

A basal carbon concentrating mechanism in plants?

Eduardo Zabaleta^{1*}, M. Victoria Martin¹ and Hans-Peter Braun²

¹Instituto de Investigaciones Biológicas IIB-CONICET-UNMdP, Funes 3250 3er nivel
7600 Mar del Plata, Argentina

²Institute for Plant Genetics, Leibniz Universität Hannover, Herrenhäuser Str. 2
D-30419 Hannover, Germany

Abstract

Many photosynthetic organisms have evolved inorganic carbon (Ci) concentrating mechanisms (CCMs) that increase the CO₂ concentration in the vicinity of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RubisCO). Several CCMs have been described in great detail, like Four carbon (C₄) and Crassulacean acid metabolism (CAM), bicarbonate accumulation systems and capsular structures around RubisCO. These systems are believed to have evolved several times as a mechanism that to acclimate organisms to unfavourable growth conditions. Based on recent experimental evidence we propose the occurrence of another more general CCM system present in all plants. This basal CCM (bCCM) is supposed to be composed of mitochondrial carbonic anhydrases (a β -type carbonic anhydrase and the γ -type carbonic anhydrase domain of the mitochondrial NADH dehydrogenase complex) and probably further unknown components. The bCCM is proposed to reduce leakage of CO₂ from plant cells by allowing efficient recycling of mitochondrial CO₂ for carbon fixation in chloroplasts.

***Corresponding author: Dr. Eduardo Zabaleta** Instituto de Investigaciones Biológicas IIB-CONICET-UNMdP, Funes 3250 3er nivel 7600 Mar del Plata, Argentina e-mail: ezabalet@mdp.edu.ar

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Abbreviations C3: Three Carbon, C4: Four Carbon, CA: Carbonic Anhydrase, CAL: Carbonic Anhydrase Like, Cam: Carbonic Anhydrase Methanosarcina sp., CAM: crassulacean acid metabolism, CCM: Carbon Concentrating Mechanism, Ci: inorganic Carbon, CMS: Cytoplasmic Male Sterility, DiT: Dicarboxylic Acid Translocase, EZA: EthoxZolAmide, gcvT: cyanobacterial plant-like glycine decarboxylase, glc: cyanobacterial glycolate dehydrogenase, HCR: High Carbon Requiring, HPR: Hydroxypyruvate Reductase, LCI: Limiting-CO₂-Inducible, odc: cyanobacterial oxalate decarboxylase, PEP: PhosphoEnolPyruvate, RubisCO: Ribulose-1,5-bisphosphate Carboxylase/Oxygenase, SSU: Small SubUnit, SHMT: Serine HydroMethyl Transferase, TCA: Tricarboxylic Acid Cycle, tsr: cyanobacterial tartronic semialdehyde reductase.

Key words: Carbon concentrating mechanism, plant, green algae, mitochondria

1. Introduction

Carboxylation of ribulose-1,5-bisphosphate by the enzyme Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RubisCO, E.C. 4.1.1.39) is the main source of organic carbon for almost all living organisms. However, due to a side activity of RubisCO, oxygenation of ribulose-1,5-bisphosphate also can take place. The ratio of the carboxylation and oxygenation reactions catalyzed by RubisCO depends on the CO₂ and O₂ concentration in the vicinity of the enzyme [1]. On a global scale, one out of four reactions catalysed by RubisCO leads to oxygenation of ribulose-1,5-bisphosphate. However, oxygenation can be much higher under certain conditions, for example high temperatures or aridity dry conditions. As a consequence of the oxygenation of ribulose-1,5-bisphosphate, one molecule of 3-phosphoglycerate and one of 2-phosphoglycolate are formed in chloroplasts.

Since 2-phosphoglycolate represents a potent inhibitor of photosynthesis, it has to be converted into other compounds. This mainly takes place by the photorespiration pathway: The process of photorespiration is based on this: The 2-phosphoglycolate is first dephosphorylated to glycolate in the chloroplast and transported into the peroxisome where it is oxidized to glyoxylate. This reaction is linked to hydrogen peroxide (H₂O₂) formation, which is then detoxified by a peroxisomal catalase. Subsequently, glyoxylate is aminated into glycine which is then transported to the mitochondrion. In mitochondria, two molecules of glycine are converted into one serine by the glycine decarboxylase (E.C. 2.1.2.10) and serine hydromethyl transferase (EC 2.1.2.1-SHMT) enzymes. This conversion is linked to reduction of NAD⁺ and liberation of CO₂ and NH₄⁺. Based on this conversion, three out of four carbon atoms are recovered for primary carbon metabolism. Serine formed in mitochondria is next transported back to the peroxisome where it is deaminated into hydroxypyruvate and reduced to glycerate by hydroxypyruvate reductase (EC 1.1.1.81-HPR). A second but cytosolic HPR has recently been discovered and was proposed to allow a bypass of this

part of photorespiration pathway and/or to be important in the context of a metabolic overflow protection mechanism [2]. Finally, glycerate is phosphorylated by glycerate kinase (EC 2.7.1.31) in the chloroplast to form 3-PGA, which can be fed back into the Calvin cycle. Since the whole metabolic pathway only takes place in the light and leads to liberation of CO₂ it is designated “photo-respiration”. The pathway of course co-exists with classical respiration (termed “dark respiration” in plants). Due to the oxygenase activity of RubisCO, it is estimated that C3 plants such as wheat or rice only fix about 55% CO₂ of what they theoretically could fix if RubisCO was solely a carboxylase. However, indirect measurements of photorespiration indicate a reduction in C_i assimilation in the range of 25-35% under present atmospheric conditions [3].

The CO₂ concentration around RubisCO is crucial for proper carbon fixation to sustain plant growth and ultimately all life on earth. During evolution, especially during periods of low CO₂ in the atmosphere, different biochemical and even anatomical strategies have independently emerged to overcome limiting carbon fixation, designated as inorganic “carbon concentrating mechanisms” (CCMs).

In addition to the previously described carbon concentrating mechanisms, we here propose, based on recent experimental findings, that all eukaryotic photosynthetic organisms contain a basal CCM (bCCM). Photosynthesis is estimated to be reduced up to 20% without such a bCCM (see below). The proposed bCCM is based on the allows recycling of mitochondrial CO₂ for carbon fixation in chloroplasts. Besides CO₂ diffusion between mitochondria and chloroplasts, we propose an active CO₂ transfer mechanism which is based on the presence of carbonic anhydrases (E.C. 4.2.1.1) and bicarbonate transporters in the two involved organelles. Carbonic anhydrases are Zn-metalloenzymes that catalyse the reversible hydration of carbon dioxide into bicarbonate. There are five types of enzymes (α , β , γ , δ and ϵ) which are not evolutionary related. they are seen as a case of convergent evolution. We propose that the proposed basal CCM system probably has evolved from

endosymbionts (α proteobacteria) during the process of chloroplast adaptation in the modern green eukaryotic cell. In this paper, experimental evidence for a bCCM is summarized. But first, the previously described CCM systems are briefly introduced and compared.

2. Types of CCMs

2.1 C4 and CAM mechanisms systems Four-carbon (C4) photosynthesis and crassulacean acid metabolism (CAM) in terrestrial higher plants were the first photosynthetic CCM to be described in detail. Both systems are based on a carbon pre-fixation step (binding of bicarbonate to phosphoenolpyruvate [PEP] by PEP carboxylase -E.C. 4.1.1.31-). The carbon pre-fixation step and the final CO₂ fixation step by RubisCO are spatially (C4) or temporally (CAM) separated.

In CAM plants, which often grow at very hot and dry locations, oxaloacetate is formed by PEP carboxylation during the night when stomata are open to allow gas exchange. Oxaloacetate next is converted into malate which can be stored in the vacuole. When stomata are closed during the day preventing loss of water, malate is decarboxylated providing CO₂ for final carbon fixation by the Calvin cycle [4].

In C4 plants, PEP carboxylation takes place in specialized mesophyll cells that also carry out the complete light reaction of photosynthesis. Malate or aspartate are transported from mesophyll cells to another type of leaf cell termed bundle sheath cells (Kranz anatomy). At this location the pre-fixed carbon is released for final CO₂ fixation by RubisCO. Due to this process, the CO₂ concentration in the bundle-sheath cells is around 10-fold higher than in normal air. Thereby, photorespiration is much reduced. At the same time, most bundle sheath cells do not carry out the complete photosynthetic light reaction [1].

The primary CO₂ fixation step catalysed by PEP carboxylase takes place in the cytosol of mesophyll cells using HCO₃⁻ as a substrate. Therefore, carbon dioxide coming from the external surroundings must be rapidly hydrated by a β-type carbonic anhydrase (βCA) and converted into HCO₃⁻. Thus, carbonic anhydrase activity is mainly found in the cytosol of mesophyll cells while in C3 plants, the highest carbonic anhydrase activity is detected within the chloroplast stroma [5].

The presence of C4 metabolism has been observed in several important crop species such as maize, sorghum and sugar cane [6] and also in submerged aquatic plants and macroalgae such as *Udotea* spp. [7] or the planktonic diatom *Thalassiosira weissflogii* [8,9], whereas CAM-like metabolism has been found mainly in terrestrial xerophytic species such as cacti, yucca and aloe but also in many aquatic plants and brown macroalgae [4,10]. In some species of *Flaveria* spp. and *Heliotropium* spp. an intermediate “C3-C4 metabolism” takes place [11]. Half of the species of *Flaveria* express intermediate traits between C3-like to C4-like forms [12]. Some C3–C4 intermediate species restrict glycine decarboxylation to the bundle sheath compartment and thereby thus concentrate CO₂ in this cell type only into the bundle sheath cells [11]. This process also was named ‘C2 photosynthesis’ because the decarboxylation of photorespiratory metabolites concentrates CO₂ around RubisCO, thereby increasing photosynthetic efficiency. Some genera have only few confirmed C3-C4 intermediate species (*Brassica*, *Alternanthera*, *Parthenium*, *Neurachne*, *Salsola*, *Cleomaceae*) [11]. Interestingly, Conversely, some amphibious species of *Eleocharis*, particularly *Eleocharis vivipara*, express C4 characteristics under terrestrial conditions and C3 characteristics under submerged aquatic conditions [13].

Three species in family Chenopodiaceae perform single-cell C4 photosynthesis without Kranz anatomy [14,15]. These species have two chloroplast types (dimorphic chloroplasts), which are biochemically and morphologically different. Each type is located in a distinct cytoplasmic domain within individual photosynthetic cells. Single cell C4 metabolism very much resembles C4

metabolism in C₄ plants with Kranz-type leaf anatomy [16]. It also is based on a CO₂ pre-fixation step catalyzed by PEP carboxylase, the occurrence of separate photosynthetic compartments domains and physiological responses typical of C₄ plants.

Photorespiration is much reduced in C₄ plants compared to C₃ plants. However, photorespiration is an essential metabolic process since mutants affecting enzymes of the 2-phosphoglycolate metabolism, e.g. glycolate oxidase (EC 1.1.3.15) of maize, are not viable in normal air but completely rescued at high CO₂ conditions. A maize *gox1* mutant rapidly accumulates glycolate when transferred to normal air, which greatly decrease efficiency of net carbon assimilation. The existence of such kind of mutants indicates that a functional photorespiratory pathway is essential for maize seedling development, most likely for detoxification of glycolate [17,18].

2.2 CCM in *Chlamydomonas* Eukaryotic green algae such *Chlamydomonas* spp. have a similar but inducible CCM in which bicarbonate is concentrated in the chloroplast stroma via several Ci transporters and α and β CAs. RubisCO is located in a specialized micro-compartment, the “pyrenoid”, where Ci fixation occurs [19].

An α CA, CAH3, is localized in the thylakoid lumen and is enriched in tubules that penetrate the pyrenoid. It plays an essential role in the rapid dehydration of the accumulated HCO₃⁻ that and thereby releases CO₂ into the pyrenoid [20]. Mutants lacking CAH3 have a non-functional CCM in which HCO₃⁻ accumulates intracellularly. However, the mutant cells cannot grow at low levels of CO₂ [21]. Furthermore, Limiting-CO₂-inducible (LCI) B and C proteins are involved in CO₂ metabolism. They form a complex of 350 kDa localized around the pyrenoid in the light [22] and were initially thought to represent Ci transporters [23,24]. However, upon genetic analyses it was proposed that these proteins may trap CO₂ released by CAH3 avoiding CO₂ leakage [22,25]. These

gene products are members of a small gene family so far only found in a few microalgae species [26]. Mutants with defects in B gene expression cannot grow in normal air but survive at very low CO₂ indicating the existence of multiple Ci transporters in different CO₂ conditions [23,25,27]. There is considerable evidence in green algae of a role in carbon capture of the plastid envelope via active transport of Ci. Photosynthetically active chloroplasts as well as intact cells grown at high or low CO₂ have low- or high-affinity Ci uptake systems, respectively [27].

Based on mathematical modeling of *Chlamydomonas* metabolism, it was proposed that an additional mitochondrial β CA could be involved in converting CO₂ from the TCA cycle or derived from photorespiration into HCO₃⁻ which is transported via anaplerotic reactions (i.e. PEP carboxylase reaction to form 4 carbon acids) back into chloroplasts for carbon fixation by RubisCO, thereby potentially limiting CO₂ leakage from mitochondria [28]. Such a role requires the presence of a bicarbonate translocase in the inner membrane of the mitochondrion, which has not yet been identified. This bicarbonate translocase would allow a controlled carbon efflux to the cytosol.

2.3 CCM in cyanobacteria The cyanobacterial CCM is based on a system that concentrates HCO₃⁻ in the matrix using a series of carbonic anhydrases and HCO₃⁻ transporters. Cyanobacterial RubisCO shows lower affinity for CO₂ than that the enzyme present in of C3 plants. The higher K_M of cyanobacterial RubisCO to CO₂ is compensated by an efficient CCM that increases the [CO₂] concentration in around the carboxylating enzyme. Furthermore, RubisCO is localized within an icosahedral proteinaceous compartment called carboxysome (α or β -type carboxysomes, see [29] for review depending on the species as reviewed in [29]). Mutants impaired in impairing functional CCM components, such as the carboxysome shell proteins or bicarbonate transporters, show very low photosynthetic affinity for external Ci. They thus have a high CO₂ requiring phenotype. These observations clearly demonstrate the importance of CCM for cyanobacterial survival in present atmospheric conditions. Both types of carboxysomes are composed of several shell proteins with

different proposed functions. The main difference between α and β carboxysomes is the RubisCO type (form A or B, respectively) and the operon encoding the shell proteins (the *csa* operon - *csaS123AB*- or by the *ccm* operon -*ccmKLMN*-, respectively) [30]. Bicarbonate dehydration is catalyzed by a specific carboxysomal carbonic anhydrase that in the case of α -carboxysome is an ϵ CA (encoded by *csaS3* gene, [31]) and in β -carboxysome a β CA, named CcaA, not integrated in the *ccm* operon [32]. One of the shell proteins of β -carboxysome, the CcmM subunit, consist of an its N-terminus which resembles a gamma type carbonic anhydrases (γ CAs) and a the C-terminus which includes consists of RubisCO small subunit-like repeats [33]. CcmM is an important component subunit of a multiprotein bicarbonate dehydration complex which also includes, together with CcaA, and CcmN (similar to CcmM). This complex, through CcmM, also interacts with CcmK and CcmL, the major shell proteins. CcmM is inactive as a carbonic anhydrase but has been proposed to be involved in the transport of bicarbonate to the inside of carboxysomes where it is converted by CcaA, into CO₂ near RubisCO [34]. However, some β cyanobacteria lack a functional *ccaA* gene. In these species, CcmM is an active carbonic anhydrase. The activity depends on an essential disulfide bond which is not conserved in other γ CA homologs [35]. *Synechocystis* spp. contain a third structurally related protein with a high similarity to bacterial and plant γ CAs. However, its function or localization is unknown.

In addition, there is evidence for the association of CA-like proteins (Chp X and Y/Cup A and B) within to the NADH dehydrogenase complex (termed NDH-1 complex) of the thylakoid and plasma membranes of cyanobacteria. These polypeptides are involved in catalysing active CO₂ uptake by converting CO₂ into bicarbonate within the cell linked to electron transport and proton translocation associated with the NDH-1 complex. Although Chp/Cup proteins have no homologies with known CA protein families, two conserved histidine residues and one conserved cysteine residue which could act as a potential Zn coordination site have been identified (a novel CA class?). Electron donation to the complex by donors such as NAD(P)H produces a reduced intermediate within the NDH-1 complex that could oxidize the Zn-H₂O to Zn-OH⁻ and H⁺. In the second step,

Zn-OH could react with CO₂ to form HCO₃ which, and together with the abstracted released proton, is are translocated across the membrane to the lumen via a proton shuttle path within the hydrophobic proton channel subunits of the NDH-1 complex [36].

Because of the presence of efficient CCM systems, the finding of a high CO₂ requiring phenotype, which completely lacks 2-phosphoglycolate metabolism (all three routes inactivated: oxalate decarboxylase *-odc-*, tartronic semialdehyde reductase *-tsr-* and the plant-like glycine decarboxylase *-gcvT-* or a double mutant in glycolate dehydrogenases *-glcD1 and D2-*) was unexpected and suggests that photosynthesis requires a functional CCM as well as a fully active glycolate detoxifying system [37]. By pulse labelling experiments using ¹³C NaHCO₃, ¹³C glycolate was detected under conditions thought to suppress photorespiration [38]. Therefore, both CCM and 2-phosphoglycolate metabolism appear to be crucial for the viability of all organisms performing oxygenic photosynthesis (cyanobacteria and plants), grown at normal CO₂ conditions.

3. Proposed basal CCM

The discovery of γ CAs in mitochondria of almost all photosynthetic eukaryotic organisms analyzed so far but not in animals or fungi led to hypothesize on their physiological role. The mitochondrial γ CAs are attached to complex I (CI) of the respiratory chain and form a spherical extra domain (named CA domain) on the matrix exposed side of its membrane arm [39].

A possible hypothesis extends a proposition originally made by Prof. John A. Raven [28] that bicarbonate translocases possibly present in the inner mitochondrial membrane in *Chlamydomonas* spp. are involved in recapturing Ci from mitochondrial decarboxylations reactions. We propose that the CA domain of complex I forms part of a mitochondrial bicarbonate export system that allows efficient transfer Ci from mitochondria to chloroplasts. The CA domain is

involved in bicarbonate formation and/or bicarbonate transfer across the inner mitochondrial membrane. Additionally, the CA domain could produce and translocate bicarbonate. Especially in the presence of high light or temperature, CO₂ in chloroplasts is usually low due to ribulose-1,5-bisphosphate carboxylation by RubisCO and due to the fact that stomata can be closed at daytime and consequently import of atmospheric CO₂ into the leaf is restricted. Chloroplast CO₂ levels are usually low due to ribulose-1,5-bisphosphate carboxylation by RubisCO and due to . At the same time, import of atmospheric CO₂ into the leaf often is restricted because the stomata are closed. and when stomata are closed, especially in the presence of high light or temperature, restricting import of atmospheric CO₂ into the leaf. At the same time, mitochondria produce excess CO₂ due to the decarboxylation reactions of the TCA cycle but especially due to glycine – serine conversion from photorespiration. While CO₂ is more or less “waste” in animal and fungal mitochondria, in plant cells it is the main substrate for photosynthetic carbon fixation in chloroplasts and, at many conditions, rate-limiting for plant growth. Part of the mitochondrial CO₂ can be re-cycled for carbon fixation by RubisCO following diffusion to the chloroplast. However, we additionally propose occurrence of a more targeted carbon transfer system which, besides other elements, is constituted by a bicarbonate export system linked to mitochondrial complex I. Bicarbonate could be transferred from the cytosol into chloroplasts by another bicarbonate translocase or by a metabolic system resembling the carbon pre-fixation steps during C₄-metabolism. In the chloroplasts, βCAs facilitate efficient re-conversion of bicarbonate into CO₂ for carbon fixation by RubisCO. Our hypothesis is summarized in [Figure 1](#). Furthermore, we propose that the CA domain of complex I has a function functions like the carboxysomal CcmM-CcmN proteins. Plant proteins could have evolved from γCAs of an ancient α protobacterium during evolution of the mitochondrial compartment. the adaptation of the first endosymbiont to be converted into a mitochondrion. These proteins are were only conserved in the plant and basal lineages of Eukarya. This could be regarded as a case of convergent evolution. Thus, We propose that all photosynthetic eukaryotic organisms contain this “basal” CCM (bCCM, Figure 1) and that this process could account for about 10 to 20% of carbon

fixation in C3 plants. In the following sections, we present recent experimental results which, according to our interpretation, support the hypothesized basal carbon transfer mechanism within plant cells.

3.1 Evidence for the basal CCM

Mitochondrial γ CAs have been described for Arabidopsis (C3, dicotyledonous), Rice (C3, monocotyledonous), Maize (C4 monocotyledonous) as well as in for some green algae such as *Chlamydomonas* (C3 with CCM), [39-46]. Surprisingly, no γ CA homologs were found in the *Ostreococcus tauri* genome, which is the smallest known eukaryotic green alga [47]. Recently, similar proteins have been found in mitochondrial complex I of *Acanthamoeba castellanii* and other aplastidic eukaryotes such as Amoebozoa, Chromalveolata and Excavata. [48,49]. It can be assumed that the function of γ CA proteins is somewhat different in plant/green algae than in aplastidic eukaryotes.

In Arabidopsis, the gammaCA family consists of five proteins encoded by five nuclear genes. Three are named γ CA1, γ CA2 and γ CA3 because they very much resemble the “prototype” γ CA from *Metanosarcina thermophila* (Cam) and two are named γ CAL1 and γ CAL2 (“gamma Carbonic Anhydrase like”) because they have a more derived sequence with respect to Cam with less conservation of important residues [50]. At the same time, the Arabidopsis CA/CAL proteins exhibit sequence similarity to cyanobacterial CcmM proteins. The CA/CAL proteins form the so called “CA domain” of complex I first discovered in Arabidopsis [44, 39]. The exact composition of this domain is not known in detail. Based on single particle electron microscopy, the CA domain most likely is a trimer [39,45,51], which nicely corresponds to the composition of Cam [52]. Since the interaction between γ CALs and γ CAs is strong and since all photosynthetic organisms analyzed so far contain at least one γ CA and one γ CAL, the CA domain most likely represents a heterotrimer

[50]. It so far is unclear if all five CA/CAL proteins of Arabidopsis are simultaneously present in individual complex I particles. All five proteins were detected by mass spectrometry within isolated complex I or within the 550 kDa membrane arm of this complex upon dissection of the holo-enzyme by low SDS (0,01%) [46]. Moreover, at slightly higher SDS concentrations (0.04%), a ~85 kDa subcomplex is detached from Complex I which includes γ CA1 and γ CA2 and the two γ CALs but surprisingly not γ CA3. Additional data suggest that γ CAL1 and γ CAL2 are not simultaneously present within individual CA subcomplexes [46]. Therefore, the CA domain of complex I seems to be composed by three CA/CAL proteins of varying identity. The physiological relevance of this heterogeneity is currently a mystery.

It so far has not been possible to measure carbonic anhydrase activity of the mitochondrial γ CAs for any plant or eukaryotic green algae although all relevant amino acids are present and properly arranged in a putative active site similar to Cam or members of a Cam subfamily termed CamH [42]. However, it was shown that cyanobacterial CcmM as well as Arabidopsis γ CA2 bind CO_2 at comparable rates [34, 53]. Amino acids essential to bind bicarbonate are also conserved [42]. Thus we propose that the CA domain of complex I could function as a carbonic anhydrase and/or bicarbonate translocator. Bicarbonate could bind the conserved Gln and Tyr residues together with a possible metal ion (Zn^{2+} or Fe^{2+}). How the bicarbonate could be transferred across the mitochondrial membrane? This currently is completely speculative. Interestingly, EM analysis of complex I from Arabidopsis revealed presence of a small cavity within its membrane arm in opposite to the point of attachment of the CA domain, possibly indicating a pore like structure and this position [44]. Furthermore, protease protection experiments indicate that the CA subunits of Arabidopsis span the membrane once and that their C-termini constitute a small domain on the intermembrane-space exposed side of the membrane arm [39]. In the micrographs of several plant-like complexes I, a cavity is clearly seen opposed to the CA domain [44] and for some experimental observations [39], the C-terminus of CA proteins would transverse the membrane exposing 2 kDa to

the intermembrane space. The C-termini of more than one subunit These C-termini could form an amphipathic channel which allows bicarbonate transfer, possibly together with protons where bicarbonate could pass possibly helped by proton transfer that could be associated as it was proposed for Cup proteins in the NDH-1 complexes in cyanobacteria [36].

Gene expression profiling data indicate that mitochondrial γ CAs are down-regulated when *Arabidopsis* is cultivated at elevated CO₂ concentrations [54]. These data indicate a possible role of the CA proteins during photorespiration [51]. Furthermore, ethoxzolamide (EZA), a strong inhibitor of γ CAs (and to a lesser extent of β CAs) impairs photosynthetic oxygen evolution in isolated protoplasts but not in isolated chloroplasts. This effect was only observed at low CO₂ concentrations with almost no effect at high CO₂ concentrations [55]. These results strongly suggest that internal CAs (outside the chloroplast) are involved in recycling of mitochondrial CO₂ in photorespiratory conditions.

Arabidopsis null mutant plants lacking the gene encoding γ CA2 contain drastically reduced amounts of complex I. A weaker effect was found in *ca3* null mutant plants. Moreover, oxygen consumption experiments carried out in the presence of different respiratory chain inhibitors using leaves and flower tissues as well as cell suspensions growing in the dark indicate that these mutant plants have increased alternative respiration. Both mutant lines did not exhibit an altered phenotype with respect of wild type plants at conditions so far tested but derived cell suspensions are growing more slowly than wild type in darkness [54].

Arabidopsis plants overexpressing γ CA2 plants show a male sterile phenotype by indehiscence of anthers due to a dramatic reduction in ROS content which seems to cause reduced lignin deposition [56] Furthermore, these plants have significantly longer roots and bigger seeds. A considerable enrichment of γ CA2 within Complex I was consistently detected (Villarreal and

Zabaleta, unpublished results) suggesting that over-expression of this protein leads to replacement of γ CA1 and γ CA3 within the trimeric CA domain attached to complex I. Homotrimers of γ CA2 bind Ci as efficiently as the cyanobacterial CcmM protein [34,53]. Both proteins are assumed to have a similar function in Ci translocation, which is especially important in the context of photorespiration [51].

Complex I abundance and activity is normal in mitochondria of plants overexpressing γ CA2. If the CA domain of complex I indeed is involved in mitochondrial bicarbonate export, a possible explanation of the observed bigger sink organs is that increased abundance of γ CA2 should allow higher rates of bicarbonate export resulting in higher CO_2 fixation rates by RubisCO and thus increasing plant growth. This increase in plant growth only can take place if γ CA2 over-expression does not impair respiration. Indeed, oxygen consumption experiments using an *Arabidopsis* cell suspension culture overexpressing γ CA2 revealed elevated oxygen consumption rates and faster growth (approx. 70% more fresh weight per week compared to wild type cells, Villarreal and Zabaleta, unpublished results).

It so far is unknown how bicarbonate gets into the chloroplasts since a cyanobacterial-like bicarbonate transporter or equivalent has not yet been identified in higher plants. One possibility is that bicarbonate exported from mitochondria reacts with PEP. The reaction is catalysed by a cytoplasmic PEP carboxylase. The resulting oxaloacetate can be converted into malate and transported into chloroplasts as in C_4 metabolism (Figure 1). All necessary enzymes are present in *Arabidopsis* (cytosolic AtPEP carboxylase [57], cytosolic malate dehydrogenase, E.C. 1.1.1.83 antiporter for malate / oxaloacetate exchange, dicarboxylic acid translocase AtDiT family, [58] and a chloroplast NADP malic enzyme, E.C. 1.1.1.40, [59,60]). The *Arabidopsis* dicarboxylic acid translocase DiT2 knockout mutant, *dct2* and tobacco DiT1 antisense plants both have a photorespiratory phenotype [57, 61]. It has also been shown that DiT1 has a high affinity for the

dicarboxylate oxaloacetate [61]. These evidences are consistent with the interpretation that malate or oxaloacetate transporters are important during photorespiration, according to our hypothesis for recycling mitochondrial C_i to carbon fixation by RubisCO. The net result is the introduction of CO_2 into the chloroplast. However, recent comparative proteome and transcriptomic analyses with C3 and C4 plants revealed that the DiT proteins specifically are enriched in the chloroplast envelopes of C4 plants [62, 63]. The DiT family members may play a role in central nitrogen metabolism [61] and for core C4 photosynthesis in maize [64]. All members of the DiT families were identified in C4 as well as in C3 plants. A recent investigation clearly showed that all enzymes required for C4 photosynthesis are present in *Arabidopsis* and that cell specific expression of the corresponding transcripts in C4 species can be explained by as yet unidentified trans-factor [65]. Several reports indicate as well that high activities of the C4 enzymes are found around the veins in C3 species and may have facilitated the polyphyletic evolution of C4 photosynthesis [66].

CMS I and II *Nicotiana sylvestris* cytoplasmic male sterile mutants have drastically reduced amounts of complex I, which affects photosynthesis under normal (photorespiratory) conditions [67]. Steady-state photosynthesis in the mutant was reduced by 20% to 30% at atmospheric CO_2 levels. The inhibition of photosynthesis was alleviated by high CO_2 or low O_2 . It was interpreted that a functional complex I is required to ensure a subcellular redox balance [3,68]. However, the importance of complex I for photosynthesis also can be explained by its proposed role in the recycling of mitochondrial C_i .

Taken together, the proposed basal CCM for efficient recycling of mitochondrial CO_2 for carbon fixation in chloroplasts, which is especially important during photorespiratory conditions, is supported by several experimental findings: (i) the CA domain of complex I is present in all plants but absent in animal and fungal mitochondria; (ii) CA proteins efficiently can bind C_i and very much resemble the cyanobacterial CcmM proteins involved in the enrichment of C_i in

carboxysomes; (iii) genes encoding CA proteins are down-regulated when plants are cultivated at elevated CO₂; (iv) mutants with reduced amounts of complex I have reduced photosynthesis rates under photorespiratory but not under non-photorespiratory conditions and (v) other components of the proposed Ci transfer system, which are necessary for Ci transfer into chloroplasts, might represent enzymes of the carbon pre-fixation step of C₄ metabolism. All necessary enzymes are also present in C₃ plants. Interestingly, some mutants of these enzymes have photorespiratory phenotypes.

4. Outlook

Photorespiration is a metabolic process of great importance. It is one of the main determining factors for biomass production in C₃ crops. Recent technological developments have allowed to address important questions concerning the photorespiratory pathway (reviewed in [3]). However, a number of aspects deserve further investigation, especially the role of metabolite transporters such as the dicarboxylic acid translocases in photorespiration and the role of the photorespiratory enzymes such as PEP carboxylase and NADP malic enzyme in C₃ plants. Last but not least, the physiological role of the CA domain of complex I, which not only occurs in C₃ plants but also in plants with other photosynthetic metabolism and in green algae, has to be further investigated. Specifically, bicarbonate export by complex I has to be analyzed, e.g. by reconstitution of plant complex I into artificial liposomes.

The proposed basal CCM involves a number of metabolites which are also part the photorespiratory [69] and other cellular pathways. Its investigation therefore is a complicated task. RNA profiling and proteomic analyses of isolated organelles [62, 70-72] may help understand this network. Isolation of organelles from different tissues or cell types will also provide interesting data about the function of the proposed basal CCM. For example, the CA domain of complex I is present

in C4 maize [45]. Do mitochondria of mesophyll as well as bundle sheath cells contain the same arrangement of complex I subunits? Future research should also investigate other organisms with differences respect to photosynthetic primary metabolism, for example green algae. The CA domain is also present in these organisms, which at the same time contain other sophisticated CCM systems. All these investigations should be of fundamental importance to achieve a better understanding of the evolution of cyanobacterial CCM to the compartmentalized (yes, this term exists and is correct in this context!!) basal CCM proposed for plants.

In the last decade, several projects have attempted to improve the photosynthetic performance of C3 crop plants. One idea is to incorporate C4 CCM into C3 crop (mainly rice) and thereby elevating CO₂ around RubisCO [73-76]. A second approach is to transfer cyanobacterial bicarbonate transporters or even other cyanobacterial CCM proteins to C3 chloroplasts to provide significant improvement in photosynthetic performance [77]. A third approach is based on the idea that - since the oxygenation reaction of RubisCO can not be eliminated - the degradation of 2-phosphoglycolate could be improved via a bacterial glycolate pathway introduced in *Arabidopsis* chloroplasts [78]. In a fourth approach, the complete design of a novel pathway to fully oxidizes 2-phosphoglycolate to CO₂ was undertaken based on the incorporation of glycolate oxidase, malate synthase and catalase into *Arabidopsis* chloroplasts [79]. And finally: alterations of the CA domain of complex I might allow to optimize photosynthesis in crops. The combination of more than one approach might one day allow developing higher yielding crops for feeding tomorrow's world.

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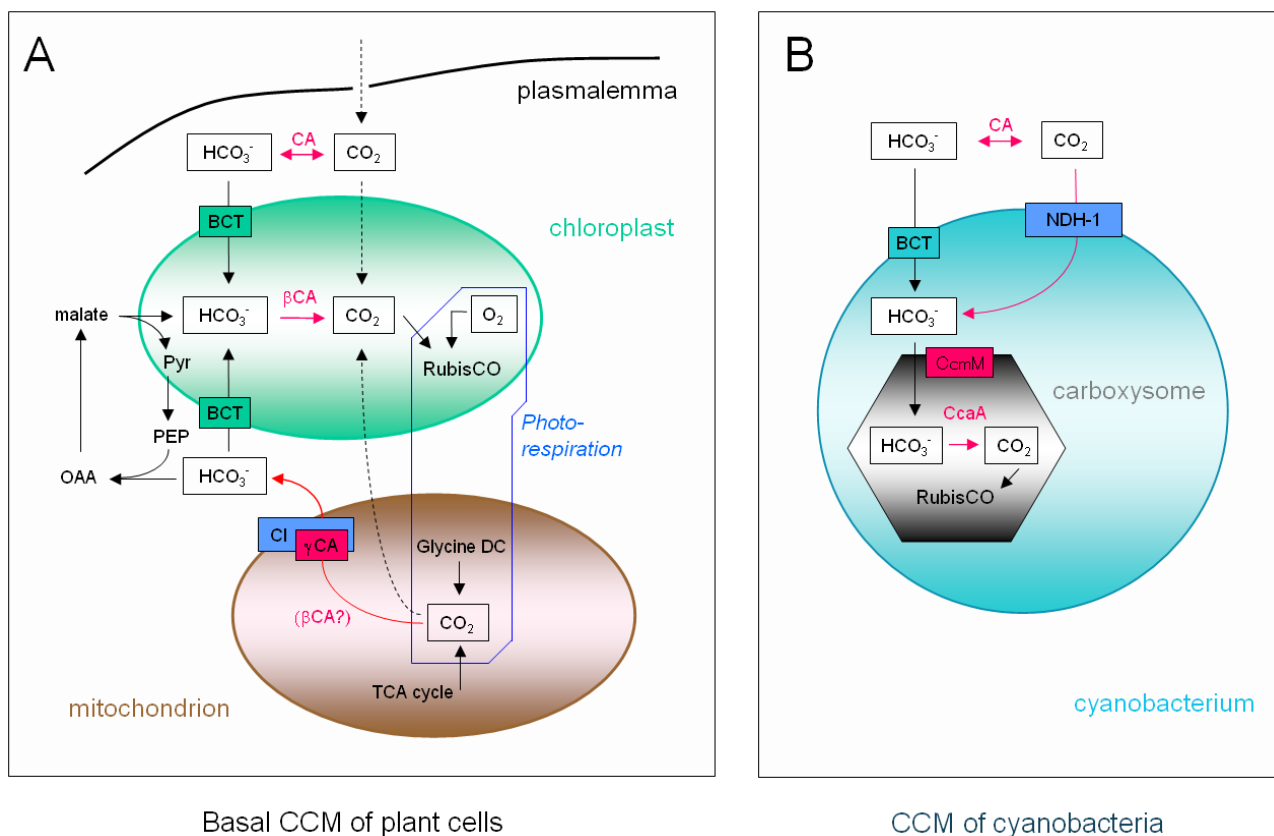


Figure 1:

Model for Carbon Concentrating Mechanism in Plants and Cyanobacteria

A- Proposed basal CCM (bCCM) in plants. The CO_2 concentration in chloroplasts often is low due to carbon fixation by RubisCO, especially if plants are cultivated under high light or at high temperatures. At the same time, large amounts of CO_2 are liberated in mitochondria due to the reactions of the TCA cycle and photorespiration. Experimental evidence indicates that mitochondrial CO_2 is recycled for carbon fixation in chloroplasts. Transfer of CO_2 could be based on diffusion but would be more efficient with an active HCO_3^- transfer mechanism. The mitochondrial CA domain (given in red) of complex I (blue) is proposed to play a role in converting CO_2 to HCO_3^- and/or transfer HCO_3^- from mitochondria into the cytosol. A matrix-localized βCA (At1g58180) might additionally be involved in HCO_3^- formation or only transfer if conversion is catalysed by a mitochondrial βCA (At1g58180). Subsequently, HCO_3^- could be transferred from the cytosol into the chloroplast by a putative bicarbonate translocator in the chloroplast envelope or by a C4-like pathway. **B-** Carbon concentration mechanism (CCM) of cyanobacteria. Cyanobacteria export a carbonic anhydrase increasing the bicarbonate concentration in their surroundings. Transporters are localized in the plasma membrane of cyanobacteria facilitating active uptake of bicarbonate or CO_2 . Besides other components, cyanobacterial complex I (NDH-1; given in blue) is involved in this process. Bicarbonate subsequently is transferred into a specialized compartment, the “carboxysome”, where CO_2 fixation occurs. Capturing of inorganic carbon within the carboxysome Translocation is based on the CcmM protein (given in red) and conversion of bicarbonate into CO_2 by on CcaA. Designations: CI: mitochondrial complex I, NDH-1: cyanobacterial complex I involved in Ci uptake, BCT: bicarbonate translocase, βCA and γCA : beta- and gamma-type carbonic anhydrases, CcmM and CcaA: carboxisomal proteins involved in Ci delivery for RubisCO, OAA: oxaloacetate, Pyr: pyruvate, PEP: phosphoenolpyruvate.

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