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The carbon concentrating mechanism in *Chlamydomonas reinhardtii*: finding the missing pieces

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Abstract The photosynthetic, unicellular green alga, *Chlamydomonas reinhardtii*, lives in environments that often contain low concentrations of CO₂ and HCO₃[−], the utilizable forms of inorganic carbon (C_i). *C. reinhardtii* possesses a carbon concentrating mechanism (CCM) which can provide suitable amounts of C_i for growth and development. This CCM is induced when the CO₂ concentration is at air levels or lower and is comprised of a set of proteins that allow the efficient uptake of C_i into the cell as well as its directed transport to the site where Rubisco fixes CO₂ into biomolecules. While several components of the CCM have been identified in recent years, the picture is still far from complete. To further improve our knowledge of the CCM, we undertook a mutagenesis project where an antibiotic resistance cassette was randomly inserted into the *C. reinhardtii* genome resulting in the generation of 22,000 mutants. The mutant collection was screened using both a published PCR-based approach (Gonzalez-Ballester et al.

2011) and a phenotypic growth screen. The PCR-based screen did not rely on a colony having an altered growth phenotype and was used to identify colonies with disruptions in genes previously identified as being associated with the CCM-related gene. Eleven independent insertional mutations were identified in eight different genes showing the usefulness of this approach in generating mutations in CCM-related genes of interest as well as identifying new CCM components. Further improvements of this method are also discussed.

Keywords Calvin–Benson–Bassham cycle · Carbon concentrating mechanism · Carbonic anhydrase · *Chlamydomonas* · Rubisco

Abbreviations

CA	Carbonic anhydrase
CBB cycle	Calvin–Benson–Bassham cycle
CCM	Carbon (dioxide) concentrating mechanism
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate

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Introduction

The vast majority of aquatic photosynthetic organisms rely on CO₂ concentrating mechanisms (CCMs) to elevate the concentration of CO₂ near the enzyme Rubisco. The CCM overcomes barriers to photosynthesis caused by the low dissolved CO₂ concentration found in most aquatic environments and the slow diffusion of CO₂ in water. Since aquatic photosynthetic organisms account for about 50 %

of the world's CO_2 fixation, the emergence of a CCM is one of the most significant photosynthetic adaptations to the low atmospheric CO_2 conditions experienced on earth over the past 30 million years (Giordano et al. 2005; Raven et al. 2011, 2012).

Photosynthesis in most terrestrial plants is limited by the CO_2 concentration, particularly in hot and dry environments. A small percentage of terrestrial plants have adapted to low CO_2 conditions using different CO_2 concentrating mechanisms, namely C_4 photosynthesis and Crassulacean acid metabolism. With improved CO_2 fixation in mind, a number of international projects have worked to introduce C_4 traits into crop plants, most notably rice (Gowik and Westhoff 2011; von Caemmerer et al. 2012). There is also considerable interest in introducing components of the CCMs from aquatic organisms into higher plants (Price et al. 2013). The underlying idea is that increasing the efficiency of CO_2 fixation in higher plants could also lead to an improved tolerance to water stress. A CCM can also be expected to reduce wasteful photorespiration (Moroney et al. 2013). Computer simulations have indicated that the introduction of CCM components into higher plants could lead to significant increases in photosynthesis and decreases in photorespiration (Zhu et al. 2008). The introduction of CCM components from aquatic organisms into crop plants might lead to elevated CO_2 levels around Rubisco, increasing photosynthesis and crop yield (Price et al. 2011; Zhu et al. 2008, 2010).

CCMs are found in cyanobacteria, chlorophytes, prasinophytes, and other types of algae, and it is thought that all of these lineages developed CCMs independently (Giordano et al. 2005; Meyer and Griffiths 2013). The best studied CCMs are from cyanobacteria and green algae. While the overall architecture of the CCMs from these two types of organisms is similar, the specific components that make up the CCMs of cyanobacteria and green algae differ greatly (Moroney et al. 2013). The CCM in prokaryotic cyanobacteria is the best understood and a number of laboratories are attempting to introduce components of the cyanobacterial CCM into higher plants (Price et al. 2004). This report will focus on the CCM from the eukaryotic green alga *Chlamydomonas reinhardtii*. *C. reinhardtii* is a eukaryote and the locations of the CCM components can be directly matched with the locations needed to improve higher plants photosynthetic efficiency. A working model of the *C. reinhardtii* CCM is shown in Fig. 1 and involves three key components. First, this model proposes that inorganic carbon transporters increase C_i within the algal cell. Next, it is postulated that the pyrenoid, a structure in the chloroplast where most of the Rubisco is localized in the *C. reinhardtii* chloroplast, is analogous to the carboxysome of cyanobacteria and that the pyrenoid is the site of elevated inorganic carbon concentration. Finally, carbonic anhydrase (CA) activity is needed to

convert accumulated bicarbonate to CO_2 , the actual substrate of Rubisco. In many ways, the CCM model for *C. reinhardtii* is similar to the models proposed for cyanobacteria, where bicarbonate transporters increase the HCO_3^- concentration within the cytoplasm, and then the accumulated HCO_3^- is converted to CO_2 in the carboxysome where Rubisco is localized. It is important that the conversion to CO_2 is confined within the carboxysome as it gives Rubisco an opportunity to fix the accumulated CO_2 before it leaks out of the cell.

The *Chlamydomonas reinhardtii* CCM and the unresolved aspects of the model

While the overall arrangement of the CCM in green algae and cyanobacteria is similar, there are some important differences. One major difference is the location of the CA that converts the HCO_3^- to CO_2 . In *C. reinhardtii*, the CA, CAH3, is localized within the thylakoid lumen (Karlsson et al. 1998). It is thought that the placement of CAH3 in the acidic lumen helps to convert HCO_3^- to CO_2 in a location that favors CO_2 production, thus increasing the CO_2 concentration. This implies that inorganic carbon transport proteins are needed on the thylakoid membrane as well as the chloroplast envelope (Fig. 1).

Inorganic carbon transporters

An important component of algal CCMs is the inorganic carbon transporter system. In cyanobacteria, three transporters are known: BicA, SbtA and Bct1 (Price 2011). Cyanobacteria lacking these transporters have very poor affinities for inorganic carbon (Price et al. 2004; Woodger et al. 2005). However, it has been found that at least two of these transporters must be knocked out before a clear growth phenotype is observed (Price et al. 2004) indicating that these CCM components may have overlapping functions. What we know about bicarbonate transporters in *C. reinhardtii* is less clear. Some possible inorganic carbon transporters found in *C. reinhardtii* are listed in Table 1 and are discussed in the next section.

LCI1

LCI1 was one of the first membrane proteins identified as being up-regulated under low CO_2 conditions (Burow et al. 1996). The transcript for this protein is only present in cells grown under low CO_2 conditions and its abundance increases dramatically when high CO_2 grown cells are switched to low CO_2 conditions. The regulation of LCI1 is also under the control of CIA5 and LCR1 (Fukuzawa et al. 2001; Miura et al. 2004). LCI1 has only weak homology to other transport proteins and no similarity with known

Fig. 1 Model of the CO₂ concentrating mechanism of *Chlamydomonas reinhardtii*. LCI1, HLA3, CCP1/2 and NAR1.2 are potential C_i transport proteins. CAH1, CAH3, CAH4/5, CAH6 and CAH8 are CAs

Table 1 Genes that encode potential inorganic carbon transporters

Gene name	Phytozome gene ID	Protein location	Up-regulated on low CO ₂	Regulated by CIA5	RNAi strain/complete knock out
<i>LCI1</i>	Cre03.g162800	Plasma membrane	Yes++	Yes	Yes/yes
<i>NAR1.2</i>	Cre06.g309000	Chloroplast envelope	Yes++	Yes	Yes/no
<i>CCP1</i>	Cre04.g223300	Chloroplast envelope	Yes++	Yes	Yes/yes
<i>CCP2</i>	Cre04.g222750	Chloroplast envelope	Yes++	Yes	Yes/yes
<i>HLA3</i>	Cre02.g097800	Plasma membrane	Yes++	Yes	Yes/no
<i>Bestrophin</i>	Cre16.g663450	Unknown	Yes	Yes	No
<i>/LCI1</i>					
<i>MITC11</i>	Cre06.g295400	Unknown	Yes	Yes	No
<i>NAR1.1</i>	AF149738 ¹	Unknown	No	No	No
<i>NAR1.3</i>	g4546				
<i>NAR1.4</i>	Cre07.g35600				
<i>NAR1.5</i>	Cre12.g541250				
<i>NAR1.6</i>	Cre01.g012050				

¹ NAR1.1 has no gene model in the current Phytozome version (Phytozome version 9.1, *Chlamydomonas* genomic information: v5.3.1 of *Chlamydomonas* annotations, based on the Augustus update u11.6 of JGI assembly v5.). The NCBI accession number is given instead for sequence information

HCO₃⁻ transporters. However, when introduced into *C. reinhardtii* in the absence of other CCM components, LCI1 elicits an increase in C_i uptake (Ohnishi et al. 2010). LCI1 has been localized to the plasma membrane but so far no LCI1 mutant has been identified or generated.

NAR1.2

NAR1.2, also called LCIA, is up-regulated under low CO₂ conditions and was one of the first up-regulated proteins with transporter properties (Miura et al. 2004). Originally annotated as a possible nitrite transporter, it belongs to a small protein family with six members that show structural similarity to the wide spread group of Formate/Nitrite

transporters (PF01226) (Mariscal et al. 2006). NAR1.2 is also considered to be a HCO₃⁻ transporter because its presence correlates well with the CCM, and its synthesis is controlled by CIA5 (Miura et al. 2004). It has been localized to the chloroplast envelope (Mukherjee 2013). Over-expression of NAR1.2 in *Xenopus* oocytes indicates it can transport both nitrite and bicarbonate (Mariscal et al. 2006). Interestingly, NAR1.2 does not have a close paralog in higher plants.

Other NAR proteins

NAR1.1 through NAR1.6 comprises a small family of proteins in *C. reinhardtii*. Like NAR1.2, all of these

proteins belong to the Formate/Nitrite family of transporters. They vary considerably in their N-terminal domain and are predicted to target to different membranes. Unlike NAR1.2, the synthesis of these proteins does not correlate well with the synthesis of the CCM (Mukherjee 2013) but their structural similarity in *C. reinhardtii* could indicate a role in the CCM.

HLA3

First identified as a transcript that increases in abundance under high light conditions (Im and Grossman 2002), HLA3 also increases under low CO₂ conditions and is regulated by CIA5. This protein is an ABC-type transport protein. RNAi strains with reduced expression of both NAR1.2 and *HLA3* grew poorly on low CO₂ (Duanmu et al. 2009).

CCP1/CCP2

CCP1 and CCP2 are two very closely related proteins localized to the chloroplast envelope (Ramazanov et al. 1993). CCP1 and CCP2 are both strongly up-regulated by growth in low CO₂, and their expression is controlled by CIA5. CCP1 and CCP2 are members of the mitochondrial carrier protein family which includes many transporters of metabolites and are found in other locations in the cell besides the mitochondria. Pollock et al. (2004) reported an RNAi line of *C. reinhardtii* that showed reduced expression of both genes. This strain grew poorly on low CO₂ but still accumulated C_i normally and had a normal affinity for external C_i as judged by photosynthesis assays (Pollock et al. 2004).

Other transport proteins

Other genes that might be part of the CCM have been identified from two recent reports using RNA sequencing (Brueggeman et al. 2012; Fang et al. 2012). These include the transmembrane proteins bestrophin and MITC11. The transcripts for both bestrophin/LCI11 and MITC11 are strongly up-regulated under low CO₂ growth conditions (Brueggeman et al. 2012) and appear to be regulated by CIA5 (Fang et al. 2012). In other systems, bestrophins function as anion channels transporting mostly chloride, but bicarbonate was also identified as a transported molecule for human bestrophins (Qu and Hartzell 2008). MITC11 shows homologies to mitochondrial carrier proteins although localization predictions for MITC11 indicate that it is a chloroplast protein.

The above mentioned proteins belong to a number of different protein families but all have the potential to function as inorganic carbon transporters. The evidence that these proteins participate in the CCM comes from the

observation that the synthesis of most of these proteins is highly correlated with the presence of the CCM in *C. reinhardtii*. In addition, the synthesis of these proteins is regulated by CIA5 (CCM1), a protein that controls the synthesis of many other CCM components (Moroney et al. 1989; Fukuzawa et al. 2001; Xiang et al. 2001). Thus, a number of promising C_i transporter candidates have been identified. However, there are no knock-out lines for any of these putative transporters and only a few RNAi strains have been generated. It is also clear that the CCM is regulated by light as there is no C_i uptake in the dark (Moroney et al. 1987). Sültemeyer et al. (1989) reported that the regulation of the CCM takes place over a very short time during a light/dark transition. This regulation of C_i transporters by light is also seen in cyanobacteria. However, we need to identify the regulating protein(s) that are required for activation of these transporting proteins especially if these regulators are necessary for the successful introduction of a CCM into higher plants. In addition, for most of the transport proteins it is not clear what C_i species they transport or whether they function as symporters or antiporters. It has been established that two of the cyanobacterial C_i transporters are Na⁺–HCO₃[–] co-transporters (Price 2011). Similarly in *C. reinhardtii*, it needs to be established which of these proteins transport HCO₃[–] and which require some other ion in addition to HCO₃[–] to function.

Rubisco and the pyrenoid

The pyrenoid is the site for CO₂ fixation by Rubisco in *C. reinhardtii*. *C. reinhardtii* mutant strains lacking Rubisco also lack an observable pyrenoid (Borkhsenius et al. 1998). In mutants in which Rubisco becomes unstable and breaks down at elevated temperatures, the pyrenoid is also lost at non-permissive temperatures (Rawat et al. 1996). Immunolocalization studies from multiple laboratories also support the conclusion that over 90 % of the Rubisco is in the pyrenoid in *C. reinhardtii* cells grown under low CO₂ conditions (Morita et al. 1997; Borkhsenius et al. 1998). Finally, there is just enough Rubisco in the cell to support the maximal rates of photosynthesis (Borkhsenius et al. 1998) indicating that Rubisco in the pyrenoid is active and that the pyrenoid is the site of CO₂ fixation (Borkhsenius et al. 1998).

What other proteins are in the pyrenoid?

A recent study indicates that none of the other CBB cycle enzymes are found in the pyrenoid of *C. reinhardtii* (Mettler and Stitt, personal communication). So the enzymatic composition of the pyrenoid is similar to the carboxysome which also lacks CBB enzymes other than Rubisco. In *C. reinhardtii*, the only other protein that has

been localized to the pyrenoid is Rubisco activase (McKay et al. 1991).

Besides the electron-dense soluble part of the pyrenoid, the pyrenoid of *C. reinhardtii* is penetrated by thylakoid tubules (Rawat et al. 1996). Little is known about the protein composition of the tubules except that CAH3 has been detected in the tubules. Recently Flor Martinez and her colleagues found that CAH3 was phosphorylated in the light and that this phosphorylation might be activating and possibly relocating CAH3 to the tubules within the pyrenoid (Blanco-Rivero et al. 2012). Based on these findings, the model of the *C. reinhardtii* CCM can be fine-tuned. Bicarbonate is enriched in the chloroplast and finally transported into the lumen of the thylakoids which also pervade the pyrenoid. This bicarbonate is then converted by CAH3 into CO₂ that is subsequently fixed by Rubisco in the pyrenoid.

The pyrenoid is a dynamic structure

The appearance of the pyrenoid of *C. reinhardtii* changes dramatically depending on whether the cells are grown on low or high CO₂. Under low CO₂ growth conditions, the pyrenoid becomes much more defined and is surrounded by a starch sheath (Mason et al. 1991). Later studies showed that Rubisco is more evenly distributed in the chloroplast stroma under high CO₂ growth conditions but becomes predominantly, if not exclusively localized to the pyrenoid under low CO₂ growth conditions (Borkhsenius et al. 1998). Furthermore it has been shown that the distribution of starch and Rubisco changes depending on the growth conditions of the cells. The starch sheath around the pyrenoid is more pronounced and denser under low CO₂ (Ramazanov et al. 1993; Borkhsenius et al. 1998). The current hypothesis, based on this finding is that the starch sheath functions as a diffusion barrier for CO₂ that prevents the leakage and therefore enhances the fixation of CO₂ under limiting conditions. More recent studies indicate LCIB also localizes to the perimeter of the pyrenoid under low CO₂ growth conditions. It has been postulated that LCIB might act as an additional barrier to the diffusion of CO₂ out of the pyrenoid (Yamano et al. 2010). Finally, it has been reported that CAH3 could move into the pyrenoid tubules when *C. reinhardtii* is grown under low CO₂ growth conditions (Sinetova et al. 2012). All of these observations underscore the dynamic nature of the pyrenoid and its important role in the CCM of *C. reinhardtii*.

Is the pyrenoid required for a functional CCM?

Studies from the Griffiths and Spreitzer research groups indicate that a functional pyrenoid might be needed for the operation of a functional CCM (Genkov et al. 2010; Meyer

et al. 2012). They substituted regions of the Rubisco small subunits of *C. reinhardtii* by swapping in higher plant subunits or parts of higher plant subunits. The substitution of the higher plant sequences did not change the catalytic properties of the Rubisco protein but still resulted in *C. reinhardtii* cells that failed to grow under low CO₂ conditions. It was notable that in many cases the pyrenoid was absent in these cells. These observations imply that Rubisco must be tightly packaged for a CCM to work. The *cia6* mutant provided an additional line of evidence that a pyrenoid structure is required for CCM function. This mutant has a poorly organized pyrenoid and grows more slowly than wild-type cells on low CO₂ (Ma et al. 2011). These observations support the idea that the pyrenoid is required for CCM function and that other proteins besides Rubisco are important in determining the pyrenoid structure.

CA proteins involved in the CCM

CAs are required for CCM function. Since CCMs accumulate HCO₃[−], the conversion of HCO₃[−] to CO₂ before fixation by Rubisco is an essential part of any CCM. The *C. reinhardtii* genome has been sequenced and we have considerable evidence on the number and location of the CA proteins in this alga (Moroney et al. 2011; Table 2). The CA genes, *CAH1*, *CAH4* and *CAH5* are strongly up-regulated under low CO₂ growth conditions (Ynalvez et al. 2008). *CAH1* is localized to the periplasmic space while *CAH4* and *CAH5* are localized to the mitochondria. *CAH1* is thought to be important in the delivery of inorganic carbon to the cell (Moroney et al. 1985). However, a mutant missing *CAH1* grows at near normal rates even under low CO₂ conditions (Van and Spalding 1999). The other two CAs up-regulated under low CO₂ are *CAH4* and *CAH5* (Villand et al. 1997). Mutants for both proteins would increase understanding of their functions.

One CA with a very well understood role in the CCM is *CAH3* which is localized to the chloroplast thylakoid lumen (Karlsson et al. 1998). A number of knock-out strains of *CAH3* exist (Karlsson et al. 1998), and all of these mutants require elevated CO₂ for growth (Hanson et al. 2003). It is thought that *CAH3* converts accumulated HCO₃[−] in the chloroplast to CO₂ for fixation by Rubisco in the pyrenoid (Sinetova et al. 2012). The roles of the other CAs, most notably *CAH6*, *CAH7* and *CAH8* are not known yet.

The identification of CCM components

CCM components have previously been identified using two approaches. The first approach uses forward genetics done by mutagenizing cells and screening for colonies that

Table 2 The CAs of *Chlamydomonas reinhardtii*

Gene name (CA type)	Phytozome gene ID	Protein location	Regulated on low CO ₂	Regulated by CIA5	Knock out
CAH1 (α)	Cre04.g223100	Periplasm	Yes+	Yes	Yes
CAH2 (α)	Cre04.g223050	Periplasm	No	Unknown	No
CAH3 (α)	Cre09.g415700	Thylakoids	No	No	Yes
CAH4 (β)	Cre05.g248400	Mitochondria	Yes+	Yes	No
CAH5 (β)	Cre05.g248450	Mitochondria	Yes+	Yes	No
CAH6 (β)	Cre12.g485050	Chloroplast	No	No	No
CAH7 (β)	Cre13.g607350	Unknown	No	Unknown	No
CAH8 (β)	Cre09.g405750	Plasma membrane/ Periplasm	No	Yes ^a	No
CAH9 (β)	g5283	Cytoplasm	No	Yes ^a	No

^a The transcript level of these two CAs are higher in the CIA5 mutant compared to the wild type according to Fang et al. (2012)

grew poorly under low CO₂ but well on high CO₂. Initially chemical or UV mutagenesis was employed and mutants of CAH3, LCIB and CIA5 were first identified using these methods (Spalding et al. 1983a; Moroney et al. 1986, 1989). More recently, insertional mutagenesis has been used as it is easier to identify the disrupted gene once a mutant has been identified. This approach has generated a number of CCM mutants (e.g. *cia6*), photosynthesis mutants and a strain defective in Rubisco activase (Pollock et al. 2003; Dent et al. 2005; Ma et al. 2011).

The second approach involves looking for genes or proteins strongly up-regulated under low CO₂ growth conditions. This strategy began by using ³⁵S-labeled proteins and has evolved through different techniques including differential display, microarray analysis and more recently, RNA sequencing (Miura et al. 2004; Yamano and Fukuzawa 2009; Brueggeman et al. 2012; Fang et al. 2012). In some cases, most notably LCIB, both the mutant screen and differential expression have identified the same protein (Spalding et al. 1983b; Duanmu et al. 2009).

The identification and characterization of CCM genes using these methods has significantly contributed to the understanding of the *C. reinhardtii* CCM. However, forward genetics screens have their limitations since only mutants with a noticeable phenotype can be isolated and characterized. Not all mutants exhibit a certain phenotype, especially in a general mutagenesis phenotype screen. For example, the *CAH1* mutants (*cah1* and CC-503) do not have dramatic phenotypes especially under normal pH growth conditions (Van and Spalding 1999; Mukherjee et al. 2012). It is also likely that disruption of some of the genes controlled by CIA5 will not lead to an obvious growth phenotype. Miura et al. (2004) showed that CIA5 controlled at least 52 genes and more recently Fang et al. (2012) demonstrated that loss of CIA5 led to expression changes in over 1,000 genes. So, while *cia5*-deficient strains grow poorly on low CO₂, the alga still survives.

Therefore it seems likely that knocking out some of the genes controlled by CIA5 would lead to only subtle growth differences and that these differences might be missed using a phenotypic screen. In addition, if two genes encode proteins with redundant functions, cells defective in one of the genes would not be expected to exhibit a growth phenotype. Finally, the identification of CCM genes based on expression studies also has its limitations. For example CAH3, a CA essential to the CCM, is constitutively expressed.

As detailed in this article, a collection of CCM mutants has already been generated and characterized. These mutants can often be acquired from the *C. reinhardtii* Center. However, a large number of proposed CCM genes do not have a corresponding mutant, which hinders understanding the specific role of each component and the development of the entire CCM network. For example, the chloroplast stroma CA, CAH6 has been postulated to play an important role in CO₂ fixation by reducing the leakage of CO₂ out of the chloroplast thus maintaining the elevated CO₂ concentration around Rubisco (Yamano and Fukuzawa 2009). CAH6 has also been proposed to be associated with the pyrenoid, and a chloroplast stroma protein LCIB (Yamano et al. 2010). The identification of a *CAH6* mutant would be most beneficial to the CCM community.

Recently, a large scale insertional mutagenesis protocol which employed a PCR-based screen to identify cells with an insertion in a gene of interest was published (Gonzalez-Ballester et al. 2011). The speed and efficiency of the PCR-based mutant screening was dramatically increased by incorporating DNA pooling from a number of transformants and a primer cocktail for each specific gene. Starting with a 100,000-mutant library, a total of 45 mutants were identified with insertions in 37 different genes out of 63 screened (Gonzalez-Ballester et al. 2011). This powerful tool can also be adapted for the identification of missing mutants for CCM genes.

Overall strategy for the new CCM mutant screen

We have conducted an insertional mutagenesis screen employing a PCR-based reverse genetics approach to obtain more mutants in which potential CCM genes are disrupted. Two different screening methods were followed. First a phenotypic screen was employed using cells grown on low CO₂ (200 ppm). Genes disrupted in mutants unable to grow well on low CO₂ were identified using iPCR. The second screen was a reverse genetics PCR-based screen where inserts were identified using PCR primer mixtures for specific genes (Supplemental Table S1) along with primers that anneal in the inserted cassette. The overall strategy in the PCR-based screen was similar to that described by Gonzalez-Ballester et al. (2011). *C. reinhardtii* cells were first transformed using an *AphVIII* cassette, which confers resistance to paromomycin. After mutagenesis, the genomic DNA from the paromomycin resistant transformants was pooled together to serve as the PCR template and screened for the presence of the *AphVIII* cassette in specific genes. A total of 22,860 insertional mutants were generated, and 71 genes that are possibly involved in the CCM were screened (Supplemental Table 1).

Experimental procedures

Generating mutants

The overall experimental procedure is shown in Fig. 2. Mutant selection was performed using paromomycin as the selective antibiotic. The paromomycin resistance cassette (*AphVIII* cassette) was obtained from the plasmid pSL18 (Depege et al. 2003). Using restriction enzymes, a DNA fragment comprised of the *Hsp70+RbcSII* dual promoter, the ORF of the paromomycin resistance gene: aminoglycoside 3'-phosphotransferase (*AphVIII*) (Sizova et al. 2001), and the *RbcSII* terminator was obtained. The linear, purified *AphVIII* cassette (100 ng linearized DNA per 300 µl cell suspension with a density of 2×10^8 cells per ml in standard TAP medium supplemented with 60 mM Sorbitol) was transformed into the strain D66 by electroporation (Shimogawara et al. 1998). Before electroporation, D66 was grown in minimal medium to midlog phase in high CO₂ and then switched to low CO₂ for 3 h prior to transformation. This was done to target insertions to genomic regions that are actively transcribed during the acclimation to a low CO₂ stress condition. Overall, 197 individual transformations were conducted, and a total of 22,860 paromomycin resistant mutants were obtained.

Culture maintenance and DNA preparation

Agar plates with a 180-colony grid were used for cell growth and kept in low light (50 µmol photons m²/s). Fungal contamination was controlled by supplementing plates with carbendazim (70 µg/ml). A total of 135 agar plates were generated and replica-plated monthly. For DNA preparation, all 180 colonies from one plate were combined and the DNA was extracted (Supplemental Materials). DNA preparations from five such plates were further combined (referred to as “pooled DNA” in the text) to allow for the simultaneous screening of 900 individual colonies in a PCR screen. Thus, DNA from 135 plates was used to produce 27 DNA pools for the PCR screen.

Low CO₂ screen

The low CO₂-growth screen was performed as previously described (Colombo et al. 2002; Pollock et al. 2003). Cells were replica plated onto minimal medium plates and grown under low CO₂ (200 ppm CO₂) for 10 days at continuous light (50 µmol photons m²/s). Colonies that grew more slowly than the wild-type strain (D66) were further characterized. Colonies were then replica plated onto two more minimal plates and grown on both high CO₂ (2 % CO₂ in air) and again on low CO₂ (200 ppm CO₂) for 10 days. This growth test was repeated. Colonies that reproducibly grew poorly on low CO₂ were analyzed via iPCR to find the insert location.

iPCR

We followed a published iPCR protocol (Colombo et al. 2002) with minor modifications. In brief, DNA was extracted (Supplemental Materials) from cells with a low CO₂ phenotype and one µg digested with blunt end cutting restriction enzymes (PmII and PvuII) that generate approximately 3,000–4,000 bp genomic fragments but do not cut within *AphVIII* cassette. The fragmented DNA was purified with phenol/chloroform, ligated to form circular DNA and used for a three step PCR protocol with three different nested Primer sets (RB1+RX2, RB2+RX1, RB3+RX1). Amplified products of the last PCR that showed a progressive enrichment at each successive nested reaction were gel purified and sequenced. Sequences were then aligned to the annotated *C. reinhardtii* genome.

Growth at low CO₂ at different pH

For testing growth on solid media and under different pH levels, equal numbers of cells from different strains were spotted on minimal medium plates maintained at different pH levels by the addition of the following buffers: 25 mM

Fig. 2 Overall strategy for the dual phenotypic and PCR screens for potential CCM mutants. A linear, purified cassette mediating paromomycin resistance (*AphVIII* cassette) was transformed into cells of the *C. reinhardtii* strain D66 by electroporation. Transformants were selected on TAP plus paromomycin and surviving colonies were maintained on large plates with 180 colonies per plate. For the phenotypic screen, colonies were replica plated and grown at low and high CO₂. Growth was compared to control strains and strains deficient in various CCM proteins. For the PCR screen, DNA from all 180 colonies on a plate was extracted and further pooled with DNA from four other plates. A number of target genes were selected and the DNA pools were subjected to multiple rounds of PCR based on primers for these target genes. The pooled DNA allowed rapid screening of the selected colonies

MES (pH 5.8 and 6.2), 25 mM MOPS (pH 7.2) and 25 mM HEPES (pH 8.2). The plates incubated in chambers maintained at high and low CO₂ conditions for 10 days under continuous illumination of roughly 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$.

PCR screen overview

A PCR-based reverse genetics screen was used to identify mutants with modifications in specific genes potentially involved in the CCM. Briefly, the potential CCM genes were selected based on previous knowledge and published bioinformatics data (Supplemental Table 1). Primers were designed and PCR screens using primer mixes were performed on DNA pools. PCR signals were validated first by

multiple rounds of PCR and then by sequencing. Finally, single colonies that produced the positive signals were identified. An example on an insert in the *CAH8* gene is shown in Fig. 3.

Gene selection

Two groups of genes were selected as PCR targets (Supplemental Table 1). The first group consisted of genes that had been previously characterized and/or proposed as essential CCM components in *C. reinhardtii* or components of the photorespiratory pathway. The second group was chosen from nearly 2,000 genes identified as up-regulated by low CO₂ in two recent publications (Brueggeman et al. 2012; Fang et al. 2012). Other criteria considered for

Fig. 3 PCR strategy for mapping the insert. At the top is a depiction of the 5' end of the *CAH8* gene showing the first two exons and introns. Beneath the gene map the locations of the primer pairs used are shown. At the bottom are the PCR products using genomic DNA from the *cah8a* strain. “C” indicates a PCR control and the numbers above the band indicates the used primer pair. The two insert specific genes RB2 (3' end of insert) and RX2 (5' end of insert) were used with the gene specific primers CAH R5 and CAH F2 respectively to analyze the regions flanking the insert. Primer sequences are given in Supplemental Table 2

the second group of PCR target genes included possible chloroplast localization and predicted transmembrane domains in addition to exhibiting large changes in transcript levels between high and low CO₂ growth conditions. The target genes were listed in the order of their relative physical locations on the chromosomes in Supplemental Table 1.

Primer designs for the selected genes

To design primers for one specific gene (all the gene models used were based on Augustus 5 models, <http://genome.jgi-psf.org/Chlre4/Chlre4.home.html>), two sets of primers covering both directions were designed along the locus, spaced approximately 1-kb from each other. Primer design followed a published method (Gonzalez-Ballester et al. 2011) with a few modifications. The first forward (F1) and the first reverse (R1) primer were positioned 20–200 bp beyond the 5'UTR or 3'UTR respectively.

PCR procedure

For a specific gene, all the forward primers were combined together (designated as “F”) and all the reverse primers were combined together (designated as “R”). For each gene, two PCRs were performed by pairing the *AphVIII* internal primer RB1 (Gonzalez-Ballester et al. 2011) with either F or R. Whenever a pool produced a PCR product and that product was verified with the second *AphVIII* internal primer, RB2, DNA from each plate used in that product was screened. Whenever the same PCR product was observed from a PCR reaction using DNA from a specific plate the exact forward or reverse primer paired with RB1 that produced the product was determined. The PCR products were verified by sequencing. Then that primer pair was used to identify the specific colony with the insert of interest. After the mutant was determined on the single colony level, single colonies from each mutant colony were further isolated. PCR analysis from five random colonies was performed to ensure the homogeneity of the mutant colony. Furthermore, the insertion bordering DNA sequence was analyzed to verify whether the inserted *AphVIII* cassette caused deletions or other DNA alteration at the insertion site (Fig. 3).

Results

Phenotypic screen on low CO₂

Thirty-seven colonies from the screened 22,000 insertional mutants consistently showed a slow-growth phenotype on low CO₂. On elevated CO₂, these colonies grew at the same rate as wild-type cells. Four examples are shown in Fig. 4 and DNA flanking the insert was obtained for these mutants using iPCR. The locations of the *AphVIII* DNA inserts were found for four mutants (M7, M16, M17 and M19) and included insertions in the following genes: a glycerol-3-phosphate dehydrogenase (Phytozome ID: Cre01.g053150, M7), a leucyl-tRNA synthase (g14211, M16) a flagellar membrane protein FMG1-B (g9144, M17), and a signal recognition particle (SRP54) associated GTPase (Cre11.g479750, M19) (Table 3). The *AphVIII* cassette was inserted into intronic regions in the mutants M7, M16 and M19 (Table 3). The sequencing information of M17 showed an insertion into the third exon of the FMG1-B gene.

PCR-based screen

Using the 22,000 insertional mutants, eleven different mutants were found with inserts in eight different targeted genes of interest (Table 4). Exonic insertions were found

Fig. 4 Growth phenotypes of the insertional mutants M 7, M16, M17 and M19 selected from the phenotypic screen. All strains were grown under three different CO₂ conditions. The mutants, *cia5*, *cia3* and *pmp1* (mutant lacking the Ci uptake facilitator, LCIB) were used as

controls on the plate. All show a growth deficiency under low CO₂ conditions. The different pH levels were pH 6.2 (25 mM MES–KOH), pH 7.2 (25 mM MOPS–KOH) and pH 8.2 (25 mM HEPES–KOH) to the plates

for the genes *ECA1* and *RDDP*. *ECA1* codes a Calcium transporting ATPase that is up regulated under low CO₂. *RDDP* codes a RNA dependent DNA polymerase. However, further PCR analysis revealed this gene *RDDP* is found in multiple copies in *C. reinhardtii*. The *CAH8* gene had two independent inserts, one in an intron (the mutant named *cah8a*, Fig. 3) and the other in the 5'UTR (named *cah8b*). Additional insertions were found in the 5'UTR of the genes *bestrophin/LCI11* and *RHP1*. Five other genes had insertions in the 3'UTRs. Interestingly, we could identify three independent *NAR1.3* mutants (named *nar1.3a*, *-b* and *-c*). The *AphVIII* cassette was located in *MITC11* and *LCI9* for the other two mutants (Fig. 5; Table 4).

Earlier studies employing insertional mutagenesis showed side effects like deletions of kilobases of DNA (Wang and Spalding 2006). Therefore we also tested the DNA upstream and downstream of the inserted *AphVIII* cassette. We amplified the junction at both sides of the insertion and found that no major deletions had occurred (Figs. 3, 5). Furthermore, we sequenced the amplified fragments and the *C. reinhardtii* sequence was only altered for 4–20 bases adjacent to the insert in some cases while

for other inserts and the genomic DNA sequence near the insert remained unchanged.

Growth on low CO₂ and different pH

Several mutants from the PCR based screen had inserts in genes encoding previously known or newly identified potential transporters. A growth study was conducted in order to determine if these insertions in potential transporters like *NAR1.3* or *MITC11* produced a reduced growth phenotype under low CO₂. The mutants were exposed to low ambient and high CO₂ at pH 6.2, 7.2 and 8.2. The pH controls the form of inorganic carbon that is predominantly available for use by photosynthesizing *C. reinhardtii* cells. A higher pH increases the amount of available bicarbonate. From our growth studies no significant differences in growth between the wild-type and the mutants were observed (Supplemental Figure S1). This is not surprising since it has been previously seen that redundancy of C_i uptake proteins often masks the absence of one protein in the cell and mutants do not show discernible growth deficiencies under low CO₂ or high bicarbonate concentrations. Furthermore, most of the

Table 3 Mutants with low CO₂ phenotype

Gene name	Phytozome gene ID	Protein location	Regulated on low CO ₂	Regulated by CIA5	Insert location
<i>Glycerol-3-phosphate dehydrogenase</i> (M7)	Cre01.g053150	Cytoplasm	Yes—	No	Intron 13
<i>leucyl-tRNA synthase</i> (M16)	g14211	Chloroplast	Yes—	No	Intron 13
<i>FMG1-B</i> (M17)	g9144	Flagella	Yes—	No	Exon 3
<i>SRP54-GTPase</i> (M19)	Cre11.g479750	Mitochondria	No	No	Intron 12

iPCR was used to determine the location of the insertion in the genome for these four mutants

Table 4 Mutants with insertion in genes that were found during the PCR based screen

Gene name	Phytozome gene ID	Protein location	Regulated on low CO ₂	Regulated by CIA5	Insert location
<i>Bestrophin</i>	Cre16.g663450	Unknown	Yes+	Yes ^a	5' UTR
<i>LCI11</i>					
<i>CAH8</i>	Cre09.g405750	Plasma membrane/ Periplasm	No	No	(b) 1 × 5' UTR (a) 1 × Intron 1
<i>MITC11</i>	Cre06.g295400	Unknown	Yes+	Yes ^b	3' UTR
<i>NAR1.3</i>	g4546	Unknown	No	No	3 × 3' UTR
<i>ECA1</i>	Cre09.g410050	ER	Yes+	Yes ^c	Last exon
<i>LCI9</i>	g9783	Mitochondrion	Yes+	Unknown	3' UTR
<i>RHP1</i>	Cre06.g284100	Unknown	No	Yes ^c	5' UTR
RDDP ^d	Cre17.g737450	Unknown	Yes+	Unknown	Exon 1

^a Cluster 14 (Fang et al. 2012)

^b Cluster 8 (Fang et al. 2012)

^c The transcript levels of these two genes are lower in the CIA5 mutant compared to the wild type according to Fang et al. (2012)

^d The RNA directed DNA polymerase (RDDP) is part of a transposon and PCR revealed that only one of the multiple copies of this gene in the *Chlamydomonas* genome was affected by the insertion

insertions were in non-coding regions of the gene and may not have an effect on the mature protein.

Discussion

The CCM of *C. reinhardtii* is the best understood CCM from eukaryotic algae (Moroney and Ynalvez 2007). Recently there has been an interest in introducing CCM components into higher plants with the goal of increasing the efficiency of CO₂ fixation (Zhu et al. 2010; Price et al. 2011). To maximize the chance of success, a greater understanding of the *C. reinhardtii* CCM is needed. Open questions include the identification of additional inorganic carbon transporters as well as a complete understanding of how these transporters are regulated. Also, while the number and locations of the CAs of *C. reinhardtii* are known, the exact role of each CA in the CCM remains to be determined. Finally, we need to determine the components and organization of the pyrenoid. This information

will help decide whether the pyrenoid or a structure similar to a pyrenoid is needed before a functional CCM can be realized in a higher plant. With these missing pieces in mind, a screen for additional CCM mutants was initiated. In this screen, cell growth was monitored at high and low CO₂ levels with the goal of identifying mutations in new CCM components. While the growth screen was underway, a PCR based screen was also performed. The advantage of this approach was the identification of insertions in target genes of interest, which may or may not have been detected through the phenotypic screen. The combination of the phenotypic and PCR-based screens ensured that insertions in a maximum number of previously identified/unidentified genes involved in the *C. reinhardtii* CCM could be recovered.

The mutants found using these screens

Insertional mutagenesis in *C. reinhardtii* involves the random integration of a foreign piece of DNA into the *C.*

Fig. 5 PCR confirmation of the insertion location for three affected genes of interest. D66 and the mutants with insertions in *MITC11*, *LCI9*, and *LCI11* were subjected to PCR analysis. **a** Models of the three genes showing the position of the inserted *AphVIII* cassette. The insert specific primer RX1 and RB2 were paired with gene specific forward (F) or reverse (R) primers. **(B)** Amplified fragments from the PCR reactions. DNA from D66 (wild-type without insert) was subjected to PCR with two gene specific primers that span the insert in the mutant (lane 1). RB2 (located near the 3' end of the insert) was paired with a gene specific primer to analyze the DNA downstream of

the insert (lane 2). RX1 (located near the 5' end of the insert) was used with the appropriate gene specific primer to amplify the DNA upstream of the *AphVIII* cassette in the mutants (lane 3). The two gene specific primers spanning the insert were used to amplify DNA from each mutant (lane 4). In each case the PCR product from the mutants was about 1.8 kb larger than the wild type PCR product which corresponds to the size of the *AphVIII* cassette (compare lanes 1 and 4). *Non-specific band. Primer sequences are given in Supplemental Table 2

reinhardtii genome, sometimes disrupting target genes in the process. It is notable that in past studies very few insertional mutants were found in low CO₂-inducible genes. The only previously reported mutants in inducible genes were in *LCIB* (Spalding et al. 1983b; Wang and Spalding 2006), *LCR1* (Yoshioka et al. 2004) and *CAH1* (Van and Spalding 1999). In this screen we obtained colonies with inserts in *MITC11*, *bestrophin/LCI11*, *LCI9* and *ECA1*, all genes that are up-regulated under low CO₂ (Table 4). For most of these mutants there was no growth difference compared to wild-type cells and these mutants would have been overlooked using the traditional phenotypic screen. Most insertions in this screen were insertions that did not cause any significant deletions close to the point of insertion (Figs. 3, 5). Our results agree with those of Gonzalez-Ballester et al. (2011) and

indicate that the electroporation method results in few large deletions.

Insertions in previously identified and potential CCM genes

One goal of this work was to obtain knockout lines in genes that might play a role in the CCM. Physiological studies on these knockout lines can then be used to determine if a particular gene is required for optimal CCM function. In this study we obtained strains with inserts in three transporter genes *bestrophin/LCI11*, *MITC11* and *NAR1.3* and the CA gene *CAH8*. Thus it is clear that PCR based screening for inserts in genes of interest can help to extend the characterization of the functional roles of many CCM proteins. Combining this with a phenotypic screen based on

growth defects under low CO₂ conditions can lead to the identification of new genes that fill some of the gaps in our understanding of the CCM machinery.

Future perspectives

The insertional mutagenesis method described here, can be used to identify new components of the *C. reinhardtii* CCM. The advantage of the PCR based screen is that no observable phenotype is needed to identify a mutant with an insert in a specific gene. However, the method is dependent on random insertion events which require a large-scale screen to have a chance of identifying mutants of interest. In addition, the PCR-based screen requires identification of a gene of interest a priori. If the PCR-based screen is coupled with a rigorous screen looking for colonies unable to grow on low CO₂, then the odds of finding new components of the CCM increase significantly.

One of the biggest limitations of insertional mutagenesis studies lies in the identification of the gene that is disrupted by the selectable marker. Methods such as iPCR, adapter PCR and map-based cloning have all been used to find the sites of DNA insertion but many interesting mutant colonies have proved intractable to analysis. An adapter-PCR method is currently being tested that should allow for the recovery of flanking DNA sequences in a high percentage of the insertional mutants (Pollock unpublished observations). A high-throughput method of identifying DNA insert locations is also needed. With improved sequencing methods, the development of high-throughput methods to locate DNA insertions appears feasible. Once refined, high-throughput methods have the potential to identify most or all of the inserts in a large population of insertional mutants, allowing the researcher to target genes of interest in a short period of time.

Conclusion

This report summarizes the progress made in generating *C. reinhardtii* mutants lacking either previously identified or novel proteins that might play a role in the *C. reinhardtii* CCM. Using a combination of forward genetics and a PCR based reverse genetics approach; mutants in genes of interest were identified (Figs. 3, 4, 5; Tables 3, 4). This dual-screen strategy provides a powerful tool to find missing links in the CCM of *C. reinhardtii*. It combines the ability to identify newer components of the CCM based on an interesting growth phenotype with the purposeful search of mutations within previously identified genes of interest. These mutants will be needed to judge which CCM components are good candidates for

improving photosynthesis in agriculturally important land plants.

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