their affinity to the other substrate, i.e. bicarbonate, is increased. The C_4 PEPC isoforms are more tolerant toward the allosteric inhibitors Asp and malate and are more strongly affected by the allosteric activators Glc-6-P or Gly. These differences in enzymatic properties were achieved by relatively small changes in primary enzyme structure. The pair of orthologous *ppcA* PEPCs from *F. trinervia* (C_4) and *Flaveria pringlei* (C_3) shares 96% identical amino acid positions and was used as an experimental system to identify some of the evolutionary changes at the amino acid level of resolution (Westhoff and Gowik, 2004). The molecular changes observed appear to be subject to certain constraints that are given by the enzyme's properties.

The lower affinity for the substrate PEP is closely related to an Ala to Ser exchange in the C-terminal part of the enzyme (Bläsing et al., 2000). This amino acid exchange is found in all C_4 PEPCs analyzed so far but not in nonphotosynthetic or Crassulacean acid metabolism PEPC isoforms (Gowik and Westhoff, 2010). Within the C_4 PEPCs of the grasses these constraints seem to be even more distinctive. Although C_4 PEPCs evolved at least eight times independently within the grass family the resulting enzymes show a surprisingly high degree of similarity. A strong positive selection was found for 21 amino acid positions (Christin et al., 2007). Only two of the 21 amino acid positions that are under positive selection in grass PEPCs are also important for the evolution of dicot C_4 PEPCs. This could indicate special requirements for grass C_4 PEPCs when compared to dicot C_4 PEPCs. Alternatively, this might also reflect the fact that most of the dicot C_4 lineages are very young compared to the first origins of C_4 photosynthesis within the grasses (Ehleringer et al., 1997; Sage, 2004). One may infer therefore, that the C_4 PEPCs of the grass family are much more optimized for their role in C_4 photosynthesis than their dicot counterparts. This might explain the higher degree of convergence within the photosynthetic PEPCs of the grasses.

The C_4 NADP-ME also acquired unique kinetic and regulatory properties during their evolution from nonphotosynthetic isoforms. Distinct enzyme regions could be identified that are involved in an altered pH-dependent inhibition by malate and differences in tetramerization of the enzyme (Detarsio et al., 2007).

Adaptation of C_4 enzymes to the new metabolic context of the C_4 pathway could also involve a change in the cellular location of the enzyme. The photosynthetic carbonic anhydrase gene of *F. bidentis* (FbCA3) is a prime example for this case. The gene is highly expressed in the mesophyll cells (Tetu et al., 2007) and evolved from an ancestral gene that encoded a chloroplast-targeted carbonic anhydrase. Due to a mutation in the chloroplast transit peptide of the ancestral enzyme, the C_4 isoform became a cytosolic enzyme (Tanz et al., 2009). Interestingly, this ancestral carbonic anhydrase gene was already highly expressed in leaves, suggesting that the intracellular localization of the protein was of minor importance and altered during evolution.

It is not clear so far to which extent other enzymes, which are not directly related to the C_4 pathway, were modified during C_4 evolution.

TRANSFER OF C_4 PHOTOSYNTHESIS INTO C_3 CROPS

The world of the 21st century will face massive problems in feeding the growing human population. Green energy from plant biomass is being developed to help cover energy demands, and might compete with food production for terrain and resources in the future. It will be a challenge to increase crop production adequately in a sustainable manner both in terms of harvestable yield and total biomass.

C_4 plants exhibit high photosynthetic capacity and efficient use of nitrogen and water resources. They have received an increasing interest in recent years and the transfer of C_4 photosynthesis into current C_3 crops is being considered (Sheehy et al., 2007). Currently there are attempts under way to implement a C_4-CO_2 concentration pathway into rice, perhaps the most important crop for human nourishment to date (http://c4rice.irri.org).

Knowledge about the genetic architecture of C_4 photosynthesis and the underlying gene regulatory networks is a prerequisite to be successful in this endeavor. To elucidate these networks different approaches are needed. Large forward-genetic screens

with mutagenized rice and *Sorghum bicolor* as well as reverse-genetic approaches are being carried out to identify genes that are related to C_4 subtraits like a reduced CO_2 compensation point, high vein density, or enlarged bundle sheath cells. The analysis of the transcriptomes, proteomes, and metabolomes of different developmental stages of C_4 leaves will help to understand how C_4 leaf differentiation and the establishment of Kranz anatomy are regulated. Comparing the transcriptomes of closely related C_3 and C_4 species from genera like *Flaveria* or *Cleome* (Bräutigam et al., 2011) will illuminate the evolutionary trajectories of C_4 photosynthesis and reveal the gene repertoire that is required for the transition of a C_3 into a C_4 plant. The successful integration of these different data, the identification of the key regulators of C_4 traits, and the generation of a strategy of how the C_3 plant rice must be genetically altered to introduce the C_4 pathway should become a milestone in the relatively young field of synthetic biology.

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