The Evolutionary Path from $C_3$ to $C_4$ Photosynthesis: A Review

Priyanka Upadhyay¹*, Neha Agrawal¹, Praveen Kumar Yadav² and Ruby Patel³

¹Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, Punjab India, 141004
²Division of Seed Science and Technology, Indian Agricultural Research Institute, New Delhi, 110012, India
³Department of Agricultural Chemistry and Soil Science, Bidhan Chandra Krishi Vishwavidyalya, Mohanpura, Nadia West Bengal India, 741252

*Corresponding author

ABSTRACT

The $C_4$ photosynthetic carbon cycle can be explained as the elaborated addition to the $C_3$ photosynthetic pathway. It is a unique series of biochemical, anatomical and regulatory gene characteristics that concentrate CO$_2$ around the carboxylating enzyme Rubisco, thereby increasing photosynthetic efficiency during high rates of photorespiration. The $C_4$ photosynthetic pathway has evolved as an adaptation to high light intensities, high temperatures and dryness, therefore in the warmer climates of the tropical and subtropical dominating the grassland floras and biomass production. $C_4$ photosynthesis originated more than 40 times independently during angiosperm evolution in over 15 families of angiosperms, representing convergent evolutionary phenomena. Probably, $C_4$ grasses evolved in the early Oligocene about 30 million years ago, while later appeared $C_4$ dicots, less than 20 million years ago. Low atmospheric CO$_2$ is pivotal factor for $C_3$- $C_4$ transition, because it is required for high rates of photorespiration. Consistently, the increasing global aridification and declining atmospheric CO$_2$ coincides with periods of the appearance of $C_4$ plants. Genetically, leading mechanism for creation of $C_4$ genome is duplications of whole genomes, genome segments, or single genes followed by non-functionalization and neo-functionalization with selection for carbon conservation traits under high photorespiration promoting conditions being the ultimate factor behind the origin of $C_4$ photosynthesis.

Keywords

$C_4$ photosynthetic pathway, $C_3$-$C_4$ transition, photorespiration, genome duplications, Rubisco

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**Introduction**

The ability of photosynthetic organisms to sequester inorganic CO$_2$ of the atmosphere into organic carbon of the biosphere via the Calvin-Benson-Bassham pathway is pivotal for the existence of life on the earth. The C$_4$ photosynthetic carbon cycle can be explained as the elaborated addition to the C$_3$ photosynthetic pathway. It is a unique series of biochemical, anatomical and regulatory gene characteristics. In most terrestrial C$_4$ species, it relies on the co-ordinated functioning of mesophyll and bundle sheath cells, except in the chenopods Borszczowia aralocaspia and Bientertia cycloptera where the C$_4$ photosynthetic mechanism operates in single cells (Freitag and Stichler 2000; Voznesenskaya et al., 2001, 2002). The C$_4$ photosynthetic pathway has evolved as an adaptation to high light intensities, high temperatures, and dryness. Therefore, grassland florlas and biomass production in the warmer climates of the tropical and subtropical regions has been dominated by C$_4$ plants (Edwards et al., 2010).

In all plants, the primary photosynthetic CO$_2$ reduction reaction is the fixation of CO$_2$ by the enzyme RuBP carboxylase/oxygenase (Rubisco). Rubisco is a ubiquitous enzyme in most autotrophic organisms from prokaryotes (photosynthetic and chemooautrophic bacteria, cyanobacteria and archaea), to eukaryotes (various algae and higher plants) and even phytoplankton in the sea (providing more than 45% of global net primary production annually). Approximately, Rubisco comprises up to 50% of the total soluble protein in the plant leaf or inside the microbe.

The RuBP carboxylase reaction catalyzes the carboxylation of ribulose-1,5-bisphosphate, leading to two molecules of 3-phosphoglycerate, while its oxygenase property adds oxygen to ribulose-1,5-bisphosphate, resulting in one molecule each of 3-phosphoglycerate and 2-phosphoglycolate. The metabolic purpose of phosphoglycolate is unknown and in higher concentrations it is toxic for the plant (a potent inhibitor of chloroplastic function) (Anderson, 1971). Therefore, it has to be processed in a metabolic pathway called photorespiration. Besides energy demanding, photorespiration leads to a net loss of CO$_2$. The catalytic activity of Rubisco with O$_2$ as a substrate is some 100-fold lower than with CO$_2$ at equivalent concentrations of the two gases (Tcherkez et al., 2006). Thus, under unfavorable conditions including high temperatures and dryness the efficiency of photosynthesis can be decreased by 40% (Ehleringer et al., 1991) by decrease in the affinity of Rubisco for CO$_2$ (Jordan and Ogren 1984). The remaining reduced level of CO$_2$ and abundant availability of O$_2$ inside the leaf results in enhanced rates of RuBP oxygenation.

The unfavorable oxygenase reaction of Rubisco evolved more than 3 billion years ago when atmospheric CO$_2$ concentrations were high and oxygen concentrations low can be explained as a relict of the evolutionary history of this enzyme. Later on, enzyme’s alteration or to exchange Rubisco by another carboxylase was impossible. Indeed, plants developed different ways to cope with this problem. Perhaps the most successful solution was C$_4$ photosynthesis. A marked and sustained decline in atmospheric $p$CO$_2$ during the Tertiary period reveals the appearance of C$_4$ plants in the fossil record (Ehleringer et al., 1991; Sage 2001). Therefore, low $p$CO$_2$ might have been an important driving force for evolution of the pathway.

A complex combination of both biochemical and morphological specialization provides elevated $p$CO$_2$ at the site of Rubisco
carboxylation in all C₄ species. This result in suppression of photorespiration and allowing Rubisco to work near to its maximal rate, such that CO₂ assimilation in C₄ plants is effectively CO₂ saturated in air (Hatch 1987). In most C₄ plants a division of labor between two distinct, specialized leaf cell types, the mesophyll and the bundle sheath cells leads to the CO₂ concentration mechanism except in some species where C₄ functions within individual cells (Edwards et al., 2004). Since, Rubisco works more efficiently in C₄ than C₃ plants and can operate under high CO₂ concentrations in the bundle sheath cells. Indeed, C₄ species are also characterized by greater nitrogen and water use efficiency relative to C₃ plants. The increased nitrogen use efficiency is largely accounted for saving nitrogen in Rubisco protein (Evans and von Caemmerer 2000) because C₄ plants need less of this enzyme, however in C₃ plants leaves it is the most abundant protein. Since the rate of photosynthesis per unit nitrogen in the leaf is increased in C₄ species (Oaks 1994). Better water use efficiency of C₄ relative to C₃ plants accounted from its CO₂ concentration mechanism even when keeping their stomata more closed. Thus reducing the water loss by transpiration (Long 1999).

Initially, in the mesophyll cells of C₄ plants CO₂ is converted to bicarbonate by carbonic anhydrase, which is then fixed by phosphoenolpyruvate (PEP) carboxylase into C₄ acids (oxaloacetate) using PEP as CO₂ acceptor. Oxaloacetate is rapidly converted to the more stable C₄ acids malate or Asp that diffuses to the bundle sheath cells and is decarboxylated there to supply CO₂ for Rubisco. Three basic biochemical subtypes of C₄ photosynthesis are defined by one of three one of three different decarboxylating enzymes: NADP-dependent malic enzyme (NADP-ME), NAD-dependent ME (NAD-ME), and PEP carboxykinase (PEPCK). Rubisco refixes the released CO₂ in the bundle sheath cells. This results in release of CO₂ and a three-carbon compound, which diffuses back to the mesophyll cells. Here, at the end the primary CO₂ acceptor PEP is regenerated by pyruvate orthophosphate dikinase by the consumption of two molecules of ATP (Hatch, 1987). In NADP-ME subtype of C₄ photosynthesis malate is the dominant transport metabolite while Asp can be used in parallel. Malate synthesis occurs in the mesophyll chloroplasts and the decarboxylation by NADP-ME in the bundle sheath chloroplasts (Fig. 2).

The other two biochemical subtypes are differentiated from the NADP-ME type by the transport metabolites used and the subcellular localization of the decarboxylation reaction. In NAD-ME plants Asp is used as transport metabolite which is synthesized in the mesophyll cytosol. Deamination and reduction of Asp results in malate which 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 decarboxylated in the cytosol of bundle sheath cells by the combined action of Asp amino transferase and PEPCK. This reaction produces NADH that is used in the mitochondria to produce the ATP needed to drive the PEPCK reaction (Hatch 1987). When Asp is used as transport metabolite, usually, pyruvate (the three-carbon decarboxylation product) is partially transported back to the mesophyll cells in Alaform to maintain the ammonia balance between the two cell types (Hatch, 1987).

Resistance of the bundle sheath to CO₂ diffusion and the relative biochemical capacities of the C₃ and C₄ cycle determine the efficiency of the C₄ pathway and the pCO₂
attained in the bundle sheath. The leakiness of the bundle sheath is intimately linked with the efficiency of the C_4 concentrating mechanisms. Leakiness is defined as that fraction of CO_2 generated by C_4 acid decarboxylation in the bundle sheath that subsequently leaks out (Farquhar 1983). Consumption of energy by C_4 cycle in ATP form during regeneration of PEP, CO_2 leakage from bundle sheath is an energy cost to the leaf. High energy demand of C_4 cycle relative to C_3 cycle was demonstrated with quantum yield measurements under varying pCO_2, pO_2 and temperature by Ehleringer and Bjorkman (1977). These data represented that at low temperatures C_4 species have lower quantum yields than C_3 species, but at high temperature superior quantum yields where in C_3 species the quantum yields decrease due to high photorespiratory rates.

The significant parameters of the C_4 concentrating mechanism, such as bundle sheath resistance to CO_2 diffusion, bundle sheath pCO_2 and leakiness of the bundle sheath measurement cannot be done directly and these estimates vary widely. It has been demonstrated through various models that a low bundle sheath conductance is pivotal feature of the C_4 photosynthetic pathway (Berry and Farquhar, 1978; von Caemmerer and Furbank, 1999). The conductance across the mesophyll/bundle sheath interface and the bundle sheath surface area to leaf area ratio (S_b) are the basis for expression of conductance bundle sheath. An estimate of S_b ranges from 0.6-3.1 m^2 m^{-2} (Apel and Peisker 1978; Brown and Byrd 1993).

Nevertheless the conductance to CO_2 diffusion across the mesophyll bundle sheath interface is also several-fold relative to equivalent conductance across the cell wall and chloroplast interface in C_3 species (Evans and vonCaemmerer 1996; von Caemmerer and Furbank 2003).

**Polyphyletic evolution and quantitative trait of C_4 photosynthesis**

C_4 photosynthesis originated more than 40 times independently during angiosperm evolution (Gowik and Westhoff, 2011). Most of the C_4 species occur in the grasses and sedges approximately 4,600 and 1,600 respectively, whereas only about 1,600 C_4 dicots species are known. They are spread over 15 families with 75% of them clustering in the four families Chenopodiaceae, Amaranthaceae, Euphorbiaceae, and Asteraceae (Muhaidat et al., 2007), representing convergent of evolutionary phenomena. Probably, C_4 grasses evolved in the early Oligocene about 30 million years ago, while later appeared C_4 dicots, 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. C_4 evolution can be assumed in genetic terms, which 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 transition from C_3 to C_4 photosynthesis pathway**

The transition from C_3 to C_4 and the occurrence of C_3-C_4 intermediate species in today’s flora provides us an evolutionary adaptive advantage for the resulting species independent of whether it will progress toward the full expression of the C_4 syndrome. Recently most widely accepted model of C_4 evolution proposes a stepwise sequence of changes leading from C_3 to C_4 plants (Fig. 3).
Genetically the C₄ syndrome may therefore be best described as a polygenic, quantitative trait. The concept of C₄ photosynthesis being as a quantitative trait immediately implies a number of questions that what is the genetic architecture of C₄ photosynthesis, i.e. how many genes are required to establish this phenotypic syndrome? Are the genes organized into functional units giving rise to distinct subphenotypes? Do these functional units form gene regulatory networks whose component genes are regulated coordinately and hence may be viewed as separate regulatory modules?

Here, we only present a short summary and elucidate how the evolutionary changes might have been realized through modifications at the molecular/genetic level.

Genetically, C₄ evolution began with duplications of whole genomes, genome segments, or single genes followed by non-functionalization and neo-functionalization (Monson 2003). Thus redundant gene copies prevent deleterious consequences of evolutionary changes that alter or switch off the specific function of a certain gene. The non- and neo-functionalization’s major targets are the promoter and enhancer region of genes to allow for altered expression and compartmentalization, and the coding region to alter regulatory and catalytic properties.

Further at anatomic level, leaf modification occurred toward Kranz anatomy. A rudimentary Kranz anatomy resulted from an increase in vein density and an enhancement and activation of the bundle sheath cell layer. The compartmentation of Gly decarboxylase in the bundle sheath cells was the next step which led to a photorespiratory CO₂ pump (Fig. 4). An elevated phosphoenolpyruvate carboxylase activity and subsequently an increase in the other C₄ cycle enzymes and transporters accompanied by their compartmentalized expression established the C₄ cycle between mesophyll and bundle sheath cells. Massive changes in gene regulation accompanied all these steps. The kinetic properties of enzymes also involved in metabolic pathways and were affected by these evolutionary changes, adapted to the new requirements.

**Kranz anatomy development**

The most significant feature towards C₄ evolution was the development of the Kranz anatomy. Shortest distance between mesophyll cell and to the next bundle sheath cell is pivotal for establishing an efficient CO₂ concentrating mechanism. Therefore, in planar leaves enhancement of the vein density is essential. A higher vein density also increased 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).

Considerably leaf architecture may vary in the various mono and dicotyledonous C₄ lineages. Typically all C₄ plants have a wreath-like structure of mesophyll and bundle sheath cells around the vascular bundles (Kranz anatomy). Location of mesophyll cells are always toward the outer face of the leaf and so remain in contact with the intercellular air space, while bundle sheath cells arrangement are internal to the mesophyll cells and hence close to the vascular tissues. The high densities of plasmodesmata lead to mesophyll and bundle sheath cells of C₄ species in close proximity (Dengler and Nelson 1999).

A comparative analysis of the leaf development in both monocot and dicot C₃ and C₄ species revealed that the close vein spacing in leaves of C₄ plants is due to changes in the initiation frequency and patterning of the minor and not the major veins (Ueno et al., 2006; McKown and Dengler...
The greater vein density observed in C₄ compared to C₃ leaves resulted from either modifications of auxin production or allocation and/or modifications of the competency of ground tissue cell to become procambial cells (McKown and Dengler 2009). Since the molecular events causing the initiation of veins are not clear in C₃ model plants, so it is presently challenging to predict the changes that led to the C₄ typical leaf anatomy.

Typically, C₃ plants bundle sheath cells have low photosynthetic activity because they possess only a few chloroplasts. Bundle sheath to mesophyll cells ratio increases with higher vein densities. Since only the mesophyll cells show high photosynthetic activity of a leaf, with a given size decreases. The evolutionary pressure could have led to an increase of the number of chloroplasts in the bundle sheath cells to maintain the overall photosynthetic activity. For metabolizing the photorespiratory Gly in bundle sheath cells an enhancement of chloroplast numbers in cells is necessary, which would also require an increase in the numbers of mitochondria and peroxisomes.

The Photorespiratory CO₂ Pump during Transition of C₃-C₄ Photosynthesis

Photorespiratory metabolites are a carbon source that can be exploited to improve the efficiency of Rubisco in C₃ leaves (Hunt et al., 1987; von Caemmerer, 1989; Rawsthorne, 1992). A photorespiratory Gly shuttle is a common feature in all extant C₃-C₄ intermediate that pumps CO₂ into the bundle sheath cells (Bauwe, 2010). This is obtained by localising 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 CO₂ fixation in two ways. Inside the leaf all photorespiratory CO₂ is set free far apart from the outer surface. Therefore several cell layers diffusion is necessary, before it could escape from the leaf. Therefore it enhances chances of refixing the photorespired CO₂ the plants and minimizes the loss of carbon due to photorespiration. Since the mitochondria concentrate adjacent to the vascular bundles thus in some C₃-C₄ intermediate species this refixation capacity is supported by the spatial distribution of the organelles within the bundle sheath cell (Rawsthorne et al., 1998). Besides, the Gly shuttle enhances the CO₂ 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). Photorespiratory CO₂ pumps occur in some two dozen species in Alternanthera (Amaranthaceae), Panicum and Neurachne (Poaceae), Parthenium (Asteraceae), Moricandia (Brassicaceae) and Flaveria (Asteraceae) (Monson, 1999). It is assumed that the establishment of such a photorespiratory CO₂ pump is an important intermediate step on the way toward C₄ photosynthesis.

A photorespiratory CO₂ pump can easily be accomplished at the molecular level. Gly decarboxylase multienzyme complex encoded by single gene expression had to be restricted to the bundle sheath cells. This might had been achieved through relatively subtle changes in the cis-regulatory elements that control the expression of these genes (Akyildiz et al., 2007).

In Moricandia arvensis (the C₃-C₄ intermediate species) for example, only the P subunit of Gly decarboxylaseis 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 the other subunit genes were also expressed specifically or at least preferentially in the bundle sheath cells (Morgan et al., 1993).

Relative to C_3 species, the intermediates also exhibit close vein spacing, enlarged bundle sheath cells, increased frequency of plasmodesmata between mesophyll and bundle sheath cells, and increased number of organelles in the bundle sheath cells (Brown et al., 1983; Rawsthorne, 1992). These developments facilitated efficient function of the glycine shuttle by reducing diffusion distances, increasing intercellular transport, and enhancing metabolic capacity in the bundle sheath. In doing so, they established the anatomical and ultrastructural framework required for the subsequent evolution of C_4 metabolism.

An increase in the levels of carbonic anhydrase and PEPC in the cytosol of the mesophyll cells might have been the next step toward true C_4 photosynthesis. This would have accompanied 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 Flaveria species (C_3 plant) which do not exhibit C_4 cycle activity yet (Ku et al., 1991; Engelmann et al., 2003).

The remaining C_4 cycle enzymes must have been elevated to establish a limited C_4 cycle activity. It is suggested that even in C_3 plants the activity of the decarboxylating enzymes NADP-ME and NADME is massively increased in vascular tissues (Hibberd and Quick 2002). Therefore the related genes expression must have been shifted to the bundle sheath cells. Enhancement of chloroplastic pyruvate orthophosphate dikinase expression might have occurred for allowing an efficient PEP regeneration and to complete the C_4 cycle. In this phase of C_4 evolution plants exhibit high activities of C_4 cycle enzymes, but still in the mesophyll cells Rubisco has high activity. Consequently, CO_2 is only partially fixed through the C_4 pathway.

The C_4 cycle establishment

The spatial separation of the two carboxylation reactions was the key step in establishing true C_4 photosynthesis and to integrate the C_4 and Calvin-Benson cycle. PEPC was restricted to the mesophyll and Rubisco to the bundle sheath cells. This step was 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 photo assimilated CO_2 in the vast majority 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. Characteristic of the C_4 photosynthetic pathway are determined by the extensive shuffling of metabolites within mesophyll and bundle sheath cells of organelles and the cytosol respectively. C_4 pathway evolution requires the proper establishment of transport capacity. For instance, in NADP-ME type plants for every fixation of CO_2 molecule, one molecule of pyruvate and oxaloacetate have to be transported into the mesophyll chloroplasts and in a countermove PEP and malate have to be translocated to the cytosol. On the other
hand, in the bundle sheath cells entry of malate leads to exit of pyruvate from chloroplast matching the CO$_2$ assimilation rate. Transcriptome and proteome analyses at large scale shows that other pathways related to sulfur, nitrogen, and carbon metabolism were also altered with respect to either overall activity or to mesophyll/bundle sheath compartmentation (Friso et al., 2010; Brautigam et al., 2011). It was might be because of energy supply difference and reduction equivalents in the different tissues and for optimization of overall integration of the various metabolic pathways.

**Alterations at Gene Expression level**

C$_4$ photosynthesis evolution was accompanied by massive alteration in quantitative and spatial gene expression. The quantitative alterations in C$_4$ evolution can be observed in *Cleome* species. When the transcriptomes of mature leaves of the C$_4$ plant *Cleome gynandra* and the closely related C$_3$ species *Cleome spinosa* were quantitatively compared by a RNA-Seq-based digital gene expression approach, then about 2.8% of the detected transcripts differed significantly in their abundance between the two species (Brautigam et al., 2011). It can be expected that the expression levels of genes in the C$_4$ cycle, the photorespiratory pathway, and the photosynthetic light reactions including several other pathways also changed. It can be seen in the C$_4$ Cleome, which showed reduced steady-state levels transcripts for the shikimate pathway, and amino acid metabolism (Brautigam et al., 2011). The C$_4$ species cytosolic and plastidic protein synthesis machinery encoding components genes are down-regulated, while the genes involved in starch metabolism, cofactor synthesis, and nitrogen metabolism showed higher steady-state transcript levels in C$_4$ leaf (Brautigam et al., 2011). The Spatial gene expression alteration patterns can be seen in Maize. Sawers et al., (2007) reported that about 18% of the genes in maize (*Zea mays*) are differentially expressed between mesophyll and bundle sheath cells. It showed that the establishment of C$_4$ photosynthesis involved a dramatic redesign and restructuring of leaf functions. At the molecular level, most of the quantitative and qualitative changes in gene expression are not yet understood and only a few have been analyzed in great detail. These things demonstrate the flexible nature in achieving the desired goal, i.e. different alteration pattern for different genes for their adaption and functioning in the C$_4$ pathway (Hibberd and Covshoff, 2010).

Transcriptional control can help to achieve cell-specific gene expression. For example, specific gene expression of the photosynthetic PEPC of the mesophyll, the C$_4$ plant *Flaveria trinervia* ppcA depends on a cis-regulatory element, the MESOPHYLL EXPRESSION MODULE1, whose location is about 1,900 bp upstream of the transcriptional initiation site (Gowik et al., 2004). In the C$_3$ *Flaverias* a very similar element was found in the promoters of the orthologous ppcA genes; however, direct mesophyll specificity was lacking in these elements. Thus, for a gene with no apparent expression specificity into a mesophyll, slight modifications within a cis-regulatory element were sufficient to convert them into a mesophyll-specific gene (Akyildiz et al., 2007).

In contrast, regulation mainly at the posttranscriptional level was reported for the bundle sheath-specific expression of one of the genes encoding the small subunit of Rubisco in the C$_4$ plant *Flaveria bidentis* (Patel et al., 2006). Most likely, in mesophyll and bundle sheath cells the FbRcS1 transcripts are differentially stable. This is controlled by stability determinants that are located in the 5’ and 3’ untranslated regions of the mRNA (Patel et al., 2006).
Figure 1. C₄ Photosynthesis

C₃ + Anatomy Change + Biochem Change + Fine Tuning = C₄

Figure 2. A. The NADP-ME grass *Themadatriandra* leaf structure and C₄ metabolic pathways of with the PCR tissue in the mestome sheath (left panel) and B. The NAD-ME grass *Panicum effusum* with the PCR tissue in the bundle sheath layer that resides outside the mestome sheath (right panel).

Figure 3. Transition from C₃ to C₄ Photosynthetic Pathway
Figure 4 The photorespiratory CO₂ pump

The transition from C₃ to C₄ photosynthesis is associated with the massive changes in gene expression that were associated with the fact that C₄ evolution must have been easy in genetic terms implies that preexisting gene regulatory networks in C₃ plants were probably the foundation for multiple evolutionary changes toward C₄ photosynthesis (Matsuoka 1995). In C₃ plants the gene regulatory networks assure a coordinated response of genes involved in photosynthesis and related metabolic pathways (Mentzen and Wurtele, 2008). The promoters driving mesophyll or bundle sheath specific gene expression in C₄ species partly maintain their cell preference of expression in C₃ species (Matsuoka et al., 993; Engelmann et al., 2008), suggesting that the C₄ plants gene regulatory networks controlling the development and differentiation of mesophyll and bundle sheath cells are not fundamentally different from those of C₃ species. Therefore, it can be concluded that the regulating networks for developmental and metabolic processes operated already in C₃ ancestral angiosperms and could serve as a platform for the establishment of C₄ leaf anatomy and metabolism.

Since our understanding of gene regulatory networks controlling the development and anatomy of a typical leaf of a C₃ angiosperm is not explicit. 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 C₃ and C₄ plants except the things discussed above. The only exceptions are the GOLDEN2-LIKE (GLK) transcription factors GLK1 and GLK2. In all land plants this pair of transcription factors occurs. The GLK proteins are largely redundant in Arabidopsis and control the expression of more than 100
genes. These genes are mainly connected with photosynthesis. In maize the two GLK genes are expressed differentially with GOLDEN2 in the mesophyll and bundle sheath, specifically affecting only chloroplast development in the bundle sheath cells (Waters and Langdale, 2009). Therefore, in mesophyll/bundle sheath differentiation in the C₄ plant maize one of the important components of the gene regulatory network appears as the GLK proteins.

**Enzyme properties optimization**

The non-photosynthetic isoforms gave rise to all C₄ cycle enzymes. The C₄ pathway ensures high fluxes, as compared to the original metabolic environment the concentration of substrates and effector metabolites is elevated in the ancestral C₃ species. Accordingly, the C₄ isoforms evolution involved changes in their kinetic and regulatory properties. Perhaps the C₄ isoform of PEPC is the best-documented example for these evolutionary processes (Gowik and Westhoff, 2010). In comparison to the nonphotosynthetic PEPCs, C₄ PEPCs bind PEP with a lower affinity, however their affinity to the other substrate, i.e. bicarbonate, is enhanced. The allosteric inhibitors of the C₄ PEPC isoforms are Asp and malate, towards which they are more tolerant and have strongly affected by the allosteric activators Glc-6-P or Gly. Relatively small changes in primary enzyme structure were responsible for these differences in enzymatic properties. In *Flaveria trinervia* (C₄) and *Flaveria pringlei* (C₃) the pair of orthologous ppcA PEPCs shares 96% identical amino acid positions. This was used as an experimental system to identify some of the evolutionary changes at the amino acid level resolution (Westhoff and Gowik, 2004). Certain constraints were subjected at the molecular alteration level that is given by the enzyme’s properties.

An Ala to Ser exchange in the C-terminal part of the enzyme is closely related to the lower affinity for the substrate PEP (Blasing *et al.*, 2000). It is found in all C₄ PEPCs analyzed so far but lacking in nonphotosynthetic or Crassulacean acid metabolism PEPC isoforms (Gowik and Westhoff, 2010). However, within the grass family C₄ PEPCs independently evolved at least eight times, then also the resulting enzymes show high degree of similarity. 21 amino acid positions showed a strong positive selection (Christin *et al.*, 2007). Out of these 21 amino acid positions only two of them are also important for the evolution of dicot C₄ PEPCs. This suggests the special requirements for grass C₄ PEPCs in comparison to dicot C₄ PEPCs. Alternatively, within grasses this can also be inferred that in comparison to the first origins of C₄ photosynthesis most of the dicot C₄ lineages are very young (Ehleringer *et al.*, 1997; Sage, 2004). Thus it indicates that the C₄ PEPCs of the grass family are much more optimized for their role in C₄ photosynthesis than their dicot counterparts. Within the photosynthetic PEPCs of the grasses the higher degree of convergence can be understood through this.

The unique kinetic and regulatory properties were also acquired by the C₄ NADP-ME during their evolution from nonphotosynthetic isoforms. The malate and differences in tetramerization of the enzyme leading to an altered pH dependent inhibition in distinct enzyme regions could be identified (Detarsio *et al.*, 2007). An alteration in the cellular location of the enzyme also involve in adaptation of C₄ enzymes to the new metabolic context of the C₄ pathway. For instance, the photosynthetic carbonic anhydrase gene of F. bidentis (FbCA3). Highly expressed gene in the mesophyll cells (Tetu *et al.*, 2007) evolved from a chloroplast-targeted ancestral carbonic anhydrase gene. The ancestral enzyme mutation in the
chloroplast transit peptide, the C₄ isoform changed to a cytosolic enzyme (Tanz et al., 2009). However, higher expression of this ancestral carbonic anhydrase gene was already reported in leaves, reflecting the minor importance of the intracellular localization of the protein and during evolution they were altered. During C₄ evolution, so far it is not explicitly understood to the extent the modification of indirect related enzymes of the C₄ pathway.

**Change of C₃ crops into C₄ photosynthesis**

Ensuring food security and protecting the environment for the world is a continuing challenge (Evans, 1998) and requires a second Green Revolution. For covering the energy demands green energy from plant biomass is being developed and that might compete with food production for feeding the 21st century growing population for terrain and resources in the future. Adequate increase of crop production in a sustainable manner will be challenging both in terms of harvestable yield and total biomass. Since C₄ plants has high photosynthetic capacity and better nitrogen and water resources use efficiency. In recent years C₄ photosynthesis has received greater interest and thus it is being considered to transfer C₄ photosynthesis into current C₃ crops (Sheehy et al., 2007). Currently in rice, attempts to implement a C₄-CO₂ concentration pathway are under way.

The prerequisite for the success of this endeavor is the knowledge about the genetic architecture of C₄ photosynthesis and the underlying gene regulatory networks. Different approaches are needed for elucidating these networks. Large scale forward-genetic (with mutagenized rice and Sorghum bicolor) as well as reverse-genetic approaches are being carried out for identifying the genes involved in C₄ subtraits like a reduced CO₂ compensation point, high vein density, or enlarged bundle sheath cells. The transcriptomes, proteomes, and metabolomes analysis of different developmental stages of C₄ leaves will help in understanding the regulation of C₄ leaf differentiation and the establishment of Kranz anatomy. The transcriptomes level comparison of closely related C₃ and C₄ species from genera like *Flaveria* or *Cleome* (Brautigam et al., 2011) illuminates the evolutionary trajectories of C₄ photosynthesis and reveals the gene repertoire requirement for the transition of a C₃ into a C₄ plant.

In conclusion, the current scenario’s complication is the manipulation of the biosphere by human beings. Particularly, increase in the atmospheric CO₂ could halt the rise of new C₄ life forms and may lead to the reduction of existing ones (Edwards et al., 2001). However, other global variables such as climate change, global warming and deforestation favor certain C₄ species (Sage and Kubien 2003). Thus, rise in CO₂ may threat many C₄ species but C₄ photosynthesis as a functional type should not be threatened in the near term (Sage et al., 1999b).

Another avenue for the rise of novel C₄ species is under way by humanity namely the of C₄ photosynthesis into C₃ crops (Sheehy et al., 2000; Miyao 2003). Research in the natural pathways for C₄ evolution may be an important endeavor for overcoming the developmental barriers to C₄ photosynthesis. The identification of the key regulators of C₄ traits, and their integration and generation of a strategy of how the C₃ plant rice must be genetically altered to introduce the C₄ pathway should become a milestone in the relatively young field of synthetic biology.

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