J Biosci (2020)45:115
DOI: 10.1007/s12038-020-00080-z

Review

Elucidation and genetic intervention of CO_2 concentration mechanism in *Chlamydomonas reinhardtii* for increased plant primary productivity

Kokkanti Mallikarjuna[1]*, Kelam Narendra[1], Ragireddypalem Ragalatha[1] and Basuthkar J Rao[2]

[1]*Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh 522510, India*

[2]*Indian Institute for Science Education and Research (IISER), Tirupati, Andhra Pradesh, India*

**Corresponding author (Email, mallikarjunaanu@gmail.com, mallikarjuna@anu.ac.in)*

The rising global population is forcing the need for adapting alternative sustainable technologies for enhanced crop productivity. The CO_2 Concentration Mechanisms (CCMs) evolved in algae to counter the inefficient CO_2 fixing enzyme, RuBisCo and slower diffusion of CO_2 in water offers good scope for the above purpose. The CCMs are single-celled CO_2 supply mechanisms that depend on multiple CO_2/HCO_3^- transporters and acclimation states and accumulate 100-fold more CO_2 than low CO_2 environments. Although some insights have been obtained regarding the CCMs of blue-green algae and green algae like *Chlamydomonas reinhardtii*, further progress needs to take place to understand the molecular and biochemical basis for intracellular transport of CO_2. In this review, complete information pertaining to the core CCM is presented and discussed in light of the available literature. In addition to this, information on CO_2/HCO_3^- sensing, photo-acclimation in low CO_2, liquid-like nature of pyrenoid, untapped potential of high CO_2 responses and high CO_2 requiring mutants, and prospects of engineering CCM components into higher plants are presented and discussed.

Keywords. *Chlamydomonas*; CO_2; concentration mechanism (CCM); ruBisCo; pyrenoid; plant productivity

1. Introduction

The global population is increasing at an alarming rate while agriculture production is not, due to several constraints like decreasing farmland, diseases, drought and dehydration, and changing agro-climatic conditions (Long *et al.* 2015; Ort *et al.* 2015). There are also innate limitations within the plants for increased plant productivity due to inefficient carboxylation enzyme, RuBisCo and less radiation conversion efficiency than theoretical maxima. The above limitations cannot be overcome by classical breeding technologies and increasing arable land, but can be increased by employing sustainable green technologies, genetic engineering, changing canopy architecture, increasing RuBisCo carboxylation efficiency and exploration of CO_2 Concentration Mechanisms (CCM) of microalgae (Wang *et al.* 2015; Mackinder 2017; Rae *et al.* 2017; Hennacy and Jonikas 2020).

1.1 *Constraints for evolution and adaptation of CCM*

CO_2 as a substrate undergoes reduction in photosynthetic reactions supplying food and energy demands of the entire biosphere. Any change in CO_2 concentrations in local environments will have a profound

This article is part of the Topical Collection: Genetic Intervention in Plants: Mechanisms and Benefits.

http://www.ias.ac.in/jbiosci
Published online: 21 September 2020

impact on the photosynthetic growth of plants (Spalding 2009). Microalgae and aquatic plants experience constant CO_2 limitations due to low CO_2 affinity and very slow catalytic turnover of CO_2 fixing enzyme, RuBisCo, slow bicarbonate into CO_2 conversion and 10^4 times slower diffusion of atmospheric CO_2 (Spalding 2009) in water. Under high O_2 concentrations, RuBisCo can fix O_2 instead of CO_2, leading to loss of photosynthetically fixed CO_2 in a photorespiratory reaction. To overcome this deleterious reaction, microalgae, aquatic plants and *Chlamydomonas reinhardtii* adapted CCM (Raven *et al.* 2017), by using which, they rapidly scavenge inorganic carbon ($Ci=HCO_3^- + CO_2$), uptake and maintain high CO_2 at the site of RuBisCo (Wang *et al.* 2015). Thus, in spite of low environmental CO_2, cells maintain high CO_2 at the site of RuBisCo by sensing, active uptake and transport of both HCO_3^- and CO_2 (Sultemeyer *et al.* 1991). The RuBisCo enzyme in *Chlamydomonas* is present in the pyrenoid of chloroplast (Borkhsenious *et al.* 1998). So as to reach the pyrenoid, inorganic carbon has to move across the plasma and green plastid membranes. Increasing global CO_2 can raise oceanic temperatures, which can in turn reduce the solubility of CO_2 in water forcing the need of CCMs even in the current scenarios (Katano *et al.* 2009). CCMs of Cyanophyceae and Chlorophyceae (*C. reinhardtii*) have been well studied. The *C. reinhardtii* CCM is very well investigated because its photo-physiology and biochemistry resembles higher plants (Spalding 2009; Salome and Merchant 2019). In this review, extensive information pertaining to *C. reinhardtii* CCM is presented and discussed in light of latest available literature.

CCMs operating in algae are known as biophysical CCMs as they transport inorganic carbon in the form of HCO_3^- to prevent diffusion of CO_2; whereas C3 and C4 plants use biochemical CCM and transport CO_2 as organic molecule (malate). CCMs are made possible in different taxa by formation of analogous structures like carboxysomes in blue-green algae, pyrenoids in algae and mosses, kranz-type anatomy in C4 plants, where CO_2, RuBisCo and carbonic anhydrases are lodged in high concentrations (Hennacy and Jonikas 2020).

1.2 *Major components of CCM*

1. Energy for active uptake and transport of CO_2^- and HCO_3^-.
2. Bicarbonate transporters or CO_2 channels at plasma membrane, chloroplast envelope and thylakoid lumen.
3. Carbonic anhydrases for rapid interconversion of HCO_3^- and CO_2 in various compartments of the cell.
4. A physical barrier for CO_2 leakage prevention.
5. A regulatory module for rapidly adjusting to variations in environmental CO_2 concentrations.

2. Induction of CCM

The induction of CCM takes place when *C. reinhardtii* cells are moved from high CO_2 (5%) to low CO_2 (air level or $\leq$0.04%) or very low CO_2 (below air level or $\leq$0.01%) (Vance and Spalding 2005) with dramatic changes at various levels of cellular organization (Miura *et al.* 2004; Yamano *et al.* 2008). Structural changes involve movement of large mitochondria from center of the cell to the periphery followed by fission into small several mitochondria forming a line in between chloroplast and plasma membranes (Geraghty and Spalding 1996). But the reasons for mitochondrial relocation and fission under low CO_2 is not known exactly. High-CO_2 grown cells and low-CO_2 grown cells show significant differences in inorganic carbon affinity and gene regulation changes. But cells in very low-CO_2 condition differ from those of low-CO_2 grown cells by very slow growth, reduced cell sizes, meager chlorophyll content per cell, and low photosynthetic rate with increased CO_2 affinity (Wang *et al.* 2015).

2.1 *Changes associated with induction of CCM*

Many CO_2-responsive genes are identified by transcriptomics and functionally characterized with aid of loss or gain function mutants are the ones that code for putative CO_2/HCO_3^- transporters, carbonic anhydrases and regulatory molecules (Miura *et al.* 2004; Yamano *et al.* 2008; Duanmua *et al.* 2009; Moroney *et al.* 2011). Recent RNA-seq-based transcriptomic analysis revealed massive global gene expression shifts (14–38%), when cells shifted from high CO_2 to limiting CO_2 (Brueggeman *et al.* 2012). The need of two Myb transcription factors, LCR1 and LCR2 for regulation of a set of CCM genes, was found by constructing genome-wide gene regulatory network (GRN) in response to limiting CO_2, but not functionally validated (Winck *et al.* 2013). Spatial-protein interactome

that operates in response to CCM induction was unveiled recently (Mackinder *et al.* 2017). Metabolic profiling (Renberg*et al.* 2010) of low-CO_2 grown cells led to the identification of differently formed metabolites under CCM conditions. Dynamic metabolic network model to explain the different phenotypes formed in response to various CO_2 levels was announced recently (Mora Salguero *et al.* 2018). The induction of CCM by the transcription factor, CIA5, the 'master regulator' was reported (Xiang *et al.* 2001), and cia5 mutant cells do not show any CCM phenotype under low CO_2 with down-regulation of all known CCM genes.

2.2 *Regulation of CCM*

The complete details of regulation of CCM are not exactly known, except the confirmed roles of two transcription factors, CIA5 (master regulator) and LCR1 in *Chlamydomonas* (Xiang *et al.* 2001; Yoshioka *et al.* 2004). Analysis of CCM transcripts under day–night cycles in synchronized *Chlamydomonas* cells exposed to air-level CO_2 revealed the up-regulation of transcripts related to HCO_3^- transporter genes (HLA3, LCI1, CCP1, CCP2 and LCIA) and mitochondrial carbonic anhydrases (CAH4 and CAH5) in light (Tirumani *et al.* 2014), whereas RNAs of chloroplast carbonic anhydrases, CAH3, CAH6 and LCIB are up-regulated in dark followed by high expression at the dawn. Although high expression of CAH3 was found in dark, CAH3 protein expression and pyrenoid localization was noticed in light (Tirumani *et al.* 2014). How transcriptional and translational regulation of CAH3 occurs in CO_2 and light–dark manner needs to be unearthed. Surprisingly, the dark induction of CAH3 is annulled after exposing of cells to high CO_2, indicating that cells are sensitive to high CO_2 even in dark. This points that though CCM is a light-dependent process, CCM relevant changes do happen in dark. Later on, Mitchel *et al.* (2014) by focusing on dark-to-light transition period of air-level CO_2-exposed *Chlamydomonas* cells found that CCM induction takes place 1 h before dawn with increased recruitment of CAH3 and RuBisCo into the pyrenoid. This means that the entire CCM machinery waits for the light signal to activate Ci uptake (Mitchel *et al.* 2014). This further supports that though CCM is a light-dependent process, dark relevant changes do happen in the cells for storing and recapture of CO_2/ HCO_3^- and for getting ready of CCM machinery just before dawn (Tirumani *et al.* 2014; Mitchel *et al.* 2014). Co-regulation and co-ordination of transcription and translation of CCM and photorespiration-related genes under light–dark cycles in low CO_2 (but not in high CO_2) and high light mixotrophic conditions were reported recently (Tirumani *et al.* 2018).

3. Sensing of HCO_3^- or CO_2 at the cell surface and CO_2 responsive elements

Unfortunately, information pertaining to $HCO_3^-$and CO_2 sensing in *Chlamydomonas* or in any other plant is very scanty. The physical interaction of HLA3 with a known CO_2 or HCO_3^- sensors, Adenylate and Guanylate Cyclases (CYG63) was recorded recently, indicating that Adenylate and Guanylate Cyclases may aid in sensing CO_2 or HCO_3^- availability at the plasma membrane (Mackinder *et al.* 2017). This observation is further supported by old literature on Adenylate and Guanylate Cyclases. Soluble Adenylate (sACs) mediated synthesis of the ubiquitous secondary messenger, cAMP in response to HCO_3^- and Ca^{+2} signals, and external Ca^{+2} acting upstream of Adenylyl Cyclase were reported. Similar to mammalian sAC, dogfish sAC is activated by HCO_3^- and is essential for maintaining systemic pH and HCO_3^- levels in the whole organism (Tresguerres *et al.* 2010). The molecular pathway for environmental CO_2 sensing in animals involves the activation of a receptor type, Guanylate Cyclase, in an evolutionary conserved mechanism (Hallem *et al.* 2011). The membrane-bound Guanylate Cyclase synthesizes cGMP for use as secondary messenger for visual detection of retinal rods and cones, which is stimulated by HCO_3^-. Sensing of CO_2 at sea surfaces controlled by cAMP in diatoms was reported (Harada *et al.* 2006). A deletion analysis of marine diatom Carbonic anhydrase (PtCa1) promoter led to identification of three cAMP/CO_2 *cis*-responsive elements (CCRE1, CCRE2 and CCRE3) (Ohno *et al.* 2012). Crosstalk of light and CO_2 signal on CCRE2 *cis*-elements for transcriptional regulation of beta carbonic anhydrase in a marine diatom was revealed (Tanaka *et al.* 2016). With the aid of reporter gene, various *cis*-elements response to high CO_2 was found to be located at -537/-370 and -724/-537 upstream region of H42 promoter (Baba *et al.* 2011). A transporter protein similar to mammalian counterpart, PtSLC4-2, was shown to function in Ci uptake at limiting CO_2 conditions in diatoms (Matsuda *et al.* 2017).

Recently, regulation of CCM genes by a Ca^{+2}-mediated signaling from chloroplast to nucleus in a reverse manner was discovered (Wang *et al.* 2016).

Low CO_2 increased Ca^{+2} concentration in the pyrenoid, which in turn caused the movement of calcium binding protein (CAS) from stroma to the pyrenoid tubules (Wang *et al*. 2016), which was later further supported by high-resolution fluorescence imaging of CAS movement (Yamano *et al.* 2018). The essential role of Ca^{+2} in CO_2 sensing via retrograde signaling in *Chlamydomonas* was discovered with a CAS mutant (H82) that displayed malfunctioned CCM with misregulation of HLA3 and LCIA HCO_3^- transporters (Wang *et al.* 2016). In another study, knock-down of CAS repressed the expression of LHCSR3 protein needed for NPQ in high light, which upon calcium supplementation de-repressed the expression, proving that CAS and $Ca^{+2}$are also needed for control of LHCSR3 expression (Petroutsos *et al.* 2011).

The control of stomatal closure by intracellular HCO_3^- ion-mediated SLAC1 channel activity was discovered in *Arabidopsis*, proving that SLAC1 anion channel is a direct sensor of CO_2/ HCO_3^- in guard cells (Zhang *et al.* 2018). The role of aquaporins in CO_2 influx was proved by showing the interaction of beta-carbonic anhydrase of *Arabidopsis* with aquaporin protein, PIP2-1. The need for bicarbonate ions in the ignition of S-type ion channels that mediate Cl^- and NO_3^- efflux in guard cells was revealed (Zhang *et al.* 2018). The above information confirms that multiple channels or transporters might be involved in CO_2 or HCO_3^- sensing at the plasma membrane.

4. Photo-acclimations under low CO_2

After sensing of low CO_2 levels, *Chlamydomonas* cells exhibits much higher photosynthetic affinity for CO_2 and uptake both forms of Ci and accumulates several folds more CO_2 than environmental CO_2, a hallmark of CCM. The increased intracellular CO_2 levels are generally measured by silicone oil centrifugation and membrane inlet mass spectroscopy (MIMS). The active uptake (Sultemeyer *et al*. 1989) and transport of both forms of Ci at cell and chloroplast levels are known very early (Sultemeyer *et al.* 1991). The operation of CCM is dependent on ATP energy formed in a cyclic electron transport that is rapidly induced under limiting CO_2 condition (Lucker and Kramer 2013). PSBP4 along with four other proteins, YCF3, YCF4, CGL71 and TAB2, are known to be essential for PSI assembly, a key step in initiation of cyclic electron transport at the periphery of the pyrenoid. PSI protein, PSAH with transmembrane domain enriched within pyrenoid tubule and binding to PSI core where PSII docks in during state transitions was discovered recently (Mackinder *et al.* 2017).

In light of lack of complete details of how light harvesting takes under various CO_2 levels, Berger *et al.* (2014) studied low CO_2-mediated regulation of PSII light harvesting, induction of cyclic electron transport and synthesis of NAB1translational repressor. The latter represses the translation of LHCBMS isoforms, while reducing the antenna size and overexcitation of PSII (Berger *et al.* 2014). In addition to this, low-CO_2-exposed *C. reinhardtii* cells contained big antenna and dissipated excess energy in high light, while high-CO_2 grown cells contained small antenna and a smaller number of pigments (Polukhina *et al.* 2016). The CO_2-limitation-imposed translational repression controls the LHCII state transitions to counter PSII overexcitation; separation of LHC-II from PSII to PSI noticed which upon CO_2 supplementation reverses LHCII from PSI and aggregates with quenching. This suggests that LHCII plays a regulatory role in acclimation to low CO_2-levels (Ueno *et al.* 2018).

5. Changes at the cell surface

In water, CO_2 and HCO_3^- are the major sources of inorganic carbon, the dominance and availability of which depends on pH and water currents. This is further hindered by very slow solubility of CO_2 in water and inter-conversion of various Ci species. Under active CCM conditions, the rapid uptake of Ci at the plasma membrane results in depletion of Ci levels, making the need of a periplasmic carbonic anhydrase, CAH1, to replenish the depleted Ci levels (Spalding 2009; Wang *et al.* 2015). Extracellular carbonic anhydrases are also needed to recover leaked CO_2 by converting it into HCO_3^- and also to maintain pH homeostasis. Carbonic anhydrase 1 is a well-investigated limiting CO_2-inducible protein, but cells without CAH1 did not contain any abnormalities except reduced photosynthetic Ci affinity and changed CO_2 gas exchange kinetics at very low CO_2 (Ohnishi *et al.* 2010). It could be due to redundancy under very low CO_2 (Wang and Spalding 2014), and studies with multiple mutants of Ci uptake systems may reveal actual function of CAH1 (Wang *et al.* 2015).

5.1 *Entry at the plasma membrane level*

The HLA3 (high light activated 3) and LCI1 (low CO_2 induced protein 1) HCO_3^- transporters have been

confirmed to be involved in the active uptake of both CO_2 and HCO_3^- at the plasma membrane (Gao *et al.* 2015). LCIB mutants with a smaller number of HLA3 transcripts displayed abnormal growth in very low CO_2 and used less Ci at high pH (Duanmu *et al.* 2009). However, gain of function mutants of HLA3 in high CO_2 exposed cells increased photosynthetic CO_2 affinity in very low CO_2 (Gao *et al.* 2015). HLA3 contains several structural features like hydrophilic and transmembrane domains of ABC transporters and its function in low CO_2 confirm that it is working as HCO_3^- transporter in very low CO_2 at plasma membrane.

Another novel protein with several transmembrane domains, without known functional motifs and homologues having Ci transporter activity on the plasma membrane, is LCI1 (Ohnishi *et al.* 2010). Gain of function of LCI1 increased HCO_3^- uptake and photosynthesis, while reduced number of LCI1 transcripts by RNAi decreased Ci uptake (Ohnishi *et al.* 2010). Immuno-fluorescence and cell fractionation studies confirmed the plasma membrane localization of LCI1 (Ohnishi *et al.* 2010). Recently, the characterization of LCI1 loss of function mutant under various CO_2 levels, genetics and pH conditions led to conclusion that LCI1 is needed for active CO_2 uptake in low CO_2 condition but not for very low CO_2 (Kono and Spalding 2020). The crystal structure of LCI1 protein revealed homotrimer organization and chances of for minga plasma membrane gated-CO_2 channel (Kono *et al.* 2020). The lcil mutant *in vivo* physiological studies further confirmed that it is essential for active CO_2 uptake (Kono *et al.* 2020).

The loss of HLA3 under low CO_2 conditions reduced Ci uptake of *Chlamydomonas* cells (Yamano *et al.* 2015), while HLA3 expressed in *Xenophus* oocytes increased HCO_3^- uptake (Atkinson *et al.* 2015). HLA3 and LCI1 are known to form a complex with a P-type ATPase (Mackinder *et al.* 2017), a H^+-exporting ATPases that can regulate HCO_3^- uptake either by forming a proton gradient or by generating localized cytosolic alkaline regions. In addition to this, HLA3 is also known to be interacted by Ca^{+2}/calmodulin-dependent protein kinase, that could post-translationally regulate HLA3. Because of the above observations, the role of HLA3 and LCI1 in HCO_3^- or CO_2 transport at plasma membrane is confirmed.

5.2 *CO_2/HCO_3^- in the cytoplasm*

Nothing is known about CCM-relevant changes in the cytoplasm and no cytosolic carbonic anhydrase could be detected possibly to minimize the leakage of CO_2 from hydration of accumulated Ci. The biochemistry and molecular biology of intracellular CO_2 transport and the role of cargo molecular motor protein in CO_2 or HCO_3^- transport and energy kinetics involved there are completely not known.

The CIA8 protein belonging to sodium acid bile symporter family was identified in a genetic screen that up-regulates under low CO_2 with the host cell having less growth and affinity for inorganic carbon (Machingura *et al.* 2017). The cia8 mutant could not survive well due to low photosynthetic rate and Ci uptake under high pH levels, proving that CIA8 is an essential part of CCM, whose molecular basis for functioning is not exactly known (Machingura *et al.* 2017).

5.3 *Moving into the chloroplast envelope*

Very early, the active Ci uptake by isolated chloroplasts of *Chlamydomonas* was demonstrated (Sultemeyer *et al.* 1989). Although many chloroplast Ci transporters are known, but LCIA alone is confirmed to function in active Ci uptake across plastid envelope in very low CO_2. It is a member of NAR and FNT protein family, but its expression is regulated by CO_2. The lcia mutant displayed less affinity to inorganic carbon at high pH (Wang and Spalding 2014). Single mutation of LCIA, or double mutation of LCIA and HLA3 in a lcib mutants caused severe growth abnormality in very low CO_2 (Duanmua *et al.* 2009; Wang and Spalding 2014). This proved that LCIA is needed for active transport of HCO_3^- in very low CO_2 at plastid envelope. The bacterial FNT proteinsare known to form pentameric complexes resembling aquaporin channels (Wang *et al.* 2009). The capability of LCIA to form a channel along with plasma membrane HLA3 for active uptake and transport of inorganic carbon against concentration gradient is experimentally proved by co-regulation, and both loss or gain of function of HLA3 and LCIA proteins (Duanmu *et al.* 2009; Wang and Spalding 2014; Wang *et al.* 2014; Gao *et al.* 2015; Yamano *et al.*, 2015). The aquaporin channel involved in CO_2 influx was reported while studying the interaction of *Arabidopsis* beta-carbonic anhydrase with the PIP2-1, an aquaporin protein (Zhang *et al.* 2018). Similar expression of LCIA and LCI20 in chloroplast stroma and thylakoids observed, whose role is not exactly known (Mackinder *et al.* 2017).

The active CO_2 uptake into chloroplast stroma by LCIB proteins was proposed as they showed stromal

localization (Yamano *et al.* 2010), but lcib mutants produced cells that die at air-level CO_2 but live normally in very low CO_2. But, later work on LCIA and LCIB double mutants without growth and photosynthesis proved that LCIB is essential for acclimation to very low CO_2 (Wang and Spalding 2014). The LCIB may function in parallel with HLA3 or LCIA in a CO_2 or HCO_3^- uptake pathway in very low CO_2 (Wang and Spalding 2014). Biochemically and molecularly it is not proven how LCIB helps in uptake of inorganic carbon, except the formation of heteromultimeric complex with LCIC (Yamano *et al.* 2010) and its localization in the chloroplast stroma under high CO_2 or dark, and in peripyrenoid under low CO_2 or light.

Jin *et al.* (2016), by structurally and functionally characterizing many LCIB homologs, proved that structurally LCIB proteins resemble classical beta-carbonic anhydrases. They purified and crystallized LCIB and LCIC from *Chlamydomonas* and discovered motifs that are very similar to catalytic sites of classical beta-carbonic anhydrases (Jin *et al.* 2016). However, recombinant LCIB/C complex did not show activity, suggesting the need of regulator or regulation. Both LCIB and LCIC are known to crosstalk with two putative bestrophins that up-regulate under low CO_2 and transfer chloride but are permeable to HCO_3^- too (Mackinder *et al.* 2017). The need of LCIB and LCIC complex as carbonic anhydrase for uptake of external CO_2 into the stromal HCO_3^-, as well as for prevention of CO_2 leakage (by forming HCO_3^-), from the pyrenoid is proposed. At this juncture, it is also essential that the LCIB and LCIC carbonic activity under high HCO_3^-/CO_2 ratio should favor the maintenance of enough quantities of Ci in the thylakoid lumen. The scope of post-translational modification of LCIB activity by glycerophosporylation is proposed as it is found to contain glycerophosporylation sites in the protein (Jin *et al.* 2016).

The abnormal LCIB localization and starch sheath formation was noticed in an isoamylase-deficient mutant (4-D1) and starch-sheathless mutant sta11-1, but not in sta 2-1mutant with a thin starch, suggesting the essentiality of starch sheath surrounding the pyrenoid for the correct positioning of LCIB (Toyokawa *et al.* 2020). In addition to this, isoamylase-deficient mutant has also showed retarded growth, lower affinity to Ci and decreased accumulation of HLA3 under very-low-CO_2 conditions.

Another stromal carbonic anhydrase, CAH6, was found by Mitra *et al.* in 2004. As it is found localized in the stroma by immunogold labelling, it is speculated that it might be having a role for converting CO_2 to HCO_3^- in the stroma to pool up high inorganic carbon concentration or for converting the leaked (from pyrenoid) CO_2 into HCO_3^- in *Chlamydomonas.* But, a recent study by Mackinder *et al.* (2017) indicated that CAH6 protein is localized to the flagella and absent in stroma. This is further supported by its presence in flagellar proteome and absence in chloroplast and mitochondrial proteome. CAH6 loss of function mutants would reveal its exact role in CCM. Recently, CAH6 has been implicated in Ci sensing as *C. reinhardtii* cells with the aid of flagella moved towards HCO_3^- (Choi *et al.* 2016). By segregating sensing machinery to the flagella, the *C. reinhardtii* cells can easily show chemotaxis to Ci (Mackinder *et al.* 2017). But the spatial-geometry of Ci sensing and uptake on *C. reinhardtii* cell surface needs to be addressed. Locating the position of bicarbonate transporters and CO_2 channels relative to flagella location or flagellar movements is very important for coupling the sensing machinery to uptake machinery, otherwise the detected Ci can diffuse away faster until or otherwise taken up. Earlier work on *Arabidopsis* also showed the sensing of inorganic carbon by carbonic anhydrases (Hu *et al.* 2009).

Until now, HCO_3^- transporters operating at the plasma membrane and chloroplast envelope have been identified but not on the thylakoid membrane. Recently, three bestrophin genes (BST1, BST2 and BST3) controlled by master regulator 'CIA5' have been known to be up-regulated in low CO_2 conditions and found in the thylakoid membrane (Mukherjee *et al.* 2019). Knockdown of BST1-3 proteins resulted in slower growth of cells at low CO_2 with reduced affinity and uptake of inorganic carbon (Mukherjee *et al.* 2019). This work proved that bestrophins are needed for delivering the accumulated HCO_3^- in the chloroplast stroma to the CAH3 present in the thylakoid lumen (Mukherjee *et al.* 2019). It will be very interesting to check whether Tat proteins interact with bestrophins (along with CAH3) in the thylakoid in a CO_2 condition-dependent manner as both show similar expression on thylakoid membranes in response to 'CIA5' master regulator (Mukherjee *et al.* 2019; Benlloch *et al.* 2015).

5.4 *Role of starch sheath in CCM biology*

Algae assemble starch granules in a shell around the pyrenoid, forming a starch sheath. Unlike globular stromal starch, the pyrenoid starch sheath has curved morphology and is made up of distinct plates that wrap around the pyrenoid and appear to form a seal

interrupted by gaps to allow the passage of pyrenoid thylakoid tubules (Meyer *et al.* 2017). Villarejo *et al.* (1996) proposed that the starch sheath was not required for operation of CCM as mutant (isa1) did not decrease Ci affinity. But, later a study on isa1 mutant found lower rates of photosynthetic O_2 evolution. Recently, the need of the starch sheath for LCIB localization around the pyrenoid and for photosynthetic affinity of CO_2 under very low CO_2 conditions was discovered (Toyokawa *et al.* 2020). Irrespective of CO_2 concentrations, both 4-D1 and sta11-1 starch mutants displayed decreased inorganic carbon affinity. Likewise, in spite of enough supply of Ci in both low and high CO_2 conditions, saga1 mutant cells having starch sheath abnormalities showed a decreased photosynthetic rate (Itakura *et al.* 2019). Interestingly, starch sheath formation was noticed even in high-CO_2-grown cells (Machinder *et al.* 2016); this points out the need of starch sheath as a physical barrier in the stroma to increase CO_2 for RuBisCo and also to protect stroma from the pyrenoid oxygenic reactions, hence spatially separating carboxylation and oxygenation reactions (Toyokawa *et al.* 2020) in all CO_2 conditions.

The starch sheath along with SBM3, CBM48, LCI9 and other protein interactors surrounding the pyrenoid may act as a scaffold for LCIB localization and may lodge LCIB in the periphery of starch sheath (Mackinder *et al.* 2017). LCI9 could degrade starch at the gaps between starch plates, possibly ensuring a close fit for adjacent starch plates and for allowing the entry of pyrenoid tubules. Therefore, the starch sheath may be needed for maintaining inorganic carbon in the surroundings of the pyrenoid by acting as a physical diffusional barrier and also as an efficient CO_2 re-capture system by clustering LCIB near chloroplasts (Itakura *et al.* 2019; Toyokawa *et al.* 2020).

The *Chlamydomonas* cells growing normally accumulates starch as a sheath around the pyrenoid, while the stressed cells show many starch granules in the plastid stroma. Under N_2 starvation, these cells accumulated stromal starch rather than pyrenoid starch and turned into mutants with many starch granules similar to photosynthetic starch (Findinier *et al.* 2019). Genetic and functional analysis led to the identification of *Bimodel Starch Granule* (*BSG1*) gene responsible for controlling the transition from pyrenoid to stroma starch under N_2 starvation (Findinier *et al.* 2019).

The stromal thylakoids extend into the pyrenoid matrix through distinct gaps in the starch sheath and fuse into reticulated network at the center (Engel *et al.* 2015). Mutants even without pyrenoid matrix still form pyrenoid tubule network at the canonical location within the chloroplast (Caspari *et al.* 2017). The process of building the pyrenoid tubule network occurs separately from the assembly of the rest of the pyrenoid, and these tubules contain the information about where a pyrenoid should be placed. Therefore, the pyrenoid tubules may actually localize the matrix and starch sheath (Hennacy and Jonikas 2020). The topology of the transition zone between the thylakoid membranes exists as stacked sheaths in the chloroplast and more cylindrical tubules that traverse the pyrenoid is complex (Engel *et al.* 2015), but how its formation is mediated molecularly is unknown (Hennacy and Jonikas 2020). The PSAH protein associated with PSI is enriched in the pyrenoid tubules and may have function there.

5.5 *Pyrenoid, an embodium of CO_2 assimilation and biological activities*

More than 50% of global CO_2 fixation takes place in a non-membrane-bound, phase-separated liquid-like droplet (LLD), pyrenoid (Freeman Rosenzweig *et al.* 2017; Wunder *et al.* 2019). In the early days, pyrenoids were perceived as a RuBisCo containing electron dense and proteinaceous bodies (Borkhsenious 1998). The total RuBisCo of the cell is recruited into the pyrenoid under low CO_2 conditions, while 50% of RuBisCo is retained in the pyrenoid under high CO_2. RuBisCo complexes are hexagonally packed within the pyrenoid with a gap of 15 nm, indicating the need of linker protein (Engel *et al.* 2015; Mackinder *et al.* 2017). Engel *et al.* (2015) revealed the three-dimensional organization of chloroplast and pyrenoid by cryo-electron tomography and revealed that thylakoids and pyrenoids are joined by pyrenoid tubules and each pyrenoid tubule contains many parallel mini-tubules that are formed by adjoining of two thylakoid membranes. These mini-tubules form a network with chloroplast stroma and the pyrenoid matrix and allow movement of metabolites like ATP, sugars and 3-PGA between those two locations. The pyrenoid thylakoid tubule network, an extension of stromal thylakoid into the pyrenoid matrix is known to contain HCO_3^- that is later dehydrated into CO_2 by thylakoid lumen CAH3. CO_2 then diffuses out into pyrenoid matrix where it is assimilated into CBB cycle by RuBisCo (Engel *et al.* 2015).

So as to reach the CAH3 present in thylakoid lumen, HCO_3^- has to cross through impermeable thylakoid membranes aided by some unknown ion channel or carrier located on thylakoid membranes (Spalding

2009). Recently, CAH3 was found to be associated with TAT2 and TAT3 proteins of Twin Arginine Translocation (Tat) pathway that delivers proteins into the thylakoid lumen (Benlloch *et al.* 2015). The CAH3 protein with Tat signal peptide (Benlloch *et al.* 2015) and localization of CAH3 to thylakoid lumen (Karlsson *et al.* 1998) supports the above observation. The direct and strong interaction of CAH3 with N-terminus of STT7 kinase that has a role in state transitions and localizations to the thylakoid lumen were revealed recently (Mackinder *et al.* 2017). Proton pumping during the light reactions of photosynthesis creates an acidic environment within the thylakoid lumen, which promotes the conversion of HCO_3^- to CO_2. It is likely to be catalysed by CAH3 which localizes to thylakoid lumen. Concentrated CO_2 produced at thylakoid lumen is thought to diffuse across the pyrenoid tubules to feed the RuBisCo in the matrix (Wang *et al.* 2015).

While both large (LSU) and small subunits (SSU) of RuBisCo are needed for pyrenoid assembly (Meyer *et al.* 2012), but small subunits contain regulatory sequences for the above purpose. Later, it was realized that two surface exposed α-helices of the SSU are mandatory for RuBisCo assembly and pyrenoid formation (Meyer *et al.* 2012). Bleomycin-resistant insertional mutant, cia6, growing poorly on limiting CO_2 and unable to accumulate Ci was generated. The cia6 mutant was found to be having a protein with SET domain methyltransferase. Cia6 mutant displayed a highly disorganized pyrenoid with lower expression of CCM genes, confirming that CIA6 (a methyl transferase) is needed for pyrenoid formation as well as for induction of CCM (Ma *et al.* 2011).

The *Arabidopsis* RuBisCo was engineered to incorporate two α-helices of the *Chlamydomonas* SSU. Leaves of the above *Arabidopsis* mutant formed hybrid RuBisCo complex and displayed similar RuBisCo, growth and photosynthetic performance (Atkinson *et al.* 2017). This proved that Rubisco SSU of *Chlamydomonas* can complement the *Arabidopsis* counterpart. By using yeast two hybrid assay, Atkinson *et al.* (2019) proved that protein–protein interactions between RuBisCo and EPYC1 are dependent on two α-helices of SSU of RuBisCo. This study also confirmed that *Chlamydomonas* EPYC1 and RuBisCo SSU can be targeted to plant chloroplasts without affecting growth. Higher-plant EPYC1 did not aggregated RuBisCo like in algal pyrenoids (Atkinson *et al.* 2019), but *Arabidopsis* RuBisCo with *Chlamydomonas* SSU could form liquid droplets with recombinant EPYC1 in a test tube, confirming the pyrenoid-like aggregation of higher-plant RuBisCo (Atkinson *et al.* 2019).

The Essential Pyrenoid Component 1 (EPYC1) that connects RuBisCos and forms pyrenoid and colocalizes to the entire pyrenoid was discovered recently (Mackinder *et al.* 2016). EPYC1 is also found to be essential for normal pyrenoid biology, and CO_2 fixation by RuBisCo at low CO_2 condition (Mackinder *et al.* 2016). Mutants with no pyrenoid showed reduced CCM proteins and failed to adapt to low-CO_2 environment (Mitchel *et al.* 2017). In addition to this, pyrenoid-less mutant could not accumulate EPYC1, and mis-regulated CCM and primary metabolism genes (Mitchel *et al.* 2017). Pyrenoid mutants from another study also exhibited severe growth and photosynthetic defects and malfunctional CCM (Caspari *et al.* 2017). The amino acid sequence of EPCY1 consist primarily of four nearly identical repeats, each with a predicted α-helical region followed by a region predicted to be highly disordered. As the aggregation of RuBisCo into pyrenoid depends on two surface-exposed α-helices found on the *Chlamydomonas* RuBisCo SSU, it is a potential site for EPYC1 binding and pyrenoid biogenesis in higher plants (Meyer *et al.* 2012; Atkinson *et al.* 2017; Hennacy and Jonikas 2020).

The pyrenoid matrix contains RuBisCo holoenzyme, RuBisCo activase and proteins of unknown function. The LCIB/C and PSB4 proteins surrounding the pyrenoid periphery in the form of puncta, Stat2 and Sbe3 proteins in the periphery, and LCI9 that localizes to mesh-like structure of the pyrenoid was discovered in a recent spatial-proteome analysis (Mackinder *et al.* 2017). By breaking the starch at starch plates, LCI9 may form a mesh-like structure at the two junctions of starch plates by interacting with CBM20. A putative methyltransferase (SMM7) located in the pyrenoid matrix along with other known methyl transferase, CIA6, are needed for pyrenoid formation (Ma *et al.* 2011; Mackinder *et al.* 2017). Pyrenoid biogenesis can be better understood after identification of functional targets of two methyl transferases, CIA6 and SMM7 (Mackinder *et al.* 2017), and a serine/threonine protein kinase, KIN4-2, that was found associated with EPYC1. EPYC1 phosphorylation in low-CO_2-condition-dependent manner was reported earlier.

EPYC1 is also known to interact with FTT1 and FTT2 proteins that are known to bind redox-regulated proteins, and hence EPYC1 phosphorylation status may regulate FTT1 and FTT2, which may in turn regulate the target protein structure, function and localization. Therefore, FTT1/2 may regulate interactions between EPYC1 and RuBisCo by changing of protein–protein interactions and protein-binding domains (Mackinder *et al.* 2017). Pyrenoid proteome

analysis by Zhan *et al.* (2018) revealed the presence of 190 proteins with functions in CCM, starch and RNA metabolism, translation, ribosomal synthesis of RuBisCo LSU, tetrapyrrole, chlorophyll and carotenoid synthesis, confirming that pyrenoid is a hub of metabolism.

The pyrenoid matrix is densely packed with RuBisCo and is believed to be crystalline or amorphous solid. But a recent study by Freeman Rosenzweig *et al.* (2017) demonstrated that pyrenoid matrix of *Chlamydomonas* is not crystalline but behaves as a liquid that dissolves and condenses during cell division. Daughter pyrenoids are formed by fission or *de novo* assembly. This discovery changed our perception of structure, synthesis and regulation of pyrenoids (Freeman Rosenzweig *et al.* 2017). This kind of liquid-liquid-like phase separation (LLPS) occurs as a protein-dense droplet formed by unique multivalent scaffold proteins like RuBisCo and EPYC1 along with other scaffold proteins (Wunder *et al.* 2019). The starch granules and P-bodies are well known to behave like liquid-like droplets. Wunder *et al.* (2018) proved the essentiality and sufficiency of RuBisCo and EPYC1 to phase separate and form dynamic functional pyrenoid liquid droplets that rapidly exchange with bulk solution. This study may help in assembly of pyrenoid components in higher plants for transfer of CCM components.

The unique nature of RuBisCo-EPYC1 phase separation was further analysed by testing RuBisCo from diverse plants and it was found that EPYC1 could interact with many RuBisCo except with that of rice (Wunder *et al.* 2018). Proper pyrenoids were not formed after replacement of endogenous RuBisCo SSU with spinach counterpart (Meyer *et al.* 2012). Chimeric RuBisCo with algal LSU and rice SSU showed different phase separation events, required high amounts of EPYC1 to demix and formed droplets with abnormalities, confirming that SSU plays a pivotal in the formation of liquid droplets (Wunder *et al.* 2019). Cyanobacterial RuBisCo demixed with *Chlamydomonas* EPYC1 as well as with its native linker protein, ccmM (Wang *et al.* 2019). Recently, *E. coli* producing a recombinant plant RuBisCo and *Arabidopsis* plant expressing algal SSUs were developed, paving the way for expression of CCM components in higher plants (Aigner *et al.* 2017; Wilson *et al.* 2019).

LLDs (pyrenoids) represent an opportunity for *in vivo* RuBisCo, RCA, CAH3, protein phosphorylation and protein–protein interaction or enzymes assays that mimic physiological concentrations of stroma. And it will also enable us to understand how metabolites like ATP, RuBP and 3-PGA affect the pyrenoid phase separation events (Wunder *et al.* 2019) in *Chlamydomonas*.

Another RuBisCo binding protein, Starch Granule Abnormal 1 (SAG1), was found to be essential for starch sheath and pyrenoid structure and function (Itakura *et al.* 2019). The saga1 mutant had an average of 10 pyrenoids per chloroplast as against to 1 in the wild-type. Starch sheath of mutants are thinner and more elongated than wild-type. SAGA1 with starch binding motif may control starch sheath surface area by interacting with SSU and LSU of RuBisCo and expressing in the pyrenoid (Itakura *et al*. 2019).

5.6 *CAH3, a thylakoid lumen protein with a dual function?*

Karlsson *et al.* (1998) identified and sequenced thylakoid localized α-CAH, CAH3, that co-purified with PSII. The cia3 mutant has reduced PSII activity and overproduced PSII reaction centers. Later on, CAH3 protein associated with PSII donor side was found to be needed for functional stabilization of Mn cluster and water oxidizing complex (Villarejo *et al.* 2002). They have also demonstrated that purified thylakoids of cia3(cah3) mutants on HCO_3^- supplementation formed functional water oxidizing complex of PSII. Cia3 mutants are impaired in ATP synthesis as compared with wild-type. Shutova *et al.* (2008) discovered that the cia3 mutant showed malfunctional O_2 evolution in the absence of HCO_3^-. Time-resolved florescence and O_2 evolution experiments indicated that CAH3 facilitate proton removal from Mn complex by providing HCO_3^- as a source of proton carrier. Thus, proton removal by CAH3on the donor side of PSII during photolysis of water is very important for O_2 liberation (Shutova *et al.* 2008).

Markelova *et al.* (2009) found the Western blot lacks cross-reactivity of antibodies to CAH3 in the mutant lacking the PSII RC in contrast to mutant deficient in LHC of PSII. Immuno-electron microscopy revealed that CAH3 is localized to the pyrenoid in low CO_2. The PSII O_2 evolution activity was lower in cia3 mutant than in wild-type under low CO_2 conditions, confirming that CAH3 is involved in CCM (Markelova *et al.* 2009). This result supported the hypothesis that carboxylation reaction occurs in the pyrenoid that acquires CO_2 from intrapyrenoid thylakoids (Markelova *et al.* 2009). This result also confirmed that CAH3 is needed for dehydration of HCO_3^- and supply of CO_2 to RuBisCo (Markelova *et al*. 2009) in the pyrenoid. The

immuno-electron microscopic observation has shed light on CAH3 distribution in thylakoids especially in the intra-pyrenoid thylakoid membranes. Its presence in the above location may favor its function in generating CO_2 from HCO_3^- in the thylakoid lumen; the high CO_2 thus generated diffuses out into the pyrenoid matrix where RuBisCo is lodged (Sinetova *et al.* 2012). CAH3 protein phosphorylation with increased activity was observed when *Chlamydomonas* cells shifted from high-CO_2 to low-CO_2 conditions. It is also correlated with its relocation from PSII in the stroma to pyrenoid (Blanco-Rivero *et al.* 2012). This indicated the need of operating post-translational regulations in CCM while adjusting to limiting CO_2. Dark induction and up-regulation of CAH3 just 1 h before dawn in synchronized and photoautotrophically grown cells was reported (Mitchel *et al.* 2014; Tirumani *et al.* 2014). Although high CAH3 transcription was noticed in dark, activated translation, protein accumulation and pyrenoid localization was noticed with light (Tirumani *et al.* 2014).

Benlloch *et al.* (2015) with the aid of MIMS proved that CAH3 was active at acidic pH present in thylakoid lumen under light. The crystal structure of CAH3 revealed that it consisted of a dimer with a disulphide bond. Titration with DTT revealed redox regulation of CAH3 with a structural feature to increase PSII function at low pH and CO_2.

The *Chlamydomonas* wild-type and cia3 mutants were found to have the same amount of reaction centers, cytochrome, subunits of WOC, Mn^{2+} and carotenes (Terentyev *et al.* 2019), but differ in carotenoids, redox forms of cytochromeb559 and outer LHCs. Functionally, PSII fractions from both mutant and wild-type showed similar photosynthetic activity, but cia3 (cah3) mutants contained more closed reaction centers even at pH 6.5 and showed more pronounced suppression of PSII photosynthetic activity (Terentyev *et al.* 2019).

So as to have a comprehensive understanding of the CCM pathway, the *Chlamydomonas* whole cell along with one big chloroplast having two flagella is shown in figure 1, and a schematic representation of the CCM pathway along with number of new molecules implicated thereof has been depicted in figure 2 for better appreciation of CCM pathway.

6. Advantages of algal CCMs

The algal CCMs are unicellular CO_2 supply mechanisms relying on many and diverse Ci uptake systems and CO_2 acclimations that can increase intracellular Ci several fold. Half of the global primary productivity comes from microalgae (Raven and Beardall 2015). Algae are 2–10 times more efficient at absorbing CO_2 and produce 15–300 times more biomass (Beardall and Stojkovic 2006) than land-based plants due to short life cycle and fast growth rate (Chisti 2008; Tredici 2010). In addition to this, algae can fix CO_2 from different sources, from the atmosphere, industrial exhaust gas and in the form of soluble carbonates. Algae can be grown in many climates, harvested year around and can be used to absorb CO_2 near emission sources and buried as biomass to reduce our carbon footprint (Zeiler *et al.* 1995). As it is well known that CO_2 is the causative agent of the greenhouse effect, using microalgal CCMs, it is possible to sequester CO_2 into biological systems, developing sustainable green technologies with beneficial CO_2 sequestration (Lam and Lee 2013; Zheng *et al.* 2014). In addition to this, it is of great significance to understand how changes in global climate like high heat, CO_2, UV radiation and temperature are impacting microalgal CCM as an important component of the global carbon cycle.

6.1 *Untapped potential of high-CO_2-requiring mutants (HCRMs)*

Biomass obtained from microalgae is considered a suitable renewable energy source and many studies have been conducted to develop superior strains for this purpose. *Chlamydomonas* with active CCM is a potential target for drawing down of environmental CO_2 through sustainable green CO_2^- sequestration technologies. But the application of the same for CO_2 sequestration was hindered by failure to induce CCM under high-CO_2 and high-light conditions. The mutants of *Chlamydomonas* with 'constitutively active CCM' will be a highly desirable phenotype as it fixes more CO_2 in high-CO_2 and high-light environments and will be resistant to photo-damage and ROS production as high CO_2 consumes reducing power generated by high light (Hwangbo *et al.* 2018). Recent studies also indicated that addition of inorganic carbon sources to the culture medium removed oxidative stress by increasing the ratio of CO_2 to O_2, thereby reducing the generation of toxic ROS (Peng *et al*. 2016). The *C. reinhardtii* mutant(s) that need high CO_2 can accumulate more biomass and offer a cheap raw material for production of industrially important molecules through environmentally sustainable CO_2 sequestration green technologies (Salome and Merchant 2019). In the event of lack of complete details on regulation of CCM,

Table 1. List of CCM-related genes expressed in higher plants along with their transgenic phenotype

S. No.	CCM gene/Host Cell	Target plant transformed	Phenotype of transformed plant	Reference
1	ictB of *Synechococus*	*Arabidopsis* and tobacco	Faster photosynthetic rate at limiting CO_2	Lieman-Hurwitz *et al.* (2003)
2	ictB of Cyanobacterium	Rice	Increased photosynthetic growth and yield	Yang *et al.* (2008)
3	RuBisCo, RbcX, and ccMM35 of Cyanobacterium	Tobacco	RuBisCo formed complex in stroma with higher rates of CO_2 fixation (fasterRuBisCo)	Lin *et al.* (2014)
4	CCM components of *Chlamydomonas*	Transiently in tobacco and stably in *Arabidopsis*	No growth differences noticed between control and modified plant	Atkinson *et al.* (2015)
5	Improved Cyanobacteria RuBisCo	Tobacco	Photosynthesis with higher carboxylation rate than control plant	Occhialini *et al.* (2016)
6	ictB of Cyanobacterium	Soybean	Increased photosynthetic CO_2 uptake and dry mass	Hay *et al.* (2017)
7	LCIA and CAH3 of *Chlamydomonas*	Tobacco	Enhanced CO_2 uptake rates, increased PSII, shoot biomass and chlorophyll content	Nolke *et al.* (2019)
8	BicA bicarbonate transporter of *Synechococus*	Tobacco chloroplast	BicA localized into chloroplast without impacting growth and development	(2014)
9	ictB/FBP of Cyanobacteria	Rice	Higher mesophyll conductance and net photosynthetic rate	Gong *et al.* (2015)
10	S1,6BP, F16B and ictB	Tobacco	Increased the rate of CO_2 assimilation, leaf area, and biomass yield Fixed 12–19% more CO_2 than wild-type	Simkin *et al.* (2015)

HCRMs thus formed may be the basis for identification of key regulatory molecules involved in CO_2 signal transduction pathway (Wang *et al*. 2014a).

6.2 *High CO_2 response of Chlamydomonas cells*

Although *Chlamydomonas* cells response to low CO_2 was extensively studied, but very little or no information is available on the molecular and biochemical basis for cells response and adaptation to high CO_2 conditions. Understanding this process is very important to know how higher plants respond to high CO_2, which will also be used in modeling of crops to high-CO_2 future environments. As *Chlamydomonas* cells receive enough number of carbon skeletons under high CO_2 concentration, the same can be diverted into diverse metabolic pathways to produce several high-value compounds under specific stress conditions. The successful exploration of high-CO_2-grown cells for production of several industrially important compounds was demonstrated (Solovchenko and Khozin-Goldberg 2013; Ruiz *et al.* 2016; Sathasivam *et al.* 2019).

The high-CO_2-exposed *Chlamydomonas* cells induce the expression of several proteins (Miura *et al.* 2004) like Rhp1 and Rhp2 (Soupene *et al.* 2004) and periplasmic proteins, CAH_2 and H43 (Hanawa *et al.* 2007). Later work on H43 proved that it is a multitarget protein and shows response to high CO_2, Fe and cadmium stresses (Hanawa *et al.* 2007; Baba *et al.* 2011) and same has been developed as marker for high CO_2 sensing mechanism. By fusing H43 to reporter gene, *cis*-elements response to high $CO_2$was found to be located at the -537/-370 and -724/-537 upstream region of H42 promoter (Baba *et al.* 2011).

6.3 *Prospects of engineering Chlamydomonas CCM components into higher plants*

The current world population and changing agro-climatic conditions indicate that there is an imminent need to develop crop plants that require limited space and inputs but give higher yields with less environmental impacts (Ruan *et al.* 2012; Long *et al.* 2015; Mackinder 2017; Rae *et al.* 2017). CCMs of microalgae represent the best option in that direction as they show high photosynthetic rate coupled with good

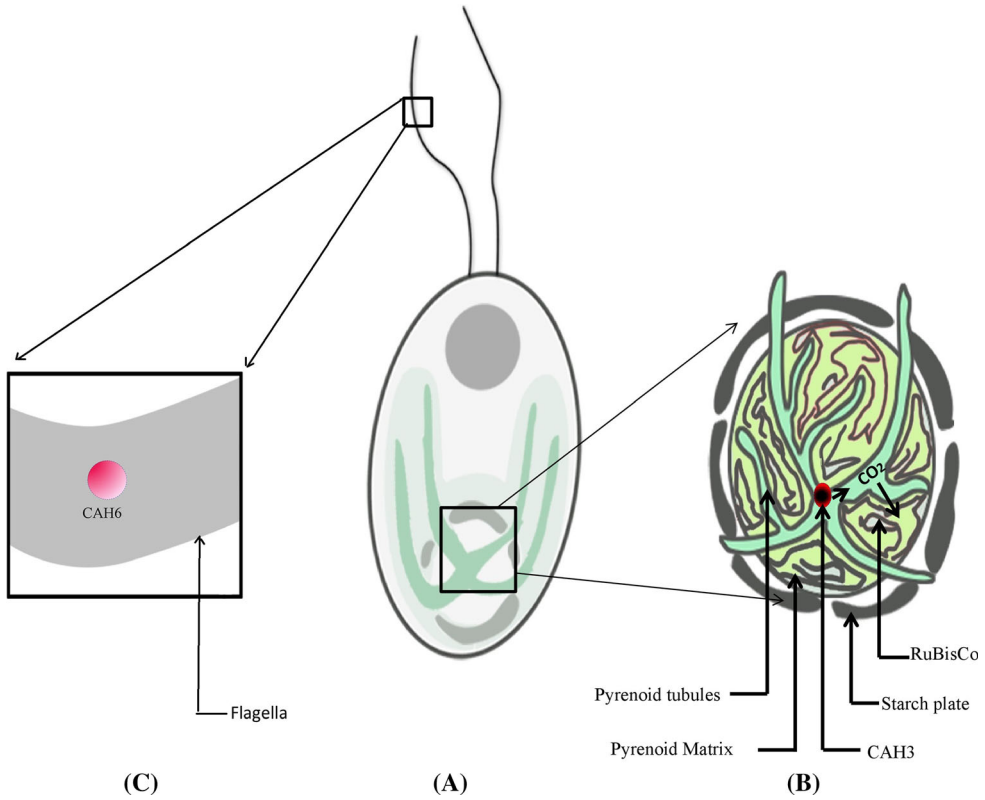

Figure 1. *Chlamydomonas* whole cell. (**A**) General view of *Chlamydomonas reinhardtii* cell with a single big cup shaped chloroplast and two flagella. (**B**) Enlarged view of chloroplast pyrenoid. (**C**) Magnified view of flagella with CAH6 protein.

growth in low-CO_2 environments. So as to realize this potential, Atkinson *et al.* (2015) took 10 major genes of *Chlamydomonas* CCM pathways and did localization studies in *Chlamydomonas*, expressed briefly in tobacco and stably in *Arabidopsis*. Expression of tagged CCM proteins in *Chlamydomonas* revealed that all CCM proteins except CAH3 and CAH6 had similar intracellular localization as in wild-type *Chlamydomonas*. By fusing to chloroplast transit peptide, they are retargeted to the chloroplast. The chloroplast carrier proteins, CCP1 and CCP2, showed expression in mitochondria of both *Chlamydomonas* and tobacco. The expression of LCIA and HLA3 in *Arabidopsis* plant displayed growth similar to wild-type plants (Atkinson *et al.* 2015). Individual transgenic tobacco plants were generated by using CrCAH3 in the thylakoid lumen or bicarbonate transporter CrLCIA in the chloroplast membrane (Nolke *et al.* 2019). The homozygous transgenic plants displayed increased CO_2 uptake, PSII activity and chlorophylls. In addition to this, they accumulated more shoot biomass, carbohydrate and amino acidsthan wild-types, reflecting the high photosynthetic rates (Nolke *et al.* 2019).

This confirmed that individual CCM genes can be transferred into C3 plants for increased biomass productivity. The present data on CCM genes targeted to higher plants indicates that by stably targeting into their appropriate locations in chloroplast and mitochondria, several CCM genes can be expressed in C3 plants for enhanced productivity. Although limited work (Atkinson *et al.* 2015; Nolke *et al.* 2019) has been done in the direction of transferring *Chlamydomonas* CCM components into higher plants, several CCM genes of prokaryotic cyanobacteria were targeted into many crops with promising results (table 1). This points out that more work needs to be done with the objective of transferring eukaryotic *Chlamydomonas* CCM components into higher plants as the physiology and biochemistry of C3 plants resemble that of *Chlamydomonas*. Engineering pyrenoid biogenesis in higher plants seems to be an attractive option as several *in vitro* studies mentioned earlier offer promising scope. *Chlamydomonas* mutant library, systems and synthetic biology approaches coupled with metabolic pathway engineering could help in transfer of core-CCM components in non-

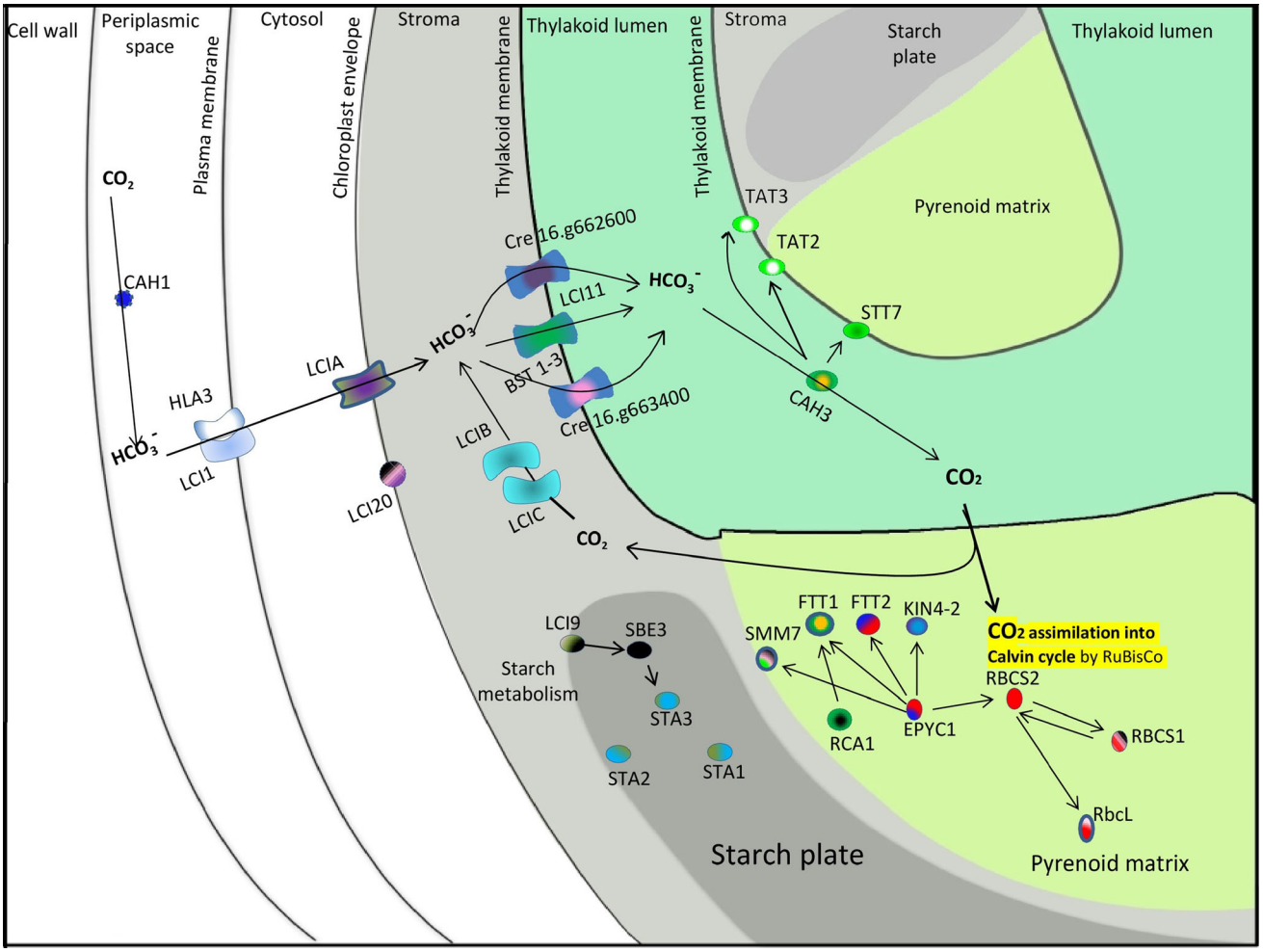

Figure 2. Schematic view of CO_2 concentration mechanism (CCM) pathway in *Chlamydomonas reinhardtii* (modified from Engel *et al.* 2015; Wang *et al.* 2015; Mackinder *et al.* 2017).

CCM host plant and later into higher plants (Mackinder 2017).

7. Future perspectives

Although some crucial leads have been obtained in understanding the mechanistic details of CCM by yeomen services of scientists working in CCM field, further work needs to be done to understand the complete details of CCM. Listed below are some lacunas to be filled in by the future projects.

1. What is the exact biochemical role of LCIB as a directional carbonic anhydrase?
2. Mechanisms of LCIB migration around the pyrenoid is not clear and may respond to changes in CO_2 irrespective of starch sheath.
3. The role of mitochondria in CCM regulation (CCP1/2 and CAH4/5) needs to be unearthed.
4. Comparison of RNA sequencing results of all known mutants and wild-type cells may lead to the finding of missing information.
5. Biochemical and molecular basis for intracellular Ci transport, the role of molecular motors, cytoskeletal proteins and energy involvements for Ci transport need to be understood.
6. Although extensive information is available on epigenetic regulation in stress response and adaptation, the role of microRNAs and methylation in CCM regulation are not completely known.
7. Modulation of CCM by other environmental factors like heat, UVB and temperature should be unearthed.
8. It will be very interesting to know how different types (colors) and fluences of light regulate the CCM process.
9. Studying the pH changes as a function of CCM will be useful in understanding the conversion of

various forms of Ci in different compartments of the cell.

10. Unraveling the complete molecular mechanistic details of pyrenoid biogenesis is very essential for manipulation of CCM.
11. The exact functional role of starch sheath in the CCM needs to be unearthed.
12. More research is needed to understand the molecular details of how pyrenoid tubules are formed from thylakoid membranes as constructing pyrenoid tubules will likely to be an important step towards engineering of algal CCM in the land plants.
13. Studying the CCM by multi-omics approach may offer insights into simultaneous changes associated with operation of CCM mechanism and it also help in picking the missing pieces and crosstalk among various systems.
14. Although it is very well known that CAH3 supplies CO_2 to RuBisCo by dehydrating HCO_3^-, how it delivers and where it is exactly localized are not known completely.
15. Although signatures pertaining to post-translational regulation of HLA3 and LCIB are known, it is not yet proven.
16. Recent studies on mutants, differential expression and localization patterns has implicated many new molecules with a possible role in CCM, but are not validated functionally.

Acknowledgements

We acknowledge the funding from CSIR (No. 38 (1429)/17-EMRII), and INSA, New Delhi, to KM. KM also acknowledges Dr M Raghuram, head of the Botany and Microbiology Department, ANU, for providing administrative support. We also thank the Department of Science and Technology, New Delhi, for the DST-FIST programme.

References

Aigner H, Wilson RH, Bracher A, Calisse L, Bhat JY, Hartl FU and Hayer-Hartl M 2017 Plant RuBisCo assembly in *E. coli* with five chloroplast chaperones including BSD2. *Science* **358** 1272–1278

Atkinson N, Feike D, Mackinder LC, Meyer MT, Griffiths H, Jonikas MC, Smith AM and McCormick AJ 2015 Introducing an algal carbon concentrating mechanism into higher plants: location and incorporation of key components. *Plant Biotechnol J.* **14** 1302–1315

Atkinson N, Leitao N, Orr DJ, Meyer MT, Carmo-Silva E, Griffiths H and McCormick AJ 2017 RuBisCo small subunits from the unicellular green alga *Chlamydomonas* complement RuBisCo-deficient mutants of *Arabidopsis*. *New Phytol*. **214** 655–667

Atkinson N, Velanis CN, Wunder T, Clarke DJ, Mueller-Cajar O and McCormick AJ 2019 The pyrenoidal linker protein EPYC1 phase separates with hybrid *Arabidopsis–Chlamydomonas*RuBisCo through interactions with the algal RuBisCo small subunit. *J. Exp. Bot*. **70** 5271–5285

Baba M, Hanawa Y, Suzuki I and Shiraiwa Y 2011 Regulation of the expression of H43/Fea1 by multi-signals. *Photosynth Res.* 109 169–177

Beardall J and Stojkovic S 2006 Microalgae under global environmental change: implications for growth and productivity, populations and trophic flow. *Sci. Asia* **32** 001

Benlloch R, Shevela D, Hainzl T, Grundstrom C, Shutova T, Messinger J and Sauer Eriksson AE 2015 Crystal structure and functional characterization of photosystem II-associated carbonic anhydrase CAH3 in *Chlamydomonas reinhardtii*. *Plant Physiol*. **167** 950–962

Berger H, Blifernez-Klassen O, Ballottari M, Bassi R, Wobbe L and Kruse O 2014 Integration of carbon assimilation modes with photosynthetic light capture in the green alga *Chlamydomonas reinhardtii. Mol. Plant* **7** 1545–1559

Blanco-Rivero A, Shutova T, Roman MJ, Villarejo A and Martinez F 2012 Phosphorylation controls the localization and activation of the lumenal carbonic anhydrase in *Chlamydomonas reinhardtii*. *PLoS one.* **7** e49063

Borkhsenious ON, Mason CB and Moroney JV 1998 The intracellular localization of Ribulose-1,5-bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. *Plant Physiol.* **116** 1585–1591

Brueggeman AJ, Gangadharaiah DS, Cserhati MF, Casero D, Weeks DP and Ladunga I 2012 Activation of the carbon concentrating mechanism by CO_2 deprivation coincides with massive transcriptional restructuring in *Chlamydomonas reinhardtii. Plant Cell* **24** 1860–1875

Caspari OD, Meyer MT, Tolleter D, Wittkopp TM, Cunniffe NJ, Lawson T and Griffiths H 2017 Pyrenoid loss in *Chlamydomonas reinhardtii* causes limitations in CO_2 supply, but not thylakoid operating efficiency. *J Exp Bot.* **68** 3903–3913

Chisti Y 2008 Biodiesel from microalgae beats bioethanol. *Trends Biotechnol*. **26** 126–131

Choi HI, Kim JYH, Kwak HS, Sung YJ and Sim SJ 2016 Quantitative analysis of the chemotaxis of a green alga, *Chlamydomonas reinhardtii*, to bicarbonate using diffusion-based microfluidic device. *Biomicrofluidics*. **10** 014121

Duanmu D, Miller AR, Horken KM, Weeks DP and Spalding MH 2009 Knockdown of limiting-CO_2–induced gene HLA3 decreases HCO_3^- transport and photosynthetic Ci affinity in *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA* **106** 5990–5995

Engel BD, Schaffer M, Kuhn Cuellar L, Villa E, Plitzko JM and Baumeister W 2015 Native architecture of the *Chlamydomonas* chloroplast revealed by *in situ* cryo-electron tomography. *eLife* **4** eo4889

Findinier J, Laurent S, Duchene T, Roussel X, Lancelon-Pin C, Cuine S and Dauvillée D 2019 Deletion of BSG1 in *Chlamydomonas reinhardtii* leads to abnormal starch granule size and morphology. *Sci Rep.* **9** 1–3

Freeman Rosenzweig ES, Xu B, Kuhn Cuellar L, Martinez-Sanchez A, Schaffer M, Strauss Mand Jonikas MC 2017 The eukaryotic CO_2—concentrating organelle is liquid-like and exhibits dynamic reorganization. *Cell* **171** 148–162

Gao H, Wang Y, Fei X, Wright DA and Spalding MH 2015Expression activation and functional analysis of HLA3, a putative inorganic carbon transporter in *Chlamydomonas reinhardtii. Plant J.* **82** 1–11

Geraghty AM and Spalding MH 1996 Molecular and structural changes in *Chlamydomonas* under limiting CO_2 (apossible mitochondrial role in adaptation). *Plant Physiol*. **111** 1339–1347

Gong HY, Li Y, Fang G, Hu DH, Jin WB, Wang ZH and Li YS 2015 Transgenic rice expressing Ictb and FBP/Sbpase derived from cyanobacteria exhibits enhanced photosynthesis and mesophyll conductance to CO_2. *PLoS One* **10** e0140928

Hallem EA, Spencer WC, McWhirter RD, Zeller G, Henz SR, Ratsch G and Ringstad N 2011 Receptor-type guanylate cyclase is required for carbon dioxide sensation by *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **108** 254–259

Hanawa Y, Watanabe M, Karatsu Y, Fukuzawa H and Shiraiwa Y 2007 Induction of a high-CO_2-inducible, periplasmic protein, H43, and its application as a high-CO_2-responsive marker for study of the high-CO_2-sensing mechanism in *Chlamydomonas reinhardtii. Plant Cell Physiol.* **48** 299–309

Harada H, Nakajima K, Sakaue K and Matsuda Y 2006 CO_2 sensing at ocean surface mediated by cAMP in a marine diatom. *Plant Physiol.* **142** 1318–1328

Hay WT, Bihmidine S, Mutlu N, Hoang KL, Awada T, Weeks DP and Long, SP 2017 Enhancing soybean photosynthetic CO_2 assimilation using a cyanobacterial membrane protein, ictB. *J. Plant Physiol.* **212** 58–68

Hennacy JH and Jonikas MC 2020 Prospects for engineering biophysical CO_2 concentrating mechanisms into land plants to enhance yields. *Annu. Rev. Plant Biol*. **71** 461–485

Hu H, Boisson-Dernier A, Israelsson-Nordstrom M, Bohmer M, Xue S, Ries A and Schroeder JI 2009 Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells. *Nat. Cell Biol*. **12**87–93

Hwangbo K, Lim JM, Jeong SW, Vikramathithan J, Park YI and Jeong WJ 2018 Elevated inorganic carbon concentrating mechanism confers tolerance to high light in an arctic *Chlorella* sp. ArM0029B. *Front. Plant Sci*. **9** 590

Itakura AK, Chan KX, Atkinson N, Pallesen L, Wang L, Reeves G and Jonikas MC 2019 A RuBisCo-binding protein is required for normal pyrenoid number and starch sheath morphology in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **116** 18445–18454

Jin S, Sun J, WunderT, Tang D,Cousins AB, SzeSK, Mueller-Cajar Oand Gao YG 2016 Structural insights into the LCIB protein family reveals a new group of β-carbonic anhydrases. *Proc. Natl. Acad. Sci. USA* **113** 14716–14721

Karlsson J, Clarke AK, Chen ZY, Hugghins SY, Park YI, Husic HD, Moroney JV, and Samuelsson G 1998 A novel alpha -type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. *EMBO J*. **17** 1208–1216

Katano T, Lee J, Lee YJ, Kim MK, Lee CG, Jin ES and Han MS 2009. Effect of temperature on inorganic carbon acquisition of *Chlamydomonas reinhardtii. J. Freshw. Ecol*. **24** 255260

Kono A and Spalding MH 2020 LCI1, a *Chlamydomonas reinhardtii* plasma membrane protein, functions in active CO_2 uptake under low CO_2. *Plant J*. **102** 1127–1141

Kono A, Chou TH, Radhakrishnan A, Jani Reddy B, Kannan S, Sayane S, Chih-Chia S, Robert L J, Carol VR, Edward WY and Spalding, MH 2020 Structure and function of LCI1: a plasma membrane CO_2 channel in the *Chlamydomonas* CO_2 concentrating mechanism. *Plant J.* **102** 1107–1126

Lam MK and Lee KT 2013 Effect of carbon source towards the growth of *Chlorella vulgaris* for CO_2 bio-mitigation and biodiesel production. *Intl. J. Greenh. Gas Con*. **14** 169–176

Lieman-Hurwitz J, Rachmilevitch S, Mittler R, Marcus Y and Kaplan, A 2003 Enhanced photosynthesis and growth of transgenic plants that express ictB, a gene involved in HCO_3^- accumulation in cyanobacteria. *Plant Biotechnol. J.* **1** 43–50

Lin MT, Occhialini A, Andralojc PJ, Parry MAJ and Hanson, MR 2014 A faster rubisco with potential to increase photosynthesis in crops. *Nature* **513** 547–50

Long SP, Marshall-Colon A and Zhu, XG 2015 Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. *Cell* **161** 56–66

Lucker B and Kramer DM 2013 Regulation of cyclic electron flow in *Chlamydomonas reinhardtii* under fluctuating carbon availability. *Photosynth. Res*. **117** 449–459

Ma Y, Pollock SV, Xiao Y, Cunnusamy K and Moroney JV 2011 Identification of a novel gene, CIA6, required for normal pyrenoid formation in *Chlamydomonas reinhardtii. Plant Physiol.***156** 884–896

Machingura MC, Bajsa-Hirschel J, Laborde SM, Schwartzenburg JB, Mukherjee B, Mukherjee A and Moroney JV 2017 Identification and characterization of a solute carrier, CIA8, involved in inorganic carbon acclimation in *Chlamydomonas reinhardtii*. *J Exp Bot*. **68** 3879–3890

Mackinder LCM 2017 The *Chlamydomonas* CO_2-concentrating mechanism and its potential for engineering photosynthesis in plants. *New Phytol*. **217** 54–61

Mackinder LCM, Chen C, Leib RD, Patena W, Blum SR, Rodman M, Ramundo S, Adams CM, Jonikas MC 2017 A spatial interactome reveals the protein organization of the algal CO_2-concentrating mechanism. *Cell* **171** 133–147

Mackinder LCM, Meyer MT, Mettler-Altmann T, Chen VK, Mitchell MC, Caspari O and Jonikas M C 2016 A repeat protein links RuBisCo to form the eukaryotic carbon-concentrating organelle. *Proc. Natl. Acad. Sci. USA* **113** 5958–5963

Markelova AG, Sinetova MP, Kupriyanova EV and Pronina NA 2009 Distribution and functional role of carbonic anhydrase Cah3 associated with thylakoid membranes in the chloroplast and pyrenoid of *Chlamydomonas reinhardtii. Russian J. Plant Physiol.* **56** 761–768

Matsuda Y, Hopkinson BM, Nakajima K, Dupont CL and Tsuji Y 2017 Mechanisms of carbon dioxide acquisition and CO_2 sensing in marine diatoms: a gateway to carbon metabolism. *Philos. TR Soc. B: Biol. Sci*. **372** 20160403

Meyer MT, Genkov T, Skepper JN, Jouhet J, Mitchell MC, Spreitzer RJ and Griffiths H 2012 RuBisCosmall-subunit helices control pyrenoid formation in *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* **109**19474–19479

Meyer MT, Whittaker C and Griffiths H 2017 The algal pyrenoid: key unanswered questions. *J. Exp. Bot*. **68** 3739–3749

Mitchell MC, Metodieva G, Metodiev MV, Griffiths H and Meyer MT 2017 Pyrenoid loss impairs carbon-concentrating mechanism induction and alters primary metabolism in *Chlamydomonas reinhardtii*. *J. Exp. Bot*. **68** 3891–3902

Mitchell MC, Meyer MT and Griffiths H 2014 Dynamics of carbon-concentrating mechanism induction and protein relocalization during the dark-to-light transition in synchronized *Chlamydomonas reinhardtii*. *Plant Physiol*. **166** 1073–1082

Mitra M, Lato SM, Ynalvez RA, Xiao Y and Moroney JV 2004 Identification of a new chloroplast carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant physiol*. **135** 173–82

Miura K, Yamano T, Yoshioka S,Tsutomu Y, Yoshihiro K, Taniguchi F, Asamizu E, Nakamura Y, Katsuyuki T, Yamato, Kanji OK and Fukuzawa H 2004 Expression profiling- based identification of CO_2 responsive genes regulated by CCM1 controlling a carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiol*. **135** 1595–1607

Mora Salguero DA, Fernandez-Nino M, Serrano-Bermudez LM, Paez Melo DO, Winck FV, C Caldana and Barrios AFG 2018 Development of a *Chlamydomonas reinhardtii* metabolic network dynamic model to describe distinct phenotypes occurring at different CO_2 levels. *Peer J*. **6e** 5528

Moroney JV, Ma Y, Frey WD, Fusilier KA, Pham TT, Simms TA and Mukherjee B 2011 The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles. *Photosynth. Res*. **109** 133–149

Mukherjee A, Lau CS, Walker CE, Rai AK, Prejean CI, Yates G, Thomas EM, Spencer GL, David JV, Mackinder LCM and Moroney JV 2019 Thylakoid localized bestrophin-like proteins are essential for the CO_2 concentrating mechanism of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **116** 16915–16920

Nolke G, Barsoum M, Houdelet M, Arcalís E, Kreuzaler F, Fischer R and Schillberg S 2019 The integration of algal carbon concentration mechanism components into tobacco chloroplasts increases photosynthetic efficiency and biomass. *Biotechnol. J*. **14** 1800170

Occhialini A, Lin MT, Andralojc PJ, Hanson MR and Parry, M A J 2016 Transgenic tobacco plants with improved cyanobacterial RuBisCo expression but no extra assembly factors grow at near wild-type rates if provided with elevated CO_2. *Plant J.* **85** 148–160

Ohnishi N, Mukherjee B, Tsujikawa T, Yanase M, Nakano H, Moroney JV and Fukuzawa H 2010 Expression of a low CO_2-inducible protein, LCI1, increases inorganic carbon uptake in the green alga,*Chlamydomonas reinhardtii*. *Plant Cell* **22** 3105–3117

Ohno N, Inoue T, Yamashiki R, Nakajima K, Kitahara Y, Ishibashi M and Matsuda Y 2012 CO_2/cAMP-responsive cis-elements targeted by a transcription factor with CREB/ATF-like basic zipper domain in the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol*. **158** 499–513

Ort DR, Merchant SS, Alric J, Barkan A, Blankenship RE, *et al.* 2015 Redesigning photosynthesis to sustainably meet global food and bioenergy demand. The integration of algal carbon concentration mechanism components into tobacco chloropl **112** 8529–8536

Peng L, Zhang Z, Lan CQ, Basak A, Bond N, Ding X and Du J 2016 Alleviation of oxygen stress on *Neochloris oleoabundans*: effects of bicarbonate and pH. *J. Appl. Phycol*. **29** 143–152

Pengelly JJL, Förster B, Von Caemmerer S, Badger MR, Price GD and Whitney SM 2014 Transplastomic integration of a cyanobacterial bicarbonate transporter into tobacco chloroplasts. *J. Exp. Bot.***65** 3071–3080

Petroutsos D, Busch A, Janben I, Trompelt K, Bergner SV, Weinl S and Hippler M 2011 The chloroplast calcium sensor CAS is required for photoacclimation in *Chlamydomonas reinhardtii*. *Plant Cell* **23** 2950–2963

Polukhina I, Fristedt R, Dinc E, Cardol P and Croce R 2016 Carbon supply and photoacclimation cross talk in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol*. **172** 1494–1505

Rae BD, Long BM, Forster B, Nguyen ND, Velanis CN, Atkinson N and McCormick AJ 2017 Progress and challenges of engineering a biophysical CO_2-concentrating mechanism into higher plants. *J. Exp. Bot*. **68** 3717–3737

Raven JA & Beardall J 2015 The ins and outs of CO_2. *J. Exp. Bot.* **67** 1–13

Raven JA, Beardall J and Sanchez-Baracaldo P 2017 The possible evolution and future of CO_2-concentrating mechanisms. *J. Exp. Bot.***68** 3701–3716

Renberg L, Johansson AI, Shutova T, Stenlund H, Aksmann A, Raven JA, Gardestrom P, Moritz Tand Samuelsson G 2010 A metabolomic approach to study major metabolite changes during acclimation to limiting CO_2 in *Chlamydomonas reinhardtii*. *Plant Physiol*. **154** 187–196

Ruan CJ, Shao HB and Teixeira da Silva JA 2012 A critical review on the improvement of photosynthetic carbon assimilation in C3 plants using genetic engineering. *Crit. Rev. Biotechnol.* **32** 1–21

Ruiz C, Pla M, Company N, Riudavets J and Nadal A 2016 High CO_2 concentration as an inductor agent to drive production of recombinant phytotoxic antimicrobial peptides in plant biofactories. *Plant Mol. Biol*. **90** 329–343

Salome PA and Merchant SS 2019 A series of fortunate events: Introducing *Chlamydomonas* as a reference organism. *Plant Cell* **31** 1682–1707

Sathasivam R, Radhakrishnan R, Hashem A and Abd Allah EF 2019 Microalgae metabolites: A rich source for food and medicine. *Saudi J Biol Sci*. **26** 709–722

Shutova T, Kenneweg H, Buchta J and Nikitina J 2008 The Photosystem II-associated Cah3 in *Chlamydomonas* enhance the O_2 evolution rate by proton removal. *EMBO J.* **27** 782–9

Simkin AJ, Mc Ausland L, Headland LR, Lawson T and Raines CA 2015 Multigene manipulation of photosynthetic carbon assimilation increases CO_2 fixation and biomass yield in tobacco. *J. Exp. Bot.* **66** 4075–4090

Sinetova MA, Kupriyanova EV, Markelova AG, Allakhverdiev SI and Pronina NA 2012 Identification and functional role of the carbonic anhydrase Cah3 in thylakoid membranes of pyrenoid of *Chlamydomonas reinhardtii*. *BBA–Bioenergetics* **1817** 1248–1255

Solovchenko A and Khozin-Goldberg I 2013 High-CO_2 tolerance in microalgae: possible mechanisms and implications for biotechnology and bioremediation. *Biotechnol. Lett.* **35** 1745–1752

Soupene E, Inwood W and Kustu S 2004 Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO_2. *Proc. Natl. Acad. Sci. USA* **101** 7787–7792

Spalding MH 2009 The CO_2-concentrating mechanism and carbon assimilation. In the *Chlamydomonas* Sourcebook: Organellar and Metabolic Processes 2nd edition Edited by EH Harris and DB Stern Academic publishers 257–301

Sultemeyer DF, Fock HP and Canvin DT 1991 Active uptake of inorganic carbon by *Chlamydomonas reinhardtii*: evidence for simultaneous transport of HCO_3^- and CO_2 and characterization of active CO_2 transport. *Can. J. Bot*. **69** 995–1002

Sultemeyer DF, Millar AG, Espie GS, Fock HP and Canvin DT 1989 Active CO_2 transport by the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* **89** 1213–1219

Tanaka A, Ohno N, Nakajima K and Matsuda Y 2016 Light and CO_2/cAMP signal cross talk on the promoter elements of chloroplastic β-carbonic anhydrase genes in the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol*. **170** 1105–1116

Terentyev VV, Shukshina AK and Shitov AV 2019 Carbonic anhydrase CAH3 supports the activity of photosystem II under increased pH. *BBA - Bioenergetics* **1860** 582–590

Tirumani S, Gothandam KMand Rao BJ 2018 Coordination between photorespiration and carbon concentrating mechanism in *Chlamydomonas reinhardtii*: transcript and protein changes during light-dark diurnal cycles and mixotrophy conditions. *Protoplasma* **256** 117–130

Tirumani S, Kokkanti M, Chaudhari V, Shukla M and Rao B J 2014 Regulation of CCM genes in *Chlamydomonas reinhardtii* during conditions of light–dark cycles in synchronous cultures. *Plant Mol. Biol*. **85** 277–286

Toyokawa C, Yamano T andFukuzawa H 2020 Pyrenoid starch sheath is required for LCIB localization and the CO_2-concentrating mechanism in green algae. *Plant Physiol.* **182** 1883–1893

Tredici MR 2010 Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. *Biofuels* **1** 143–162

Tresguerres M, Buck J and Levin LR 2010 Physiological carbon dioxide, bicarbonate, and pH sensing. *Pflüg. Arch. Eur. J Physiol*. **460** 953–964

Ueno Y, Shimakawa G, Miyake C and Akimoto S 2018 Light-harvesting strategy during CO_2-dependent photosynthesis in the green alga *Chlamydomonas reinhardtii*. *J. Phys. Chem. Lett*. **9** 1028–1033

Vance P and Spalding MH 2005 Growth, photosynthesis, and gene expression in *Chlamydomonas* over a range of CO_2 concentrations and CO_2/O_2 ratios: CO_2 regulates multiple acclimation states. *Can. J. Bot*. **83** 796–809

Villarejo A 2002 A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution. *EMBO J.* **21** 1930–1938

Villarejo A, Martinez F, Pino Plumed M and Ramazanov Z 1996 The induction of the CO_2 concentrating mechanism in a starch-less mutant of *Chlamydomonas reinhardtii*. *Physiol Plant*. **98** 798–802

Wang H, Yan X, Aigner H, Bracher A, Nguyen ND, HeeWY, Long BM, Price GD, Hartl FU and Hayer-Hartl M 2019 RuBisCo condensate formation by CcmM in β-carboxysome biogenesis. *Nature* **566** 131–135

Wang L, Yamano T, Kajikawa M, Hirono M and Fukuzawa H 2014a Isolation and characterization of novel high-CO_2-requiring mutants of *Chlamydomonas reinhardtii*. *Photosynth. Res*. **121** 175–184

Wang L, Yamano T, Takane S, Niikawa Y, Toyokawa C, Ozawa S and Fukuzawa H 2016 Chloroplast-mediated regulation of CO_2-concentrating mechanism by Ca^{2+}-binding protein CAS in the green alga *Chlamydomonas reinhardtii*. *Proc. Natil. Acad. Sci. USA* **113** 12586–12591

Wang Y, Huang Y, Wang J, Cheng C, Huang W, Lu P, Xu YN, Wang P, Yan N and Shi Y 2009 Structure of the formate transporter FocA reveals a pentameric aquaporin-like channel. *Nature* **462** 467–472

Wang Y and Spalding MH 2014 Acclimation to very low CO_2: contribution of limiting CO_2 inducible proteins, LCIB and LCIA, to inorganic carbon uptake in *Chlamydomonas reinhardtii*. *Plant Physiol.***166** 2040–2050

Wang Y, Stessman DJ and Spalding MH 2015 The CO_2 concentrating mechanism and photosynthetic carbon assimilation in limiting CO_2: how *Chlamydomonas* works against the gradient. *Plant J.* **82** 429–448

Wilson RH, Thieulin-Pardo G, Hartl FU and Hayer-Hartl M 2019 Improved recombinant expression and purification of functional plant RuBisCo. *FEBS Lett*. **593** 611–621

Winck VF, Arvidsson S, Riaño-Pachón DM, Hempel S, Koseska A, Nikoloski Zand Mueller Roeber B 2013 Genome-wide identification of regulatory elements and reconstruction of gene regulatory networks of the green alga *Chlamydomonas reinhardtii* under carbon deprivation. *PLoS one* **8** e79909

Wunder T, Cheng SL, Lai SK, Li HY and Mueller-Cajar O 2018 The phase separation underlying the pyrenoid-based microalgal RuBisCo supercharger. *Nat. Commun*. **9** 5076

Wunder T, Oh ZG and Mueller Cajar O 2019 CO_2 fixing liquid droplets: towards a dissection of the microalgal pyrenoid. *Traffic*. **20** 380–389

Xiang Y, Zhang J and Donald P 2001 The Cia5 gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii. Proc. Natil. Acad. Sci. USA* **98** 5341–5346

Yamano T, Miura K and Fukuzawa H 2008 Expression analysis of genes associated with the induction of the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiol*. **147** 340–354

Yamano T, Sato E, Iguchi H, Fukuda Y and Fukuzawa H 2015 Characterization of cooperative bicarbonate uptake into chloroplast stroma in the green alga *Chlamydomonas reinhardtii*. *Proc. Natil. Acad. Sci. USA* **112** 7315–7320

Yamano T, Toyokawa C and Fukuzawa H 2018 High-resolution sub organellar localization of Ca^{2+}-binding protein CAS, a novel regulator of CO_2-concentrating mechanism. *Protoplasma* **255** 1015–1022

Yamano T, Tsujikawa T, Hatano K, Ozawa S, Takahashi Y and Fukuzawa H 2010 Light and low CO_2 dependent LCIB/LCIC complex localization in the chloroplast supports the carbon concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Cell Physiol*. **51** 1453–1468

Yang S M, Chang CY, Yanagisawa M, Park I, Tseng TH and Ku, MSB 2008 Transgenic rice expressing cyanobacterial bicarbonate transporter exhibited enhanced photosynthesis, growth and grain yield; in *Photosynthesis. Energy from the Sun* (Eds.) JF Allen,E Gantt, JH Golbeckand BOsmond (Dordrecht, Springer) pp 1243–1246

Yoshioka S, Taniguchi F, Miura K, Inoue T, Yamano T and Fukuzawa H 2004 The novel Myb transcription factor LCR1 regulates the CO_2-responsive gene Cah1, encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Cell* **16** 1466–1477

Zeiler KG, Heacox DA, Toon ST, Kadam KL and Brown LM 1995 The use of microalgae for assimilation and utilization of carbon dioxide from fossil fuel-fired power plant flue gas. *Energy Convers. Manag*. **36** 707–712

Zhan Y, Marchand CH, Maes A, Mauries A, Sun Y, Dhaliwal JS and Zerges W 2018 Pyrenoid functions revealed by proteomics in *Chlamydomonas reinhardtii*. *PLoS One* **13** e0185039

Zhang J, Wang N, Miao Y, Hauser F, McCammon JA, Rappel WJ and Schroeder JI 2018 Identification of SLAC1 anion channel residues required for CO_2/bicarbonate sensing and regulation of stomatal movements. *Proc. Natil. Acad. Sci. USA* **115** 11129–11137

Zheng YL, Yuan C, Liu JH, Hu GR, and Li FL 2014 Lipid production by a CO_2-tolerant green microalgae *Chlorella* sp. MRA1. *J. Microbiol. Biotechnol*. **24** 683–689