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The Evolutionary Path from C_3 to C_4 Photosynthesis: A Review

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A B S T R A C T

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_2around 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 forC_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_4plants. 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.

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 floras 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 chemoautotrophic 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 pCO_2during the Tertiary period reveals the appearance of C_4 plants in the fossil record (Ehleringer *et al.,* 1991; Sage 2001). Therefore, low pCO_2 might have been an important driving force for evolution of the pathway.

A complex combination of both biochemical and morphological specialization provides elevated pCO_2 at the site of Rubisco

carboxylation in all C_4 species. This result in suppression of photorespiration and allowing Rubisco to work near to its maximal rate, such that CO_2 assimilation in C_4plants is effectively CO_2 saturated in air (Hatch 1987).In most C_4 plants a division of labor between two distinct, specialized leaf cell types, the mesophyll and the bundle sheath cells leads to the CO_2 concentration mechanism except in some species where C_4 functions within individual cells(Edwards *et al.*, 2004). Since, Rubisco works more efficiently in C_4 than C_3 plants and can operate under high CO_2 concentrations in the bundle sheath cells. Indeed, C_4 species are also characterized by greater nitrogen and water use efficiency relative to C_3 plants. The increased nitrogen use efficiency is largely accounted for saving nitrogen in Rubisco protein (Evans and von Caemmerer 2000) because C_4 plants need less of this enzyme, however in C_3 plants leaves it is the most abundant protein. Since the rate of photosynthesis per unit nitrogen in the leaf is increased in C_4 species (Oaks 1994). Better water use efficiency of C_4 relative to C_3 plants accounted from its CO_2 concentration mechanism even when keeping their stomata more closed. Thus reducing the water loss by transpiration (Long 1999).

Initially, in the mesophyll cells of C_4 plants CO_2 is converted to bicarbonate by carbonic anhydrase, which is then fixed by phosphoenolpyruvate (PEP) carboxylase into C_4 acids(oxaloacetate) using PEP as CO_2 acceptor. Oxaloacetate is rapidly converted to the more stable C_4 acids malate or Asp that diffuses to the bundle sheath cells and is decarboxylated there to supply CO_2 for Rubisco. Three basic biochemical subtypes of C_4 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 CO_2 in the bundle sheath cells. This results in release of CO_2 and a three-carbon compound, which diffuses back to the mesophyll cells. Here, at the end the primary CO_2 acceptor PEP is regenerated by pyruvate orthophosphate dikinase by the consumption of two molecules of ATP (Hatch, 1987). In NADP-ME subtype of C_4 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 CO_2 diffusion and the relative biochemical capacities of the C_3 and C_4 cycle determine the efficiency of the C_4 pathway and the pCO_2

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 Sb ranges from 0.6-3.1 m^2 m^{-2} (Apel and Peisker 1978; Brown and Byrd 1993).

Neverthless 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 grassesand 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), representsing 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 C_4 syndrome may therefore be best described as a polygenic, quantitative trait. The concept of C_4 photosynthesis being as a quantitative trait immediately implies a number of questions that what is the genetic architecture of C_4 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, C_4 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 CO_2 pump (Fig. 4). An elevated phosphoenolpyruvate carboxylase activity and subsequently an increase in the other C_4 cycle enzymes and transporters accompanied by their compartmentalized expression established the C_4 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 C_4 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 CO_2 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 C_4 lineages. Typically all C_4 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 C_4 species in close proximity (Dengler and Nelson 1999).

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).The greater vein density observed in C_4 compared to C_3leaves 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 Dengler2009). 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 C_4 typical leaf anatomy.

Typically, C_3 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 photorespiratoryGly in bundle sheath cells an enhancement of chloroplast numbers in cellsis necessary, which would also require an increase in the numbers of mitochondria and peroxisomes.

The Photorespiratory CO_2 Pump during Transition of C_3-C_4 Photosynthesis

Photorespiratory metabolites are a carbon source that can be exploited to improve the efficiency of Rubisco in C_3 leaves (Hunt *et al.*, 1987; von Caemmerer, 1989; Rawsthorne, 1992). A photorespiratoryGly shuttle is a common feature in all extant C_3–C_4 intermediate that pumps CO_2 into the bundlesheath 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 CO_2 fixation in two ways. Inside the leaf all photorespiratory CO_2 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 $CO_2$the plants and minimizes the loss of carbon due to photorespiration. Since the mitochondria concentrate adjacent to the vascular bundles thus 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 (Rawsthorne *et al.*, 1998). Besides, the Gly shuttle enhances the CO_2 concentration within thebundle 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 CO_2 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 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. 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* (theC_3-C_4 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 werealso 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_4photosynthesis. This would have accompanied in recapturing the photorespiratoryCO_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_4cycle 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 C4 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_4cycle 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, thephotorespiratory 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_3Flaverias 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 Rubiscoin 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 C_4 Photosynthesis

Figure.2 A. The NADP-ME grass *Themadatriandra* leaf structure and C_4 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)

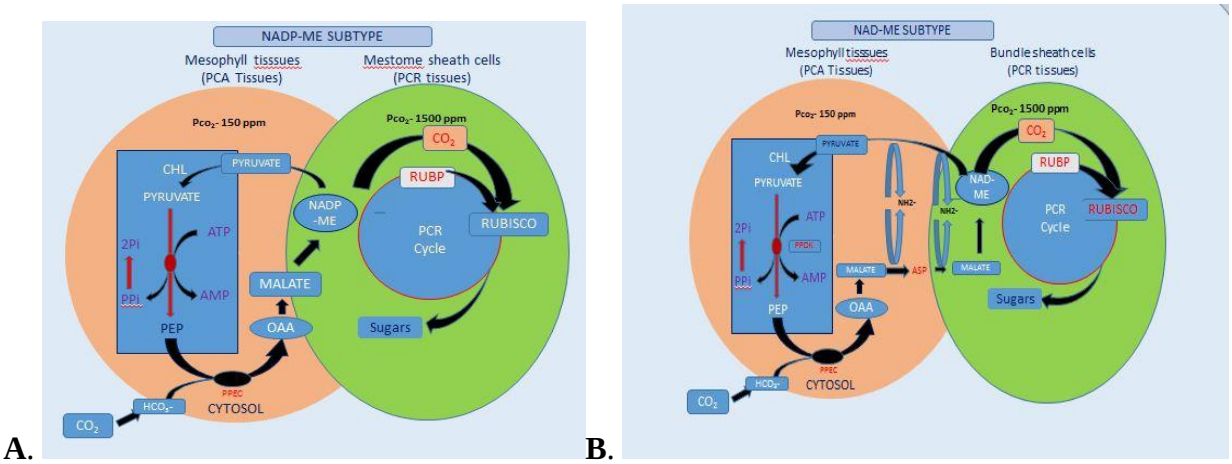


Figure.3 Transition from C_3 to C_4 Photosynthetic Pathway

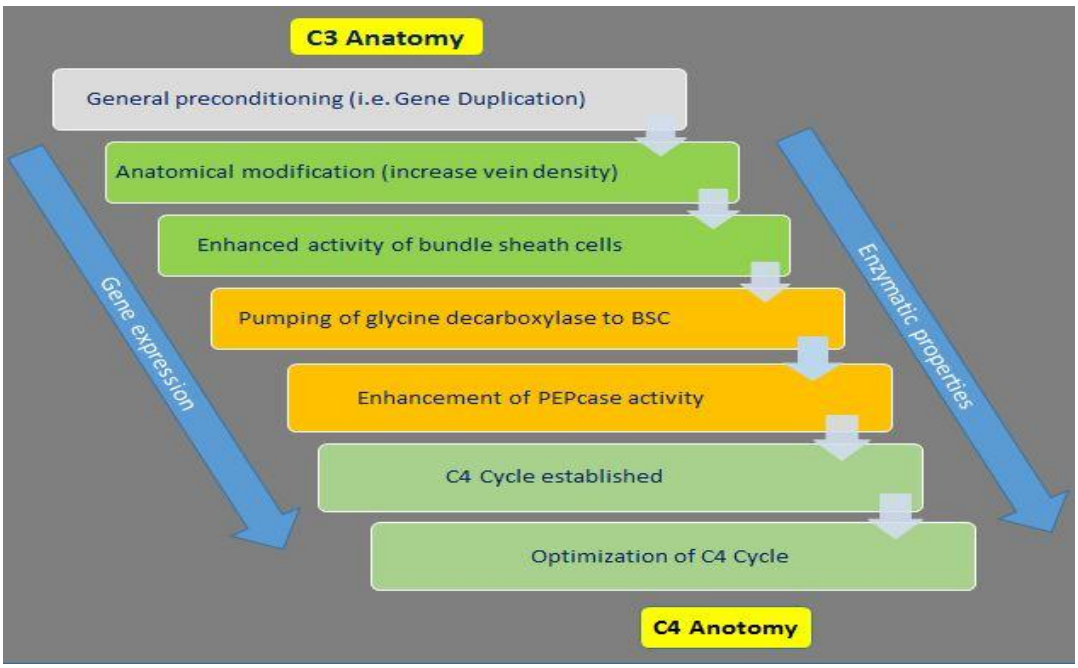
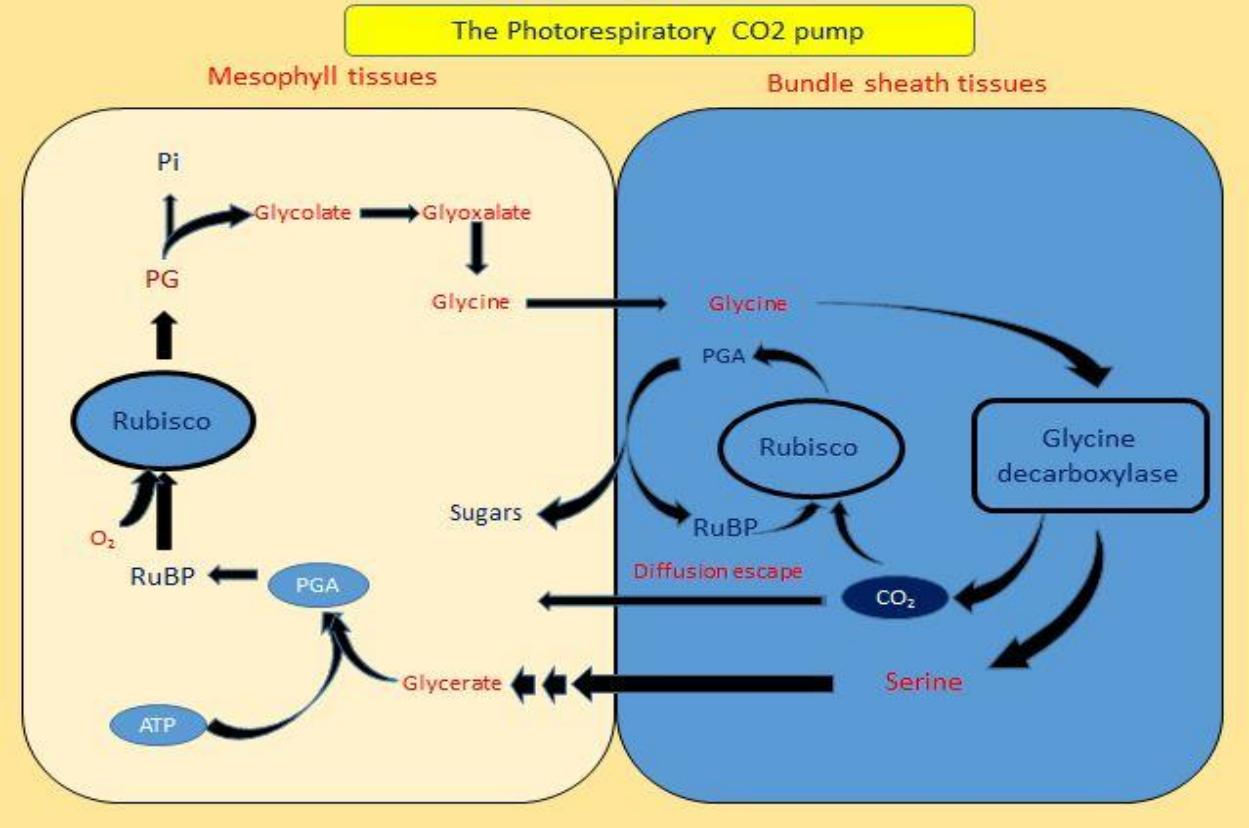


Figure.4 The photorespiratory CO_2 pump



The transition from C_3 to C_4 photosynthes is associated with the massive changes in gene expression was associated 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_4photosynthesis (Matsuoka 1995). In C_3 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 C_4 species partly maintain their cell preference of expression in C_3 species (Matsuoka *et al.,* 993; Engelmann *et al.,* 2008), suggesting that the C_4 plants gene regulatory networks controlling the development and differentiation of mesophyll and bundle sheath cells are not fundamentally different from those of C_3 species. Therefore, it can be concluded that the regulating networks for 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.

Since our understanding of gene regulatory networks controlling the development and anatomy of a typical leaf of a C_3 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 C_3 and C_4 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 C_4 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 C_4 cycle enzymes. The C_4 pathway ensures high fluxes, as compared to the original metabolic environment the concentration of substrates and effector metabolites is elevated in the ancestral C_3 species. Accordingly, the C_4 isoforms evolution involved changes in their kinetic and regulatory properties. Perhaps the C_4 isoform of PEPC is the best-documented example for these evolutionary processes (Gowik and Westhoff, 2010). In comparison to the nonphotosynthetic PEPCs, C_4 PEPCs bind PEP with a lower affinity, however their affinity to the other substrate, i.e. bicarbonate, is enhanced. The allosteric inhibitors of the C_4 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* (C_4) and *Flaveria pringlei* (C_3) 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 C_4 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 C_4 PEPCs. This suggests the special requirements for grass C_4 PEPCs in comparison to dicot C_4 PEPCs. Alternatively, within grasses this can also be inferred that in comparison to the first origins of C_4 photosynthesis most of the dicot C_4 lineages are very young (Ehleringer *et al.,* 1997; Sage, 2004). Thus it indicates that the C_4 PEPCs of the grass family are much more optimized for their role in C_4 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 C_4 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 C_4 enzymes to the new metabolic context of the C_4 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 C_4 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 C_4 evolution, so far it is not explicitly understood to the extent the modification of indirect related enzymes of theC_4 pathway.

Change of C_3 crops into C_4 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 C_4 plants has high photosynthetic capacity and better nitrogen and water resources use efficiency. In recent years C_4 photosynthesis has received greater interest and thus it is being considered to transfer C_4 photosynthesis into current C_3 crops (Sheehy *et al.*, 2007). Currently in rice, attempts to implement a C_4-CO_2 concentration pathwayare under way.

The prerequisite for the success of this endeavor is the knowledge about the genetic architecture of C_4 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 C_4 subtraits like a reduced CO_2 compensation point, high vein density, or enlarged bundle sheath cells. The transcriptomes, proteomes, and metabolomes analysis of different developmental stages of C_4 leaves will help in understanding the regulation of C_4 leaf differentiation and the establishment of Kranz anatomy. The transcriptomes level comparison of closely related C_3 and C_4 species from genera like *Flaveria* or *Cleome* (Brautigam *et al.*, 2011) illuminates the evolutionary trajectories of C_4 photosynthesis and reveals the gene repertoire requirement for the transition of a C_3 into a C_4 plant.

In conclusion, the current scenario's complication is the manipulation of the biosphere by human beings. Particularly, increase in the atmospheric CO_2 could halt the rise of new C_4 life forms and may lead to the reduction of existing ones (Edwards *et al.*, 2001). However, other global variables such as climatechange, global warming and deforestation favorscertain C_4 species (Sage and Kubien 2003). Thus, rise in CO_2 may threat many C_4 species but C_4 photosynthesis as a functional type should not be threatened in the near term (Sage *et al.*, 1999b).

Another avenue for the rise of novel C_4 species is under way by humanity namely the of C4 photosynthesis into C_3 crops (Sheehy *et al.*, 2000; Miyao 2003). Research in the natural pathways for C_4 evolution may be an important endeavor for overcoming the developmental barriers to C_4 photosynthesis. The identification of the key regulators of C_4 traits, and their integration and generation of a strategy of how the C3 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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