Origins and diversity of eukaryotic CO$_2$-concentrating mechanisms: lessons for the future

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Abstract

The importance of the eukaryotic algal CO$_2$-concentrating mechanism (CCM) is considered in terms of global productivity as well as molecular phylogeny and diversity. The three major constituents comprising the CCM in the majority of eukaryotes are described. These include: (i) likely plasma- and chloroplast-membrane inorganic carbon transporters; (ii) a suite of carbonic anhydrase enzymes in strategic locations; and usually (iii) a microcompartment in which most Rubisco aggregates (the chloroplast pyrenoid). The molecular diversity of known CCM components are set against the current green algal model for their probable operation. The review then focuses on the kinetic and crystallographic interactions of Rubisco, which permit pyrenoid formation and CCM function. Firstly, we consider observations that surface residues of the Rubisco small subunit directly condition Rubisco aggregation and pyrenoid formation. Secondly, we reanalyse the phylogenetic progression in green Rubisco kinetic properties, and suggest that Rubisco substrate selectivity (the specificity factor, $S_{rel}$, and affinity for CO$_2$, $K_c$) demonstrate a systematic relaxation, which directly relates to the origins and effectiveness of a CCM. Finally, we consider the implications of eukaryotic CCM regulation and minimum components needed for introduction into higher plants as a possible means to enhance crop productivity in the future.

Key words: aquatic photosynthesis, C$_4$, CO$_2$-concentrating mechanism, Chlamydomonas, crop engineering, pyrenoid, Rubisco.

Introduction

Inorganic CO$_2$ fixed by Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) in the Calvin–Benson–Bassham (CBB) cycle is the source of $\geq$99% of all organic carbon supporting life on earth (Berg, 2011). Nearly half of the 105 petagrams of carbon net primary production arising from oxygenic photosynthesis is attributable to the hydrosphere, in approximately equal measure to cyanobacterial and eukaryotic algae (Field et al., 1998; Falkowski and Raven, 2007). Algae achieve this extraordinary feat with a standing biomass less than one-hundredth that of land plants (Huston and Wolverton, 2009). Whereas marine and freshwater environments are often replete with dissolved inorganic carbon (DIC), it is present mainly in the form of bicarbonate. Access to free CO$_2$ in solution can also be limited by low diffusivity and extent of surface boundary layers. Therefore, many algae are capable of inducing a CO$_2$-concentrating mechanism (CCM) (Giordano et al., 2005), although entire groups of eukaryotic algae have been found to dispense with a CCM in environments favouring high concentrations of free CO$_2$ or a minimal diffusive boundary layer, for example in acidic environments or high currents (Raven et al., 2005; Diaz and Maberly, 2009). In cyanobacteria, however, the CCM appears to be obligatory. The algal CCM, whether prokaryotic or eukaryotic, is classically described from a combination of inorganic carbon transporters (which have to be energized, by ATP, NADPH, or an ion gradient), carbonic anhydrases (CAs),...
and a microcompartment for the delivery of CO₂ to Rubisco. Transporters help in building up intracellular pools of DIC, usually in the form of HCO₃⁻. Carbonic anhydrases interconvert HCO₃⁻ and CO₂, the direction being dependent on subcellular compartment and pump/leak processes. The microcompartment packages the majority of Rubisco and helps to minimize CO₂ leakage. In cyanobacteria, Rubisco is confined to multiple capsid-like carboxysomes, an apparently obligatory feature for the operation of the prokaryotic CCM. In eukaryotic algae, Rubisco is generally, but not always, localized to a single or several pyrenoids. Finally, proton pumps are required to maintain pH homeostasis following the dehydration of bicarbonate. Proton pumps have also been shown to substitute for active transport of DIC (Raven and Beardall, 2003).

Whereas the molecular basis and physiology of the cyanobacterial CCM are being increasingly better resolved (reviewed by Espie and Kimber, 2011; Price, 2011), many aspects of the eukaryotic algal CCM remain contentious, despite more than 30 years of research in the model green alga *Chlamydomonas reinhardtii*. Here, we explore whether molecular components currently associated with the *Chlamydomonas* biophysical CCM are unique to this alga or are ubiquitous, and we review evidence providing insights into the molecular structure of the pyrenoid. We then revisit the timing of origin of biophysical CCMs. These are thought to have evolved many times, perhaps some 400–300 million years ago (MYA), when atmospheric CO₂ concentrations fell below ten times the present levels, concurrent with the rise of terrestrial photosynthesis (Badger et al., 2002). Functionally, improving the operating efficiency of Rubisco under locally elevated CO₂ suggests that biophysical CCMs are analogous to biochemical CCMs, whereby crassulacean acid metabolism and C₄ have evolved independently in many terrestrial plant lineages (Edwards and Ogburn, 2012). We offer new insights for the possible relaxation of Rubisco kinetics with the presence of both biophysical and biochemical CCM systems. We conclude by discussing briefly how, and why, a better understanding of the eukaryotic CCM is important in the current context of photosynthesis reengineering.

### Physiological components of extant eukaryotic algal CCM systems

To date, at least 16 proteins have been implicated in the operation of the biophysical CCM in *Chlamydomonas*: six integral membrane channels or transporters (reviewed by Wang et al., 2011), six CAs (reviewed by Moroney et al., 2011), two soluble proteins forming a putative CO₂-recapturing barrier inside the chloroplast (Yamano et al., 2010), and two nuclear regulatory factors (Fukuzawa et al., 2001; Xiang et al., 2001; Kohinata et al., 2008). The current model for their integration and operation is summarized in Fig. 1 (after Moroney and Ynalvez, 2007; Moroney et al., 2011; Wang et al., 2011). It shows the probable coupling of distinct inorganic transporters, proton pumps, and associated CAs required for the successive conversion and transport of inorganic carbon from an external medium, across the typical cellular compartments of a eukaryotic cell such as *Chlamydomonas*.

The integration of these transport processes results in a CCM that usually produces an internal CO₂ concentration around Rubisco ~40× above ambient (Badger et al., 1980), which is at least one order of magnitude lower than that usually associated with a fully induced cyanobacterial CCM (Badger and Gallagher, 1987). As we discuss below in more detail, theoretical modelling (Raven, 1997) and the probable location of a pyrenoidal CA (Sinetova et al., 2012) suggest that some additional inorganic carbon transporter should be located on the thylakoids extending into the pyrenoid. The magnitude of the leak process, which can be determined quantitatively from carbon isotope discrimination measurements (Berry, 1989; Meyer et al., 2008), is thought to be limited partly by a starch sheath surrounding the pyrenoid, as well as low CO₂-inducible protein B and C (LCIB/LCIC) complexes (Miura et al., 2004; Wang and Spalding, 2006; Yamano et al., 2010).

The other usual feature of the algal CCM is the chloroplast pyrenoid, and some form of pyrenoid seems ubiquitous in most eukaryotic CCM systems (Raven et al., 2012). There are exceptions: a pyrenoid is not found in the genus *Chloromonas*, where there is evidence for some CCM activity in some species (Morita et al., 1998, 1999), with the genus closely associated phylogenetically to *Chlamydomonas* (Nozaki et al., 2002). Other examples of non-pyrenoidal CCM systems are associated with reduced carbon accumulation capacity (Zenviron et al., 1985; Badger et al., 1998; Giordano et al., 2005; Raven et al., 2012). The advantage of such a microcompartment is to allow inorganic carbon accumulation and limit leakage, in the close proximity to tightly packaged Rubisco. The pyrenoid seems to represent an essential component allowing carbon concentration, particularly when modelled for either algal or higher plant systems (Badger et al., 1998; Price et al., 2011, 2013), as discussed below.

### Molecular diversity of the Chlamydomonas CCM system

The sequencing of a number of algal genomes, from species that diverged at least since the Mesoproterozoic, makes it now possible to explore whether molecular components implicated in the *Chlamydomonas* CCM are unique or ubiquitous amongst aquatic photoautotrophs. The presence/absence survey shown in Table 1 is the result of a BLASTP search with a cut-off criterion of <E−10 (identity and similarity percentages are given in Table S1 at JXB online). Although there is a large body of physiological evidence for the operation of CCM in algae other than *Chlamydomonas* (Table 1, last column; Raven et al., 2012), the identity of the molecular components is generally not known. Furthermore, the ongoing annotation of most genomes makes it difficult at this stage to determine whether CCM candidates identified here are unique or redundant (hence a presence/absence scoring in Table 1). If such a survey supports the notion that functional elements required
to operate a *Chlamydomonas*-type biophysical CCM are easily recruitable, it is not evidence for their actual implication in a biophysical CCM in other species. Genes coding for enzymes involved in C4 photosynthesis are also ubiquitous, for example in diatoms (Armbrust et al., 2004; Kroth et al., 2008), yet only *Thalassiosira* seems to have a form of C3–C4 intermediate pathway in addition to a biophysical CCM (Roberts et al., 2007). *Chlamydomonas* has proven irreplaceable for molecular-level studies, but the ecological importance of this (soil-dwelling) alga is questionable. There is now an urgent need to develop the molecular characterization of the CCM components, particularly DIC transporters, in globally significant algae such as the Prasinophytes *Micromonas* and *Ostreococcus*, diatoms, and coccolithophores. Proteins listed in Table S1 may offer candidates for complementation experiments in DIC uptake-impaired *Chlamydomonas* mutants.

**Candidate inorganic carbon transporters at the plasma membrane: HLA3 and LCI1**

HLA3 (high light-activated 3, identified by Im and Grossman, 2001) has a high sequence similarity to full-sized ABC transporters of the ABCC subfamily (formerly known as multidrug resistance-associated proteins, MRP). ABCC transporters are present in all eukaryotes and perform many different transport functions. Examples in vascular plants include vacuolar sequestration of toxic metabolites, transport of chlorophyll catabolites during senescence, and shuttling of folate, but not active transport of DIC (Klein et al., 2006). Functional annotation and predicted topology of the 147 kDa protein is that of a classical ABCC transporter, with two globular domains for ATP hydrolysis connected by two hydrophobic domains each containing long transmembrane helices, responsible for substrate recognition and transport across the lipid bilayer (Fig. S1, at JXB online). It is so far the only energy-dependent candidate transporter in *Chlamydomonas*. The protein is predicted to be localized at the plasma membrane, consistent with evidence that plasma-membrane ATPase inhibitors decrease photosynthetic capacity (Karlsson et al., 1994). Further genetic and physiological evidence supporting the hypothesis that HLA3 is important for bicarbonate uptake in *Chlamydomonas* was reviewed by Wang et al. (2011). There is a 40-fold increase in *HLA3* transcripts when *Chlamydomonas* is shifted to low-CO2 for at least 3 h, i.e. when the CCM is fully induced (Brueggeman et al., 2012). There are seven ABCC-type transporters in the *Chlamydomonas* proteome with up to 59% similarity, but only HLA3 has been implicated in the CCM. All surveyed algal proteomes contain ABCC transporters, with up to nearly 50% similarity in Prasinophytes (Table 1 and Table S1), although the similarity was often highest with ABCC transporters other than HLA3. However, this is not evidence for functional equivalence. The near-identical...
Table 1. Presence (+) or absence (−) of molecular components currently associated with the Chlamydomonas reinhardtii CCM.

<table>
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<th>β-CA</th>
<th>α-CA (ABC)</th>
<th>LCIA-like (NAR)</th>
<th>CCP1/2-like (MCP)</th>
<th>LC11-like</th>
<th>LC1B/C-like</th>
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Positive hits were inferred from BLASTP search (cut-off <E–10) in 17 proteomes (databases, see Supplementary data): 15 unicellular algae, one filamentous brown alga (Ectocarpus), and the model plant Arabidopsis. References for genome and presence/absence of CCM and pyrenoid are given in the last column. References supporting the presence/absence of a pyrenoid were selected for the quality of published transmission electron micrographs rather than primacy. Note that the absence of CCM in Coccomyxa was established experimentally for a phylogenetically close lichen photobiont but not yet in the free-living marine Coccomyxa subellipsoidea.

Abbreviations and protein family database number (after Finn et al., 2010): ABC, ATP-binding cassette transporter (PF00005; PF00664); CA, carbonic anhydrase (PF00194); CCP, chloroplast carrier protein; CEM, chloroplast envelope membranes; LCI, low CO2-induced; LCR, low CO2-response regulator; MCP, mitochondrial carrier protein (PF00153); Myb, myeloblastosis-like DNA-binding domain (PF00249); NAR, nitrite accumulation rate (=formate/nitrite transporter) (PF01226).

References: 1, Merchant et al., 2007; 2, Goodenough, 1970; 3, Blanc et al., 2010; 4, Němcová and Kalina, 2000; 5, Blanc et al., 2012; 6, Peveling and Galun, 1976; 7, Palmqvist et al., 1994; 8, Derelle et al., 2006; 9, Chretiennot-Dinet et al., 1995; 10, Palenik et al., 2007; 11, Worden et al., 2009; 12, Manton and Parke, 1960; 13, Igelas-ROdriguez et al., 1998; 14, Moreau et al., 2012; 15, Eikrem and Throndsen, 1990; 16, AGI, 2000; 17, Matsuizaki et al., 2004; 18, Zenvirth et al., 1985; 19, Price et al., 2012; 20, Fathinejad et al., 2008; 21, Armburst et al., 2004; 22, McGinn and Morel, 2008; 23, Hopkinson et al., 2011; 24, Bowler et al., 2008; 25, Koth et al., 2008; 26, Cock et al., 2010; 27, Nimer and Merrett, 1996; 28, Tsuji et al., 2012; 29, Moestrup and Sengco, 2001.
Arabidopsis ABCC1 and ABCC2 (93% similarity), which are both localized to the same subcellular compartment, perform different physiological functions (Wanke and Kolukisaoghlu, 2010). The result of our BLASTP search is perhaps unsurprising, given the ubiquity of ABCC proteins, and their implication in inorganic carbon uptake remains to be investigated in all surveyed algae.

LCI1 (low-CO2 inducible 1, identified by Burow et al., 1996) is a small protein seemingly unique to Chlamydomonas. There appears to be no close homologue in any other algae (a less restrictive search by Ohnishi et al., 2010, identified a number of homologues including a yeast osmosensor and the Chlamydomonas flagellar-associated protein FAP292, which is transcriptionally associated with LCI1; see below). If the predicted protein topology (Fig. S1) is accurate, then the hydrophobic domains are too short to provide a transmembrane channel in a monomeric form. There is a 3000-fold increase in transcripts when the CCM is induced (Brueggeman et al., 2012). This makes LCI1 the second most inducible gene implicated in the Chlamydomonas CCM. Translational fusion with green fluorescent protein did not resolve the localization at the plasma membrane unequivocally (Ohnishi et al., 2010). However, if the functional importance of LCI1 in DIC uptake and uniform localization were confirmed, this would imply that transport of inorganic carbon is not preferentially located where the diffusion path length to Rubisco (and the pyrenoid) is shortest. Some representations of the Chlamydomonas CCM have implied an asymmetric organization of the inorganic carbon-uptake apparatus (Grossman et al., 2007; Moroney et al., 2011).

Candidate inorganic carbon transporters at the chloroplast membrane: LCIA and CCP1/2

LCIA (also known as NAR1.2, identified by Miura et al., 2004) belongs to the formate/nitrite transporter family. Bacterial members of this family form a pentameric aquaporin-like transmembrane channel, believed to facilitate anion fluxes rather than active transport (Wang et al., 2009). LCIA is so far the only candidate transporter that has been tested for substrate specificity in a heterologous system (Mariscal et al., 2006). Whilst capable of shuttling bicarbonate in Xenopus oocytes, a low response in the presence of micromolar concentrations implies a low affinity, and the associated voltage depolarization could also infer additional cation entry. LCIA is predicted to be targeted to the chloroplast membrane, although this also awaits experimental validation. There is a 4000-fold increase in LCIA transcripts when the CCM is induced (Brueggeman et al., 2012), making this the most highly CO2-responsive gene of all CCM genes. There are five other proteins of this family in Chlamydomonas (Mariscal et al., 2006) and close homologues with high sequence similarity (~50%) were found in all surveyed proteomes except for Arabidopsis and the red alga Cyanidioschyzon.

CCP1 and CCP2 (chloroplast carrier protein 1 and 2, identified by Spalding and Jeffrey, 1989; Chen et al., 1997) encode two members of the large family of mitochondrial carrier proteins, located primarily but not exclusively at the mitochondrial inner membrane and involved in ADP/ATP translocation. Similar-sized mammalian ADP/ATP translocators (belonging to the solute carrier subfamily SLC25) are functional as monomers (Pebay-Peyroula et al., 2003). Proteins with high sequence similarity to CCP1/2 are present in all surveyed proteomes. The closest homologue in Arabidopsis is the mitochondrial transporter BOU (à bout de souffle). BOU mutants have a phenotype similar to plants deficient in fatty acid oxidation, suggesting a role in carnitine–acylcarnitine translocation (Lawand et al., 2002). CCP1/2 are believed to be located at the chloroplast rather than mitochondrial membrane. However, relevance to the algal CCM has been questioned after RNAi knockdown provided no evidence of impaired inorganic uptake (Pollock et al., 2004). If Chlamydomonas has parallel, high/low-affinity DIC uptake systems, deleterious effects of knocking out a single transporter would be difficult to resolve. CCP1/2 are 96% identical, resulting from a recent gene duplication. They are part of an ~100 kb gene cluster on chromosome 4, which also encodes two CAs (CAH1 and CAH2) and two low CO2-inducible proteins of unknown function (LCID/LCIE) (Merchant et al., 2007). The latter are highly homologous to LCIB/LCIC, which form a putative CO2-recapture protein complex (see below). Transcripts of CCP1 increase 2000-fold (CCP2 120-fold) when the CCM is induced (Brueggeman et al., 2012). This makes CCP1 the third most highly induced gene implicated in the CCM.

CemA (chloroplast envelope membrane protein A, encoded by the chloroplast gene CemAycf10, located three genes away from rbcL) is a proton pump at the inner chloroplast envelope. It is believed to maintain the stromal pH balance, as the dehydration of bicarbonate releases hydroxyl anions that have to be exported from the chloroplast or balanced by an equimolar influx of protons (Rolland et al., 1997). Active proton transport, with conversion of bicarbonate to CO2 in an acidic compartment containing Rubisco, has been shown to substitute entirely for CCMs based on active transport of DIC (Raven, 1997; Raven and Beardall, 2003) but is unlikely to be the modus operandi in Chlamydomonas (see below).

Other candidate transporters

Conversely, it remains to be investigated whether anion transporters belonging to other SLC subfamilies are also active in the Chlamydomonas CCM. For example, bona fide sodium-dependent chloride/bicarbonate exchangers (SLC4) and sulphate co-transporters (SLC26) have been implicated in the CCM of cyanobacteria, coccolithophores, and diatoms (Price et al., 2004; Kroth et al., 2008; Price, 2011; Richier et al., 2011) but not green algae. Phaeodactylum has two SLC4 proteins and a Na+-anion driven exchanger, all predicted to be targeted to the chloroplast membrane (Kroth et al., 2008). Thalassiosira has one predicted plasma-membrane Na+-dependent bicarbonate transporter (Kroth et al., 2008). The role of the SLC4 transporter identified in Emiliania huxleyi might be either in inorganic carbon uptake for photosynthesis, calcium carbonate precipitation in coccoliths, or pH homeostasis (Richier et al., 2011). Yet, there are homologues of some of these transporters in green algal proteomes (Pootakham et al., 2010).
Finally, novel candidate transporters can also be mined by comparing transcriptomes. Timme and Delwiche (2010) identified a set of 114 genes unique to two Charophytes (Coleochaete and Spirogyra) and Chlamydomonas but absent in Arabidopsis. The three algae operate a CCM with a pyrenoid (Raven and Gbddell, 1978; Meyer et al., 2008), but Arabidopsis has C3 photosynthesis with diffusive CO2 entry. We found that this set includes HLA3 and LCIB (Table S2 at JXB online), suggesting that these two genes may code for bona fide CCM components. The set also included four other ABC transporters, of classes other than ABCC, which should be investigated for a possible involvement in the algal CCM. RbcS1, which encodes one of two Rubisco small subunit (SSU) isoforms, and a SSU post-translational methyltransferase (RMT2), were also unique to the three algae, a finding intriguingly consistent with evidence that the SSU is implicated in the formation of the pyrenoid (Meyer et al., 2012; see below).

Carbonic anhydrases

The identity, localization, and function of all 12 CAs identified to date in Chlamydomonas have been exhaustively reviewed by Moroney et al. (2011). The current consensus is that only the constitutively expressed chloroplastic α-CAH3 and β-CAH6 are essential to the operation of a CCM in Chlamydomonas. The highly expressed extracellular α-CAH1 (~1% of total proteins, Moroney et al., 2011; 660-fold transcriptional increase when the CCM is induced, Brueggeman et al., 2012) probably contributes to a positive influx of CO2. By comparison, the β-cyanobacterial CCM is reliant on a single CA, residing inside the carboxysome (Price and Badger, 1989). In Chlamydomonas, CO2 is likely to be delivered to Rubisco via CAH3, localized in the lumen of thylakoids traversing the pyrenoid (reviewed by Moroney et al., 2011). When cells are shifted to low CO2, CAH3 becomes phosphorylated and the fraction residing in intrapyrenoid thylakoids doubles, without de novo synthesis (Blanco-Rivero et al., 2012; Sinetova et al., 2012). Several laboratories have proposed that CAH3 is also associated with the O2-evolving photosystem II (PSII), supplying the acceptor side with bicarbonate, which facilitates the electron flow by binding to the non-heme iron complex between Qa and Qb (reviewed by Shevela et al., 2012). This seemingly conflicts with evidence that intrapyrenoid thylakoids are enriched in PSII and depleted in PSII (McKay and Gibbs, 1991), which would otherwise reduce the efficiency of the CCM by releasing O2 in the vicinity of Rubisco. The activation and relocalization of CAH3, demonstrated by Blanco-Rivero et al. (2012), reconciles the apparent incompatible functions of this CA. The model remains, however, contingent to the identification of a bicarbonate transporter located at the thylakoid membrane, and no such transporter has yet been found.

There are five known classes of CAs, differing among other properties in the metal co-factor. α- and β-CAs are ubiquitous throughout the green lineage, although predicted proteins with high sequence similarity to α-CAs were often closer to isoforms other than CAH3 (Table S1). α- and β-CAs are absent in red algae and Thalassiosira, but diatoms have multiple γ- and δ-CA isoforms (Moroney et al., 2011). The two Phaeodactylum α-CA isoforms (Satoh et al., 2001; Harada and Matsuda, 2005) are localized to a subpyrenoidal compartment, and may be functionally analogous to CAH3 (Tachibana et al., 2011).

Candidate regulatory factors

Whole-transcriptome shotgun sequencing experiments in Chlamydomonas (Brueggeman et al., 2012; Fang et al., 2012) have refined earlier studies (Miura et al., 2004) and shown that the expression of more than one out of three genes is CO2 responsive. These studies confirmed the central role of the nuclear transcriptional regulator CIA5 (also known as CCM1 or the CCM master switch; Fukuzawa et al., 2001; Xiang et al., 2001). All CCM candidates (HLA3, LCI1, LCI2, CCPI/2, CAH1, CAH3, CAH6, and LCIB/LCIC) are under the control of this protein. CIA5, however, does not bind directly to DNA, and its expression is independent of CO2 concentration. Therefore, additional activation systems must exist, such as via LCR1, a bona fide nuclear transcription factor of the Myb family. LCR1 activates CAH1 and LCI1 (Yoshioaka et al., 2004). Whereas CIA5 appears to be conserved only in Trebouxiophyceae (Chlorella and Coccomyxa), the Chlamydomonas LCR1 is an outlier in all known Myb transcription factors, stressing how little is known about the regulation of the algal CCM (let alone the sensing of CO2).

These two transcriptome-wide studies have also made important contributions towards helping to identify novel CCM candidates. Firstly, there appears to be a CCM-specific cluster with 35 genes sharing a common expression pattern (Fang et al., 2012). This cluster includes CAs (CAH1 and CAH4/5), candidate transporters (LCI1, LCI2, and CCPI), low CO2-inducible proteins of unknown function (LCIE), and LCR1, perhaps suggesting an even wider regulation spectrum for this transcription factor. Secondly, Brueggeman et al. (2012) discovered that several CCM genes are arranged into a head-to-head conformation and are transcribed by a common bidirectional promoter. These include CCPI/LCIE, CCPI/LCID, CAH4/5, LCI1/FAP292 (the latter was found to be somewhat homologous to LCI1 by Ohnishi et al., 2010), and other as-yet-uncharacterized low CO2-inducible proteins.

Updating our understanding of the pyrenoid

Molecular composition

Pyrenoids are electron-dense, semi-crystalline protein aggregates present in the chloroplast of many, if not all, eukaryotic algae. They are common in unicellular algae but often absent in seaweeds. Pyrenoids are also found in a single group of land plants, the hornworts (Vaughn et al., 1990; Villareal and Renner, 2012). Early biochemical isolation of pyrenoids suggested a relatively simple composition, with only a dozen different proteins, of which Rubisco accounts for >90%
Subsequent work on green and brown algae found that all pyrenoids have a similar composition (Kerby and Evans, 1978, 1981; Salisbury and Floyd, 1978; Satoh et al., 1984, 1985; Kuchitsu et al., 1988, 1991; Okabe and Okada, 1988; del Rio et al., 1996). Immunogold labeling further showed that Rubisco is preferentially localized to the pyrenoid in all probed species (green algae: McKay and Gibbs, 1989; Villarejo et al., 1997; Charophytes: McKay et al., 1991; Dinoflagellates: Jenks and Gibbs, 2000; Nassoury et al., 2005; Euglenozoa: Osafune et al., 1989; hornworts: Vaughn et al., 1990). Borkhensis et al. (1998) proved that in *Chlamydomonas* the pyrenoidal Rubisco must be active to account for CO₂ assimilation rates. This is generally taken to be the true for all pyrenoids, and established the pyrenoid as the site of CO₂ fixation in the algal CCM, which invites the question of how CO₂ is delivered to the active sites.

The pyrenoid matrix is crossed by a network of tubules 40–60 nm wide, containing thylakoid lamellae in continuity with stromal thylakoids (Ohad et al., 1967), and some micrographs suggest that these tubules may coalesce into a central fibrous knot (Lee et al., 1979), perhaps providing a nucleation or anchoring site for Rubisco. Unlike prokaryotic carboxysomes, pyrenoids are not delineated by a protein shell or membrane. No homologue of any carboxysomal shell protein has ever been found in any eukaryotic genome. The absence of a starch sheath has been shown to have little effect on CCM efficacy (Villarejo et al., 1996). By packaging Rubisco into a confined volume (the near-spherical pyrenoid in *Chlamydomonas* is 1.5–2 μm across, occupying <10% of the chloroplast volume) and by delivering CO₂ into that compartment, CO₂ leakage is minimized (Badger et al., 1998; Price et al., 2011, 2013). In *Chlamydomonas*, Rubisco accounts for only ~5% of total proteins. In cyanobacteria and other algae with a CCM, Rubisco rarely exceeds 10% of total proteins, five times less than the amount in photosynthetic tissues of C₃ higher plants (Evans, 1989; Raven, 1991).

The nature of pyrenoid proteins other than Rubisco has only been partly elucidated, and there is as yet no report of native pyrenoids having been analysed with mass spectrometric tools. In green algae, these include candidate proteins, which models predict to be necessary for pyrenoid function: the luminal CAH3 (Mitra et al., 2005; Markelova et al., 2009; Sinetova et al., 2012) and Rubisco activase (McKay et al., 1991). It is reasonable to assume that the density of packaging of pyrenoidal Rubisco should not impede the free circulation of substrates and products, and should permit access by Rubisco activase (RA) if and where required. RA is a large multimeric protein (135 × 56 Å; Stotz et al., 2011). It is seemingly required by form IB (green) Rubisco for maximal CO₂ fixation, even when a CCM operates, whether biophysical or biochemical (Pollock et al., 2003; von Caemmerer et al., 2005). The need for activation by other forms of Rubisco, including cyanobacterial, is not clear. The purple bacterium *Rhodobacter sphaeroides* has an ATPase-associated protein (CbbX), which releases ribulose-1,5-bisphosphate from its form IC Rubisco, even if the mechanistic details are somewhat different from the green RA (Mueller-Cajar et al., 2011). CbbX is present on the chloroplast genome of all Rhodophyta and algae with a secondary or tertiary red chloroplast (http://chloroplast.ocean.washington.edu/cpbase), but whether it activates the associated form ID Rubisco is not known. Components of PSI have also been found in pyrenoids, suggesting that intrapyrenoid thylakoids could provide ATP to the compartment via cyclic electron transport, perhaps to meet the energetic demands of RA, whilst avoiding the evolution of inhibitory O₂ (McKay and Gibbs, 1991).

As described in Fig. 1 (see above), a recent addition to the *Chlamydomonas* model is a barrier formed by the high-molecular-weight complexes LCIB/LCIC, which under CCM-induced conditions surround the pyrenoid, either to prevent or recapture CO₂ leaking from the pyrenoid (Yamano et al., 2010; Wang et al., 2011). Also, a protein with a methyltransferase-like domain, CIA6, has been shown to be required for normal formation of the pyrenoid in *Chlamydomonas* (Ma et al., 2011).

**Pyrenoid biogenesis and functional importance in the CCM**

Chloroplasts and cells divide synchronously in *Chlamydomonas* (Goodenough, 1970; Ettl, 1976). Whilst the molecular details of pyrenoid biogenesis have yet to be investigated, these early observations showed that, during cell division in *Chlamydomonas*, the pyrenoid is retained. *De novo* pyrenoid formation has been reported in other green algal lineages (Bisalputra and Weier, 1964).

Given that Rubisco accounts for >90% of pyrenoid proteins, it is conceivable that the formation of this microcompart ment primarily requires Rubisco–Rubisco interactions. As few as 7 aa differences between the *Chlamydomonas* and *Chloromonas* Rubisco large subunit (LSU) have been correlated with the presence and absence of a pyrenoid (Nozaki et al., 2002). However, with only one of these residues located at the protein surface (arginine/lysine 32), and the implication of the LSU in catalysis and interaction with RA, it is difficult to see how the LSU could provide an additional function in pyrenoid assembly. This hypothesis could easily be tested via site-directed mutagenesis.

Evidence from recombinant Rubisco expression in cyanobacteria and *Chlamydomonas* has demonstrated the importance of the SSU in correctly packaging form IB Rubisco within the CCM microcompartment. When the native form IB Rubisco of *Synechocystis* is replaced with a form II (SSU-less) of *Rhodospirillum rubrum*, mutants lose the ability to form carboxysomes (Pierce et al., 1989). Furthermore, null mutants of *ccmM*, an open reading frame on the β-carboxysomal CCM operon with some sequence similarity with the gene coding for the SSU, lack carboxysomes and grow poorly under air-level CO₂ (Ludwig et al., 2000). Researchers at the Australian National University and University of Toronto subsequently found that a multienzyme complex, with bicarbonate dehydration activity, is formed around CcmM (Long et al., 2007, 2010, 2011; Cot et al., 2008). CcmM is believed to act as a scaffold, organizing Rubisco and a CA (CcaA) inside β-carboxysomes. The nature of these interactions is not yet fully clarified, but the C
Enzyme complexes (Sainis et al., 2010) of the CBB cycle, including Rubisco, form functional multi-protein linkers. In higher plants and algae, enzymes that span two SSU (Süss et al., 1995), with the cohesion of these complexes being ensured by small protein linkers. The best documented protein linker is the ubiquitous 8 kDa highly flexible molecular adaptor CP12, which transiently complexes glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, and phosphoribulokinase. It is possible that pyrenoids are formed on the basis of a similar mechanism, through the action of a hitherto undiscovered linker protein, interacting with SSU. Alternatively, the pyrenoid could be a simple biophysical assemblage of Rubisco, based for example on hydrophobic interactions (Meyer et al., 2012).

It is also relevant to consider whether the pyrenoid system found in *Chlamydomonas* is representative of all other members of the Chlorophyceae, or indeed Prasinophytes or other phytoplankton lineages. For instance, among a number of unique features, peridinin-containing dinoflagellates have a form II Rubisco (Morse et al., 1995; Whitney and Andrews, 1998) and could be considered to have an obligate requirement for a CCM (Raven et al., 2012). Rather than discounting the importance of the Rubisco SSU, this suggests multiple independent mechanisms of pyrenoid formation. Subsequent studies on the circadian control in the dinoflagellate *Gonyaulax polyedra* (Nassoury et al., 2001) have demonstrated the plasticity of pyrenoid formation. Not only does Rubisco aggregate at the centre of the cell to form a pyrenoid in the light, but oxygen evolution from the peripheral chlorophyll *protein linker complex* has a different circadian periodicity to maximal CO$_2$ fixation, which coincides with pyrenoid formation (Nassoury et al., 2001). It is therefore tempting to speculate that, just as there may be at least 66 independent origins of the terrestrial C$_4$ pathway (Sage et al., 2011), there could be an equivalently high number of eukaryotic/CCM systems. In Fig. 2, we indicate pyrenoid presence/absence in 60 green algae, which all have a green form IB Rubisco inherited from a single endosymbiotic event, but diverged up to 1.3 billion years ago (BYA). The distribution supports the notion that the pyrenoid has been gained and lost many times independently. In comparison, the hornwort pyrenoid arose from a presumably pyrenoidless ancestral condition at least five times independently over the past 100 million years (Villareal and Renner, 2012).

**Ancestry of the aquatic CCM**

Given the molecular diversity of CCM constituents and potential range of origins, it is pertinent to consider the molecular genealogy of oxygenic photosynthesis, which spans 2.8 billion years or more (Farquhar et al., 2011). Whilst the origin of the Calvin cycle is still conjectural (Martin and Schnarrenberger, 1997), Rubisco probably derived from a methionine-scavenging pathway enzyme (Ashida et al., 2003, 2005). Cyanobacteria dominated the photosynthetic stage for nearly 1 billion years, triggering the great oxidation event, and maybe even a global snowball Earth around 2.3–2.2 BYA (Kopp et al., 2005). Around 1.6 BYA, a probable single primary endosymbiotic event with a cyanobacterium gave rise to the chloroplast. Then, around 1.5 BYA, the red and green chloroplast lineages split, and 200 million years later, a secondary endosymbiosis of a red plastid gave rise to algal lineages that have come to dominate contemporary oceans (diatoms, coccolithophores, and dinoflagellates). Whereas green algae (and their land plant descendants) retained the native cyanobacterial form IB Rubisco, the red lineage secondarily acquired a proteobacterial form ID Rubisco (Delwiche and Palmer, 1996). At what point was a CCM first required? Raven et al. (2012) considered the range of physiological and environmental drivers that could have promoted CCM expression during periods of low atmospheric CO$_2$ concentration or retained the CCM during subsequent periods of higher CO$_2$ availability. Thus, the energetic cost of sustaining a CCM could in theory be offset against a varying catalytic demand for nitrogen, phosphorus, iron, zinc and manganese, and the probable impact of oxygenation and ocean surface warming (Raven et al., 2012). The fossil record of oceanic primary producers in the Palaeozoic (for details, see Raven et al., 2012) provides no evidence helping to date the origin of the algal CCM. And there is (as yet) no trace of conserved carboxysomes or pyrenoids to attest to an emergence of aquatic CCM before, during, or after the Devonian–Late Permian interval. There have been occasional reports of pyrenoid-like intracellular structures, notably in the 850 million-year-old Bitter Spring formation of Australia (Oehler, 1977), but even modern techniques can at best only confirm the kerogenous nature of these inclusions (Schopf and Kudryavtsev, 2005).

A case has been made for the independent, late appearance of both the cyanobacterial and eukaryotic CCM systems in the Carboniferous period (Badger et al., 2002; Price et al., 2005).
(Berner and Kothavala, 2001; Berner, 2006) and shows concentrations 17–25 times the present levels during the Cambrian and Ordovician periods (550–450 MYA), followed by a steep and rapid decline to near-present-day levels, coinciding with the extensive colonization of land by plants in the Middle Devonian period, 370 MYA (Stein et al., 2007). However, as shown in Fig. 3, an equivalent reduction in CO₂ levels very probably occurred at least 300 MYA earlier, during the Proterozoic, 750–1250 MYA (Riding, 2006). When considering the gaseous diffusive barriers in stromatolites and other microbial mats, in which cyanobacteria have been thriving for nearly 3 billion years (Raven et al., 2008), one could argue for an even more ancient origin of the CCM. Additionally, Young et al. (2012) proposed that evidence from rbcL sequences suggest positive selection during periods of declining CO₂, consistent with diversification of Rhodophyta (500–600 MYA), Haptophytes (e.g. coccolithophores, 300–400 MYA), and diatoms (50–100 MYA).

**Phylogenetic progression in Rubisco kinetic properties: is this cause or effect of a CCM?**

Compared with cyanobacteria and algae, Rubisco from extant land plants reliant on diffusive CO₂ entry has a higher turnover rate per active site \(k_{cat}\), a higher affinity for CO₂
(1/Kc), and a higher specificity for CO2 (S_{rel} = Vc/Kc/Vo/Ko, where Vc and Vo are the maximum velocities for carboxylation and oxygenation, and Kc and Ko are the relative Michaelis constants for CO2 and O2) (reviewed by Badger et al., 1998).

Although the kinetic properties of ancestral Rubisco forms are unknown, these results are generally interpreted as evidence that modifications increasing carboxylation efficiency (via k_{cat}, Kc, and/or S_{rel}) have occurred during the course of evolution from cyanobacteria to land plants, perhaps supporting the requirement for a CCM when CO2 became limiting.

Developing the analysis of André (2011), Fig. 4 presents kinetic data from form IB green Rubisco isoforms from 43 different species, covering a range of CO2 acquisition mechanisms. Granted that care needs to be taken when comparing analyses in different laboratories using different analytical procedures, we propose a different interpretation of the data. The progression suggests that the evolution of CCMs, which increase intracellular DIC concentration by several orders of magnitude, has removed some of the pressure to enhance carboxylation kinetic efficiency. In C3–C4 intermediates, as represented by the genus Flaveria (Kubien et al., 2008), which perhaps represent a progression towards C4 and intermediate stages therein (Sage et al., 2011), mean affinity for CO2 and Vc/Vc are not different from C3 species (Fig. 4). But in full C4 plants, which perhaps developed from C3–C4 intermediate types relatively recently (30 MYA: Sage et al., 2012), affinity for CO2 (as 1/Kc) is nearly as low as in green algae. In the case of a few C4 monocots, S_{rel} (as Vc/Vc under the current atmosphere) also tends towards algal-like values. This relationship is consistent with S_{rel} data for the green alga Coccomyxa (Fig. 4), which lacks a CCM and pyrenoid (Palmqvist et al., 1994). Thus, we propose that selection for improvements in Vc in form IB Rubisco isoforms has been relaxed under the saturating CO2 provided by a CCM, in proportion to the extent of time that a CCM has been operating, with C4 < Chlorophyta < cyanobacteria. An alternative view might be that the CCM, and reduced competition from O2, has improved Vc, with concomitant elevation in Kc but without any change in S_{rel}.

**Manipulating crop productivity: prospects for engineering an algal CCM into higher plants**

By 2050, the world’s population will exceed 9 billion people. Food production, exacerbated by the food-for-fuel...
conundrum, must increase by 70% to meet the forecasted demand (Bruinsma, 2009). It is now widely recognized, if not accepted, that closing the yield gap may require more bioengineering of field crops to improve resistance to biotic stresses and resilience to climate change impacts (Zhu et al., 2010). One biochemical route to limit the competitive inhibition of carboxylation by O₂ has long been to manipulate Rubisco (Zhu et al., 2004), yet with little success so far. Other approaches suggested to enhance productivity include enhancing ribulose-1,5-bisphosphate regeneration or light use within canopies (Zhu et al., 2010; Price et al., 2011), a photorespiratory bypass (Peterhansel and Offermann, 2012), and introducing elements of the C₄ pathway (Covshoff and Hibberd, 2012). Other lines of research, which relate more specifically to eukaryotic algal systems, are discussed below.

Targeted engineering of Rubisco LSU and SSU residues, or entire Rubisco assemblies, has been informed by phylogenetic comparisons between low- and high-specificity enzyme families (Spreitzer and Salvucci, 2002). A considerable number of residues have already been altered, primarily in Chlamydomonas (reviewed by Wostrikoff and Stern, 2009), without any significantly positive effect on S\textsubscript{rel}. Alternatively, C₃ crop Rubisco could be replaced entirely by a high S\textsubscript{rel} Rubisco from higher plants (Zhu et al., 2010). However, whilst plastid transformation has been an informative tool in Nicotiana, and has identified many of the complexities and challenges ahead, the goal of improving Rubisco catalysis by this engineering strategy remains elusive (see Parry et al., 2013).

Attempts to introduce the C₄ pathway into target crops such as rice will need to upregulate or introduce gene expression, directed in a cell-specific manner across bundle sheath and mesophyll cells, which themselves may not be well differentiated (Hibberd and Covshoff, 2010). A simpler route to enhance Rubisco operating efficiency is the prospect of expressing a cyanobacterial or algal biophysical CCM in higher plants, rather than a full-edged C₄ biochemical concentrating mechanism. A theoretical approach that develops the modelled advantages of a biophysical CCM for algae (Badger et al., 1998) has been used to advance the prospect of ingressing elements of the cyanobacterial system into higher plants (Price et al., 2011). These approaches have been refined to advance a four-stage strategy to introduce such a system into a crop plant, successively incorporating selected inorganic carbon transporters into the chloroplast membrane, followed by reorganizing Rubisco into a cyanobacterial carboxysome (Price et al., 2013). More effective carbon accumulation would eventually be realized in subsequent stages by suppressing any stromal CA, and potential chloroplast aquaporins, and ultimately utilizing a cyanobacterial thylakoid membrane transporter to elevate inorganic carbon significantly (Price et al., 2013).

What then are the prospects for introducing elements of a eukaryotic algal CCM into higher plants? In principal, a selection of algal membrane transporters (HLA3, LCI1, LCIA, or CCP1/2) could be introduced into higher plant cells or plastids. Whilst the practical limitations (pump energetics and orientation, plastid ion/pH balance, stability of transformation, etc.) have been reviewed effectively by Price et al. (2013), there are additional issues, or perhaps advantages, which relate to the eukaryotic system. However, more research is required to provide a definitive molecular and structural basis to the chloroplast pyrenoid, as a microcompartment.

Fig. 4. Evolution of kinetic properties from form IB green Rubisco isoforms. S\textsubscript{rel} and affinity for CO₂ data for some C₄ monocots and the CCM-less green alga Coccomyxa may suggest that the selection for improvements in kinetic properties of form IB Rubisco has been relaxed under the saturating CO₂ provided by a CCM. V\textsubscript{c}/V\textsubscript{c} was calculated from S\textsubscript{rel}[C]/[O], where S\textsubscript{rel} is the specificity for CO₂ over O₂, and [C] and [O] the concentrations of CO₂ and O₂ in the present-day atmosphere (taken to be 13 and 258 µM, respectively). The affinity for CO₂ was calculated from 1/K\textsubscript{c} (also noted as K\textsubscript{CO₂}). Calculated after André (2011), with additional data from Read and Tabita (1994), Palmqvist et al. (1995), Kubien et al. (2009), and Carmo-Silva et al. (2010).
will be essential to ensure that inorganic accumulation is not
negated by CO₂ leakage (Price et al., 2011). In addition, the
mechanism by which inorganic carbon is delivered to the
pyrenoid, and the interplay between intrapyrenoid thylakoid
carbonic anhydrases and the putative inorganic carbon trans-
porter (Fig. 1), also need to be determined.

Of course, there is already one group of terrestrial plants
displaying a full biophysical CCM, the hornworts (Smith and
Griffiths, 1996a, b), which are believed to be a direct descend-
ant of Charophyte green algae and a direct ancestor of higher
plants in the green lineage (Griffiths et al., 2004; Meyer et al.,
2008; Ligrone et al., 2012; Villareal and Renner, 2012). If it
proves to be the case that the pyrenoid is defined by simple
sequence variations in the Rubisco SSU (Meyer et al., 2012),
and as many of the other biophysical CCM components are
already genetically defined, this presents the tantalizing pos-
sibility that transformation of a biophysical CCM into crop
plants could prove to be a tractable approach for the future.

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Supplementary data

Supplementary data are available at JXB online.

Table S1. Percentage of identity (left) or similarity (right)
calculated from pair-wise alignment.

Table S2. Functional annotation of 114 unigenes identified
by Timme and Delwiche (2010).

Fig. S1. Predicted size, folding, and function of DIC trans-
porter candidates in Chlamydomonas.

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diagram.png


