A novel gene required for pyrenoid formation in *Chlamydomonas reinhardtii*
Identification of a novel gene, CIA6, required for normal pyrenoid formation in *Chlamydomonas reinhardtii*

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FOOTNOTES

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Abbreviations: high CO₂, air supplemented with CO₂ to a final CO₂ concentration of 5% (v/v); low CO₂, air with 0.038% (v/v) CO₂; very low CO₂, air with 0.01%(v/v) CO₂; CCM, carbon dioxide concentrating mechanism; CA, carbonic anhydrase; C₅, inorganic carbon; DIC, dissolved inorganic carbon; MBP, maltose-binding protein; TAP medium, tris-acetate-phosphate medium
ABSTRACT

*Chlamydomonas reinhardtii* possesses a CO₂ concentrating mechanism (CCM) that allows the alga to grow at low CO₂ concentrations. One common feature seen in photosynthetic organisms possessing a CCM is the tight packaging of Rubisco within the cell. In many eukaryotic algae, Rubisco is localized to the pyrenoid, an electron dense structure within the chloroplast. In order to identify genes required for a functional CCM, insertional *Ble*R mutants were generated and screened for growth on minimal medium under high (5% CO₂ in air) but only slow or no growth under very low (0.01% CO₂ in air) CO₂ conditions. One mutant identified from this screen was named *cia6*. Physiological studies established that *cia6* grows poorly on low levels of CO₂ and has an impaired ability to accumulate inorganic carbon. The inserted *Ble*R disrupted a gene encoding a protein with sequence similarity to proteins containing SET domain methyltransferase, although experiments using overexpressed CIA6 failed to demonstrate the methyltransferase activity. Electron microscopy revealed that the pyrenoid of *cia6* mutant cells is highly disorganized. Complementation of the mutant restored the pyrenoid, the ability to grow under low CO₂ conditions and the ability to concentrate inorganic carbon. Quantitative-RT PCR data from a low CO₂ induction time course experiment demonstrated that the up-regulation of several CCM components is slower in *cia6* compared to the wild-type. This slow induction was further confirmed at the protein level using western blots. These results indicated that *CIA6* is required for the formation of the pyrenoid and further supported that the pyrenoid is required for a functional CCM in *Chlamydomonas reinhardtii*. 
INTRODUCTION

Most aquatic photosynthetic organisms have a CO\textsubscript{2} concentrating mechanism (CCM) that increases the CO\textsubscript{2} concentration around the carboxylating enzyme Rubisco when CO\textsubscript{2} is limiting (Badger et al., 1998; Giordano et al., 2005; Moroney and Ynalvez, 2007). Two problems faced by organisms with CCMs are the potential leakage of CO\textsubscript{2}, which can readily cross most biological membranes (Gutknecht et al., 1977), and the rate of diffusion of CO\textsubscript{2} in water which is $10^4$ times slower than in air. Since HCO\textsubscript{3}\textsuperscript{-} crosses biological membranes at about $10^{-6}$ times the rate of CO\textsubscript{2}, a strategy that concentrates HCO\textsubscript{3}\textsuperscript{-} would greatly minimize the loss of inorganic carbon. The cyanobacterial CCM adopts a system that concentrates HCO\textsubscript{3} in the cytoplasm using a series of HCO\textsubscript{3} transporters, and packages Rubisco in a proteinaceous structure called the carboxysome (Price et al., 2008). The HCO\textsubscript{3} accumulated in the cytoplasm could then diffuse into the carboxysome through carboxysomal pores, be converted to CO\textsubscript{2} by carboxysomal carbonic anhydrases (CAs) and finally be fixed by Rubisco (Price et al., 2008). The importance of the carboxysome in the cyanobacterial CCM is illustrated by the fact that the ectopic expression of carbonic anhydrase in the cyanobacterial cytosol short circuits the CCM; as the HCO\textsubscript{3} in the cytosol is converted into CO\textsubscript{2} that diffuses out of the cell without fixation (Price and Badger, 1989).

In the eukaryotic green algae \textit{C. reinhardtii}, the CCM appears to have strong similarities to the cyanobacterial system (Moroney and Ynalvez, 2007). In \textit{C. reinhardtii}, bicarbonate is concentrated in the chloroplast stroma through the cooperation of multiple C\textsubscript{i} transporters and carbonic anhydrases. Rubisco is packaged in a specialized chloroplast micro-compartment, called the pyrenoid, where active C\textsubscript{i} fixation takes place (Borkhsenious et al., 1998). In the pyrenoid of \textit{C. reinhardtii}, Rubisco is the predominant protein (Kuchitsu et al., 1988; Morita et al., 1997; Borkhsenious et al., 1998) and nonsense mutations in the Rubisco large subunit totally abolish the formation of the pyrenoid (Rawat et al., 1996). \textit{C. reinhardtii}’s pyrenoid is also penetrated by a network of thylakoid tubules (Henk et al., 1995; Borkhsenious et al., 1998). The carbonic anhydrase, CAH3 that has been localized to the thylakoid lumen, is further enriched in the thylakoid tubules inside the pyrenoid (Karlsson et al., 1998; Mitra et al., 2005). Mutants that lack CAH3 have a non-functional CCM in which HCO\textsubscript{3} accumulates intracellularly, but the mutant cells cannot grow on low and very low levels of CO\textsubscript{2} (Spalding et al., 1983; Moroney et al., 1986; Pronina and Semenenko, 1992; Karlsson et al., 1998). In addition, a putative C\textsubscript{i}
transporter, LCIB, is localized around the pyrenoid (Yamano et al., 2010). Mutants with defects in LCIB’s expression results in the unusual air-dier phenotype, that dies at air level of CO₂ but survives under very low CO₂ (Wang and Spalding, 2006; Duanmu et al., 2009).

A number of current models have proposed that the pyrenoid is critical to the optimal function of the CCM in *C. reinhardtii*, with the structure serving a role similar to the cyanobacterial carboxysomes (Morita et al., 1999; Moroney and Ynalvez, 2007; Spalding, 2008; Yamano et al., 2010). However, very few mutations affecting pyrenoid structure have been described. Among the few reports, Rawat *et al.* (1996) demonstrated that the loss of Rubisco in *C. reinhardtii* resulted in the loss of the pyrenoid. Recently, Genkov and his colleagues described the creation of *C. reinhardtii* strains with hybrid Rubiscos (Genkov et al., 2010), in which a higher plant small subunit gene (RbcS) was transformed into a *C. reinhardtii* RbcS mutant background. The resultant *C. reinhardtii* strain contained hybrid Rubisco with native RbcS and higher plant Rubisco large subunit (RbcL). However, strains containing the hybrid Rubisco could not grow as well as wild-type cells at low CO₂ levels. Strikingly, in the resultant *C. reinhardtii* strains, although the catalytic properties of the hybrid Rubisco were similar to wild-type Rubisco, and the Rubisco was expressed, it was found that these strains lacked pyrenoids as well as an active CCM.

In this report, we describe a novel mutant of *C. reinhardtii* in which a disruption in a nuclear gene other than in Rubisco, resulted in a disrupted pyrenoid structure and a high-CO₂ requiring phenotype. This work provides further evidence that the organization of pyrenoid itself is important in the functioning of the CCM.

**RESULTS**

*cia6 Needs a High CO₂ Environment to Grow Optimally*

*C. reinhardtii* strain D66 (Schnell and Lefebvre, 1993) was transformed with the pSP124s plasmid which contains the modified *Ble*R cassette conferring bleomycin resistance (Lumbreras et al., 1998). 42,000 *Ble*R insertional mutants were selected and screened for a high CO₂-requiring phenotype (Colombo et al., 2002; Pollock et al., 2003). Strains that could grow well on elevated CO₂ but grew slowly on low CO₂ were subsequently tested for their ability to accumulate inorganic carbon. One of those transformants also had a reduced ability to accumulate inorganic carbon and was named *cia6*. Figure 1 shows the photoautotrophic growth
characteristics in high (Fig. 1A) and low (Fig. 1B) CO₂ environments of the parent strain, D66; the insertional mutant, cia6; and a known high CO₂ requiring mutant, cia5, a strain defective in a transcription factor and fails to induce the CCM (Moroney et al., 1989). In high CO₂ conditions, all three strains displayed similar growth characteristics. However, in the low CO₂ environment, compared to the D66 strain which displayed growth pattern similar to the one under high CO₂ conditions, the cia6 mutant grew poorly as did the CCM-defective strain, cia5. The results presented here showed that cia6 requires an elevated CO₂ environment to grow photoautotrophically.

cia6 Has a Reduced Affinity for Cᵢ

To determine the apparent affinity of cia6 cells for inorganic carbon (Cᵢ), the rate of photosynthesis as a function of the dissolved inorganic carbon (DIC) concentration was determined. When grown on elevated CO₂, D66, cia5, and cia6 all had maximum photosynthesis rates of greater than 100 μmoles CO₂ fixed per mg chlorophyll per hour. However, cia5 and cia6 had affinities for DIC that were 1.5 to 2 times lower than D66 (Fig. 2A, Table 1). Air-acclimated cells also exhibited similar maximum rates of photosynthesis, but in contrast, both cia5 and cia6 had an affinity for DIC approximately 10 times lower when compared to D66 cells (Fig. 2B, Table 1). The rate of photosynthesis was also lower in air-grown cia6 compared to wild type at low DIC concentrations. In order to measure the ability of cia6 to accumulate inorganic carbon, light-dependent Cᵢ uptake was estimated using the silicone oil centrifugation method (Fig. 2C). The amount of Cᵢ accumulation by cia6 was one-fifth that of D66 during a 60 second time course. In summary, these photosynthetic characteristics lead to the naming of this mutant, cia6, for inorganic carbon accumulation deficient mutant following the nomenclature described by Moroney et al (1986).

cia6 Has an Insertion in a Novel Gene

Southern blot analysis of this mutant showed that a single insertion event had taken place during transformation as evidenced by a single intense hybridization band in cia6 genomic DNA digested with different restriction enzymes that do not have cleavage sites within the predicted transgene (Fig. 3A). Using an adaptor-mediated PCR strategy (Siebert et al., 1995), the DNA flanking one side of the pSP124s insert was cloned and sequenced. Comparison of this sequence
with the Joint Genomics Initiative’s *C. reinhardtii* genome sequence database (Merchant et al., 2007) yielded a match to a region on chromosome ten. No ESTs were aligned with this region. However, the gene structure prediction programs Genewise (http://www.ebi.ac.uk/Tools/Wise2/index.html) and GeneMark (http://exon.biology.gatech.edu/) identified putative open reading frames in this region of the chromosome. Using primers designed to bracket this region, a cDNA was amplified using RNA from wild-type cells as the template. RACE was employed to obtain a full length cDNA and DNA sequencing verified its identity. The *CIA6* gene consists of 5 exons spanning 2.9 kb (Fig. 3B, GenBank Accession Number: JF288753). Subsequent sequence analysis demonstrated that the *BleR* cassette insertion disrupted the third exon. Primers spanning the *BleR* insertion (Fig. 3B) were utilized to determine whether the *CIA6* mRNA was present in high and low CO$_2$ grown D66 and *cia6*, and primers amplifying glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were used as an internal control (Fig. 3C). In D66, the *CIA6* message was present in both high and low CO$_2$-grown cells, while the message was absent in *cia6*. Tetrad analysis confirmed the linkage between the CCM deficient phenotype and the *BleR* insertion (data not shown). These results are consistent with the hypothesis that the single pSP124s insertion disrupted the *CIA6* locus and abolished the transcription of *CIA6*.

**cia6 Has a Disorganized Pyrenoid**

In *C. reinhardtii*, the pyrenoid is a spheroid, electron-dense, Rubisco containing body inside the chloroplast (Fig. 4A and 4B where the pyrenoid is labeled as “P”). The pyrenoid is penetrated by numerous thylakoid membranes and is estimated to occupy a volume of 2.4 µm$^3$, approximately 1/16 of the entire chloroplast (Griffiths, 1970; Lacoste-Royal and Gibbs, 1987). In Transmission Electron Microscopy (TEM) thin sections, a normal spherical pyrenoid is often encountered at a chance of 35% out of all wild-type cell thin sections (Henk et al., 1995). During the cell’s acclimation to a low CO$_2$ environment, the pyrenoid undergoes dramatic structural changes as a ring of starch accumulates around the pyrenoid (Fig. 4B where the starch sheath is labeled as “s”). Using TEM, *cia6* mutant cells were examined and were shown to have disorganized pyrenoids in both high CO$_2$-grown (Fig. 4C where possible pyrenoid region was labeled as “=*”) and low CO$_2$ grown (Fig. 4D where possible pyrenoid region was labeled as “=*”) cells. In contrast to the normal spheroid shaped, electron-dense pyrenoid with an average of
2.3µm² in area seen in wild-type cells (Fig. 4A and 4B), it was revealed that the pyrenoid was either absent or highly disorganized in all the cia6 ultra-thin cell sections examined. Although the electron-dense region could still be observed in the mutant cells, it was significantly smaller in size with an average of 1.2 µm², highly irregular in shape and often associated with the starch sheath. Over 100 cia6 cell thin sections were observed, but no normal pyrenoid could be detected in cia6. Under light microscopy (100X), however, the possible presence of small pyrenoid-like structures was observed in the cia6 mutant cells, and oftentimes multiple small pyrenoids were observed inside the chloroplast of a single cell (Fig. 4F and 4G).

**cia6 Can Be Complemented**

To confirm whether the CIA6 mutation is responsible for the CCM deficient phenotype and the disorganized pyrenoid, complementation was attempted by transforming a 3.7kb genomic DNA fragment containing the wild type CIA6 locus into the cia6 mutant. cia6 cells were transformed and selected under very low CO₂ conditions (70 ppm) on minimum plates. A total of 50 transformants that survived under very low CO₂ conditions were further examined for the restoration of the pyrenoid as observed under light microscopy. 22 out of 49 transformants selected in this manner showed positive pyrenoid staining as indicated by strong staining using the HgCl₂-bromonophenol blue (BPB) reagent (Kuchitsu et al., 1988) in the pyrenoid and nucleus. One transformant, named cia6-comp was selected for growth and photosynthetic kinetics study. Under both high and low CO₂ conditions, cia6-comp grew as well as the wild-type cells (Fig. 1), showing that the growth defects had been complemented. Figure 5A shows that the complemented cia6 had a higher affinity for C₁ as compared to the mutant. The calculated affinity of cia6-comp for DIC (K₁/₂(DIC)) was also close to the wild-type level (Table 1). In addition, the RNA sample isolated from cia6-comp also showed that the CIA6 mRNA is recovered in the complemented strain, based on RT-PCR analysis (Fig. 5B). The recovery of the WT pyrenoid structure (Fig. 4G and 4H) together with the restoration of its C₁ concentrating ability demonstrated that the phenotype of the cia6 mutant can be complemented with a 3.7 kb genomic fragment containing the WT CIA6 locus.

**CIA6 Is Predicted to Encode a 72 kDa Protein Containing a SET Domain**
The predicted CIA6 ORF encodes for a 72 kDa protein that has sequence similarity with a group of SET domain proteins. Using the BlastP program searching the NCBI protein database, 13 homologs of CIA6 were identified (E-value ≤ 8e⁻²⁰; Max score ≥ 100) (Figure 6). Among organisms possessing CIA6 homologs, the multicellular green alga *Volvox carteri f. nagariensis* has the highest similarity, followed by the unicellular photosynthetic green alga *Chlorella variabilis* NC64A and *Micromonas pusilla* CCMP1545 which also possess pyrenoid and an active CCM (Shiraiwa and Miyachi, 1983; Ikeda and Takeda, 1995; Worden et al., 2009). In addition, the non-vascular plant *Selaginella moellendorffii* has two genes similar to CIA6. The genomes of the early branching land plant *Physcomitrella patens* as well as the land plants *Vitis vinifera, Populus trichocarpa, Arabidopsis thaliana* and *Oryza sativa*, all contained a gene predicted to encode a protein with significant homology to CIA6. To date, among all the CIA6 homologs identified in the NCBI database, none has been characterized.

**Assessment of CIA6’s Methyltransferase Activity in vitro**

In order to test the putative methyltransferase activity of CIA6, the protein was overexpressed in *E. coli* using the pMALc2x overexpression system. The entire CIA6 ORF was fused to the maltose binding protein (MBP) in the pMALc2x and overexpressed as a fusion protein in *E. coli* cells. *C. reinhardtii* Rubisco, calf thymus histone, and *C. reinhardtii* whole cell extracts were used as possible substrates. Positive controls in which pea Rubisco large subunit methyltransferase (RLsMT) (Klein and Houtz, 1995) was incubated with either the Spinach or the *C. reinhardtii* Rubisco isolated from both the wild-type and the *cia6* mutant showed significant methyltransferase activity. In contrast, no in vitro methyltransferase activity was detected when the purified CIA6 fusion protein was incubated with the same array of substrates. The detected activity of pea RLsMT with the *C. reinhardtii* Rubisco is probably achieved by methylating on the Lys14 site as the pea RLsMT does in vivo with pea Rubisco. The observation is consistent with previous reports that the isolated Rubisco could be in vitro substrates even though their Lys14 is not methylated in vivo (Houtz et al., 1992; Raunser et al., 2009).

**cia6 Has Normal Levels of Rubisco but Rubisco Fails to Associate and form a Pyrenoid**

Rawat *et al.* (1996) demonstrated that the pyrenoid could not be found in the RbcL nonsense strain 18-7G (Spreitzer et al., 1985), which indicates that the pyrenoid formation
requires the presence of Rubisco. Thus, to address why the $cia6$ mutant cells lacked a pyrenoid, Rubisco protein levels from D66 and $cia6$ were compared using western blot (Fig 7A). However, when using anti-Rubisco polyclonal antibody (Borkhsenious et al., 1998) that recognize both the RbcL and the RbcS, it was found that the protein levels of both Rubisco subunits from both strains were not different. Another possible explanation regarding the disruption of pyrenoid in the mutant cells could be that, even though the amount of Rubisco protein was not changed, the directing of the Rubisco holoenzyme into the correct position (pyrenoid) in the mutant chloroplast was impaired. Immunolocalization of Rubisco was performed in cells from the wild-type and mutant strains, under both high and low CO$_2$ conditions. As seen for the high CO$_2$ grown (Fig. 7B) and low CO$_2$ grown (Fig. 7C) wild-type cells, the pyrenoid contained the majority of immunogold particles with almost all the immunological particles found inside of the wild-type pyrenoid upon low CO$_2$ induction (Fig. 7C). This result agrees with earlier published reports from Morita’s laboratory (Morita et al., 1997), and our laboratory (Borkhsenious et al., 1998) that more than 90% of the Rubisco resides inside the pyrenoid in wild-type cells grown under low CO$_2$ conditions. In $cia6$ however, Rubisco particles were no longer accumulating inside the pyrenoid in either high CO$_2$ (Fig. 7D) or low CO$_2$ (Fig. 7E) grown cells. Using the estimated volume of the stroma and pyrenoid (Lacoste-Royal and Gibbs, 1987), it was estimated that the fraction of Rubisco in the pyrenoid-like structures was about 35% in $cia6$, which is similar to the percentage seen in wild-type cells grown in high CO$_2$ conditions (Table 2). Hence, the total Rubisco concentration in the mutant $cia6$ was not changed while the localization of this CO$_2$ fixation enzyme was greatly altered as a result of the pyrenoid disruption.

### $cia6$ Has a Higher Chlorophyll Content Per Cell

In *C. reinhardtii* wild-type cells, the chlorophyll per cell ratio remains constant during the log phase growth, at around $2.8\times10^{-6}$ µgChl·cell$^{-1}$ and $1.7\times10^{-6}$ µgChl·cell$^{-1}$ under photoautotrophic and heterotrophic conditions respectively (Fig. 8 and Supplemental Fig. S1). However, in the mutant $cia6$, it was observed that the chlorophyll per cell ratio is significantly higher in photoautotrophic grown cells (minimal medium bubbled with air, Fig.8), especially after switching from TAP medium to minimal medium. In addition, the chlorophyll per cell ratio is also higher in minimal medium even under high CO$_2$ concentrations (Fig. S1). When $cia6$ was grown under photoheterotrophic conditions where acetate was used as a carbon source, the
chlorophyll per cell ratio was similar to wild-type cells. The complemented strain cia6-comp exhibited the same growth characteristics and chlorophyll per cell ratio as the wild-type strain in all growth conditions.

**cia6 Acclimates Slowly to Low CO₂ Conditions**

To test whether the disruption of pyrenoid structure in cia6 might have other pleiotropic effects on the expression of the CCM, changes in the expression of other key CCM components including LciB, CAH4 and CCP1 were investigated by performing Q-RT analysis using RNA samples collected during a low CO₂ induction time course (Fig. 9A). As shown in Fig 9A, the relative transcript abundance of those key CCM genes was lower in the mutant cells than in the wild-type cells. In addition, protein samples collected at the same time points were probed with CCP1, LCIB and CAH4 antibodies to estimate the protein levels by western blotting (Fig 9B). For most of the CCM related proteins, the amount of the protein was close to wild-type levels after 4 hours (Fig 9B). However, the abundance of CCP1, a chloroplast envelope protein, was reduced the most among the CCM proteins that were examined, and remained at low levels even after 4 hours.

**DISCUSSION**

In this report, we described the isolation and characterization of a novel *C. reinhardtii* mutant, in which a nuclear gene named CIA6 was disrupted. The mutation in CIA6 resulted in the dysfunction of the CCM (Fig. 1 and Fig. 2), the disruption of the chloroplast pyrenoid (Fig. 4 and Fig. 7) and a higher than normal chlorophyll concentration (Fig. 8). Transformation of the *cia6* mutant with a functional wild-type CIA6 gene restored a functional CCM (Fig. 1, Fig. 5B and Table 1), a normal pyrenoid phenotype (Fig. 4G and 4H) and its normal levels of chlorophyll (Fig. 8). This work revealed that the nuclear gene product CIA6 is required for the normal formation of pyrenoid, and also provides evidence that the presence of pyrenoid is essential for the functioning of CCM in *C. reinhardtii*.

Most eukaryotic photosynthetic algae have pyrenoids (Griffiths, 1970; Bold and Wynne, 1985), while pyrenoids are almost absent from higher plants with the exception of some species in Hornworts (*Anthocerotophyta*) (Griffiths, 1970). In electron micrographs of a *C. reinhardtii*
cell, the pyrenoid appears as an electron-dense microcompartment embedded inside the chloroplast stroma. A network of modified thylakoid tubules is present inside the pyrenoid body (Griffiths, 1970), and the accumulation of the starch plates surrounding the pyrenoid is often observed especially when the environmental CO$_2$ level is low (Ramazanov et al., 1994; Henk et al., 1995). The dense matrix of the pyrenoid mainly consists of Rubisco (Kuchitsu et al., 1988; Rawat et al., 1996; Morita et al., 1997; Borkhсенiouс et al., 1998). Rubisco activase has also been shown to be present in the pyrenoid (McKay et al., 1991).

The specific localization of Rubisco to the pyrenoid in *C. reinhardtii* is considered to be a key element in the optimal functioning of its CCM (Moroney and Ynalvez, 2007; Spalding, 2008; Yamano et al., 2010). Analogous to the packaging of Rubisco in cyanobacterial carboxysomes (Badger et al., 1998), the pyrenoid in *C. reinhardtii* could likewise be the location of the high CO$_2$ concentration generated by CCM. Since this high CO$_2$ concentration is in the vicinity of Rubisco, the carboxylation reaction will be enhanced at the expense of the oxygenation reaction, hence reducing the photorespiration. In the pyrenoid-containing hornworts, there is also good correlation between the operation of a CCM and the presence of a pyrenoid (Vaughn et al., 1990; Hemsley and Poole, 2004). Besides the role in physical compartmentalization of Rubisco away from the rest of chloroplast stroma, the dynamic nature of pyrenoid in response to environmental changes adds extra complexity to the CCM. One notable observation made using immunolocalization was that in response to a switch from high CO$_2$ to low CO$_2$ environment, the Rubisco in the wild-type chloroplast started to redistribute and accumulate in the pyrenoid after 2 h in low CO$_2$ (Borkhсенiouс et al., 1998). The percentage of the pyrenoid-Rubisco labeling over the total labeling rose from 40% on high CO$_2$ to a maximum of 90% within 4 h on low CO$_2$. This process is tightly correlated with the time required for the appearance of a starch plate surrounding the pyrenoid (Henk et al., 1995), for the induction of most of the CCM genes (Miura et al., 2004) and for the maximal increase in C$_i$ affinity (Borkhсенiouс et al., 1998). The rapid biochemical and morphological rearrangement indicates that the pyrenoid is a dynamic structure and is actively involved in the cell’s acclimation to low CO$_2$ conditions.

One prediction based on the present CCM models is that, if the pyrenoid structure is disrupted, the CCM should also be adversely affected. However, among the few mutants with disrupted pyrenoid, either Rubisco was not present (Rawat et al., 1996), or chloroplast ribosomes...
were mutated leading to the absence of Rubisco (Goodenough and Levine, 1970), or the entire chloroplast structure is highly disrupted (Inwood et al., 2008). One piece of direct evidence supporting the role of the pyrenoid in an active CCM comes from a recent study by Genkov and his colleagues (Genkov et al., 2010). In their work, the *C. reinhardtii* Rubisco holoenzyme was engineered so that the *C. reinhardtii* native RbcL assembled with higher plants RbcS. Although the hybrid Rubisco had similar kinetic properties and was present in amounts equivalent to wild-type Rubisco, the resultant *C. reinhardtii* cells were found to lack the chloroplast pyrenoid, and failed to grow on low CO₂. This is strong evidence that the pyrenoid is playing an important role in the CCM.

In this current study, the characterization of the *cia6* mutant with disorganized pyrenoids provides additional evidence that the pyrenoid is a necessary part of the CCM. Besides the *CIA6* mutation is the first mutation in a gene other than the Rubisco large or small subunit genes that disrupts the pyrenoid structure, while not affecting the entire chloroplast. Here, we show that the pyrenoid is disorganized in the *cia6* mutant cells. We also show that Rubisco is mislocalized in *cia6*, as approximately 65% of the Rubisco was found in the stroma rather than in the “pyrenoid-like” structures. This result is reminiscent of the *Synechococcus* sp. PCC 7942 strain, EK6, described by Schwarz et al. (Schwarz et al., 1995) where a 30 amino acid extension in the RbcS caused the Rubisco to mislocalize to the cytoplasm. The EK6 strain had empty carboxysomes and was unable to grow under low CO₂ conditions. However, the mutation in *cia6* is not within RbcS as in the EK6 strain, but rather in the *CIA6* gene on chromosome ten. By using both denaturing gels and non-denaturing gels, the Rubisco holoenzymes in wild-type and mutant cells have been examined. Cell extracts from both the wild-type and mutant cells were analyzed by western blot on a denaturing gel using an anti-Rubisco antibody, but no significant concentration differences in the two subunits could be revealed between the strains (Fig. 7A). Rubisco isolated from both the wild-type and mutant cells were also subjected to electrophoresis on a non-denaturing gel and stained with Coomassie blue. However, no significant differences were noticed between the two Rubisco preparations, as evidenced by the appearance of the same three high molecular weight bands at the same position, which were all recognized by anti-Rubisco antibody after using western blotting (data not shown). The combination of both the denaturing and the non-denaturing gel electrophoresis analysis indicated the normal composition of the Rubisco holoenzyme in the mutant cells. On the other hand, the appearance of the four high
molecular weight bands in both *C. reinhardtii* Rubiscos on a non-denaturing gel, is similar to the reported four bands observed when the pea RLsMT bound spinach Rubisco was subjected to non-denaturing gel (Raunser et al., 2009), with each band representing the addition of one RLsMT onto the Rubisco while the lowest band represents the native Rubisco.

In both cyanobacteria and the green algae, the arrangement of the CCM components within the cell or chloroplast is critical to the functioning of the CCM. In a current model of the *C. reinhardtii* CCM (Moroney and Ynalvez, 2007; Spalding, 2008; Yamano et al., 2010), a higher than ambient concentration of CO2 inside the thylakoid lumen is predicted to be generated by CAH3. Since the thylakoid lumen is acidified in the light, most inorganic carbon in the lumen will be converted to CO2. This CO2 will either diffuse into the pyrenoid region to be fixed by Rubisco, or diffuse back into the stroma. Since the stroma becomes more basic in the light, any CO2 that leaks out of the pyrenoid have a chance to be converted back to HCO3^- by CAH6, thus reducing CO2 leakage. In *cia6* cells, net C_i accumulation was reduced (Fig. 2C). This reduced accumulation could be due to a lower rate of C_i uptake or due to a reduction in the ability of the cells retain C_i, which we cannot discriminate between these two possibilities. It is clear that any disruption of the pyrenoid potentially disconnects the arrangement of these CCM components and reducing CO2 assimilation efficiency.

Recently there has been increasing interest in the possible interaction between the LCIB/LCIC complex and the pyrenoid (Yamano et al., 2010). Two roles for such a localization have been proposed, including a role in re-capturing CO2 using stromal carbonic anhydrase (CAH6); and a role as a structural barrier in avoiding CO2 leakage from the pyrenoid. It would be intriguing to investigate how the LCIB/LCIC complex would localize in the *cia6* mutant background in which the pyrenoid is absent and Rubisco is no longer concentrated in this specialized region of the chloroplast. For example, would low CO2 still induce the migration of LCIB/LCIC complex to the vicinity of the pyrenoid-like structure in the mutant *cia6* cell? These results might shed light on whether the aggregation of LCIB/LCIC complex needs the presence of pyrenoid or not; or whether the complex is actually associating with the thylakoids tubules or the pyrenoid.

Apparently CIA6 is required for the formation of pyrenoid in *C. reinhardtii*, however, the exact function of CIA6 in the CCM or pyrenoid formation is not clear. Predicted to contain a SET domain by Pfam (Bateman et al., 2002), the CIA6 protein is likely to act as a lysine
methyltransferase (Dillon et al., 2005; Qian and Zhou, 2006; Ng et al., 2007). In plants, other than modifying histones, SET domain containing proteins were also found to methylate Lys$_{14}$ on Rubisco large subunit (Klein and Houtz, 1995; Ying et al., 1999; Trievel et al., 2002; Trievel et al., 2003). Since in the cia6 mutant cells, the Rubisco containing pyrenoid is not well developed, it could be speculated that one possible function of CIA6 could be acting as the *C. reinhardtii* Rubisco methyltransferase, modifying the conformation of this holoenzyme in some way allowing it to organize or self-assemble into a pyrenoid. However, the Rubisco methylating activity of CIA6 could not be found in vitro. It should be noted that unlike the pea RbcL with methylated Lys$_{14}$ on the N-terminal tail, the *C. reinhardtii* RbcL’s Lys$_{14}$ is un-methylated. Instead, crystal structure data from *C. reinhardtii* Rubisco indicated the presence of methyl-Cys$_{256}$ and methyl-Cys$_{369}$ (Taylor et al., 2001), with the first Cys buried at the interface of RbcL and RbcS, and the latter Cys positioned on the external surface of RbcL. Notwithstanding the potential electrostatic problem of a cysteine fitting in the narrow cleft of the SET domain’s catalytic site, neither cysteines is thought to easily enter the catalytic channel of RLsMT, as compared to the Lys$_{14}$ on the flexible N-terminal tail. On the other hand, by examining the amino acid sequences of CIA6 and its 13 homologs, it was found that the invariant tyrosine residue on the SET domain N terminus is present, indicating the possible presence of the target lysine binding site. However, in all 13 CIA6 homologs, the highly conserved catalytic site motif NHS could not be found, which could argue that these proteins may belong to a sub-class of SET domain containing proteins that do not have catalytic functions but emphasize on structural roles in relation to Rubisco aggregation and pyrenoid formation. Alternatively, based on the fact that CIA6 homolog are only found in green algae and higher plants but not in cyanobacteria or the sequenced diatom genomes, it could be speculated that the function of CIA6 may not be limited to or directly linked to the formation of pyrenoid structure or CCM function.

A notable phenotype of cia6 *C. reinhardtii* cells was its increased chlorophyll content under photoautotrophic conditions (Figure 8). This observation implies that the function of CIA6 might be related to chloroplast organization or nuclear/chloroplast coordination. Like the chloroplast, the pyrenoid reproduces by binary fission during cell division (Goodenough, 1970; Goodenough and Levine, 1970; Harris et al., 2009). The disruption of the pyrenoid might occur if chloroplast structure or division is not well coordinated in the CIA6 deficient cell. We are presently working on obtaining an Arabidopsis knockout line with CIA6 homolog gene disrupted...
to see whether the chloroplast structure is affected in that plant. Given the fact that multiple small pyrenoids were sometimes observed in a single mutant chloroplast, the possibility that the mutant pyrenoid division problem led to the altered chlorophyll concentration could not be excluded.

In summary, CIA6 is a member of a new gene family found in green algae and in higher plants. When this gene is knocked out in C. reinhardtii, the cell has a disrupted pyrenoid and a dysfunctional CCM. When the wild-type gene is returned to cia6 mutant cells, the normal pyrenoid morphology and CCM function are restored. This is the first report of a gene outside of the Rubisco structure genes that affects pyrenoid structure. The linkage of the pyrenoid structure and the CCM provides strong support for CCM models in which the localization of Rubisco to the pyrenoid is essential.

MATERIALS AND METHODS

Cell Cultures and Growth

C. reinhardtii culture conditions were similar to those used previously (Rawat and Moroney, 1991). The strain D66 (nit2?, cw15, mt+) was obtained from Rogene Schnell (University of Arkansas, Little Rock) and CC124 (nit1?, nit2?, and mt) from the C. reinhardtii Duke University Stock Center (http://www.chlamy.org/). Tris–acetate–phosphate (TAP) and minimal (without acetate) liquid medium were prepared according to Sueoka (Sueoka, 1960). TAP plates and minimal media plates were prepared by adding 1.2% agar (w/v). Cell cultures were started from inoculating C. reinhardtii colonies from TAP plates into 100mL of TAP liquid medium and grown with continuous shaking and light (100 µmol·m⁻²·s⁻¹) until the culture reaches early log phase. TAP grown cultures were harvested and washed with minimal liquid medium twice and then split into two flasks containing minimal media and bubbled with high CO₂ (5% [v/v] CO₂ in air) until it reached a cell density of about 2-3x10⁶ cells·mL⁻¹. To induce CCM, one flask was switched from high CO₂ to low CO₂ (0.01% [v/v] CO₂ in air) bubbling for one day or otherwise as indicated in the text; the other flask was kept on high CO₂ as the control.

C. reinhardtii Mutagenesis and Electroporation
Strain D66 was mutagenized by transformation with a linearized pSP124s plasmid (Lumberaras et al., 1998) using the electroporation procedure described by Shimogawara et al. (1998) with modifications described by Colombo et al (2002). Transformants were first selected on TAP plates containing the antibiotic zeocin (7.5 µg·mL⁻¹, Invitrogen, Carlsbad, CA). Antibiotic resistant strains were then screened for a CCM deficient phenotype in low CO₂ chamber (0.01% [v/v] CO₂ in air) in light (100 µmol·m⁻²·s⁻¹).

**Nucleic Acid and Protein-blot Analysis**

Total DNA was isolated according to Newman et al. (1990). Southern blots were carried out following the guidelines in (Sambrook et al., 2001). Briefly, restriction enzyme digested DNA (2 µg in each lane) was loaded and separated on a 0.8% (w/v) agarose gel and blotted onto a nylon membrane (Schleicher & Schull, Keene, NH). ³²P-dCTP-labeled probes were prepared using a random primer procedure. For Quantitative RT-PCR analysis, RNA was first extracted using Trizol reagent following guidelines provided (Invitrogen, Carlsbad, CA). Contaminating DNA was removed by DNase treatment (Roche Applied Science, Indianapolis, IN) and was further cleaned up using an RNeasy kit (Qiagen, Tokyo, Japan). Total RNA was used for cDNA synthesis following the manufacture’s instruction (Roche Applied Science, Indianapolis, IN) using Poly (dT) primers. Synthesized cDNA was then subjected to Quantitative RT-PCR analysis using Sybr Green Premix (Takara, Shiga, Japan) using an ABI 7000 Real Time PCR System (Applied Biosystem, Foster City, CA).

**Identification of Flanking Regions and Genetic Linkage Analysis**

Adaptor-mediated PCR was used to identify the DNA flanking the pSP124s insertion using a modified version of the Genome Walker TM kit (Clontech, Mountain View, CA). Homology searches were performed using the BLAST server (Altschul et al., 1997), and the Joint Genome Institute *C. reinhardtii* database V4.0 site (Merchant et al., 2007). Genetic crosses and tetrad analysis were performed as previously described (Sears et al., 1980; Moroney et al., 1986; Harris et al., 2009). Briefly cia6 (mt⁺) and CC124 (mt⁻) cells cultures were subjected to nitrogen starvation in high light overnight and combined for mating for one hour. Aliquots (0.3mL) were plated on nitrogen-minus minimum medium containing 4% nitrogen-depleted agar and stored in the dark for zygote maturation for 10 days. The zygotes on the maturation plates
were transferred to 1.2% agar TAP medium plates incubated overnight for meiotic germination. Tetrad dissections were then carried out and linkage was determined by association of the *Blomycin resistance* with those progeny that grew poorly on low CO2 levels.

**Photosynthesis Assays**

Cultures were started heterotrophically in 100 mL of TAP media. After reaching the log phase, the cultures were then transferred to 1 L minimal medium and bubbled with 5% CO2 until they reached a cell density about 3x10^6 cells·mL⁻¹. The cultures were then subjected to CCM induction for 12 h at 100 μE·m⁻²·s⁻¹. The affinity for external inorganic carbon, K_{0.5} [DIC] was estimated as described by Pollock and Colman (2001). Briefly, cells containing 100μg chlorophyll were suspended in CO2 free HEPES buffer (pH=7.3), which was previously bubbled with N₂. The cell suspension was transferred to the electrode chamber (Rank Brothers, Cambridge, UK) and was allowed to deplete any endogenous DIC until no net O₂ exchange was observed. The DIC concentrations of the medium were controlled by injecting NaHCO₃ solution into the chamber. The light intensity for photosynthesis assay was adjusted at 300 μE·m⁻²·s⁻¹. The K_{0.5} [DIC] value is calculated where the DIC concentration required for half-maximal rates of oxygen evolution (Badger, 1985).

**Complementation**

Complementation of the mutant strain *cia6* was achieved by transforming *cia6* cells with the entire *CIA6* genomic region spanning the entire gene using the glass bead method (Kindle, 1990). Briefly, cells were grown to 3x10^6 cell·mL⁻¹ in 100 mL TAP before transferring to 1 L minimum medium bubbled with air till the cell density reaches 2x10^6 cell·mL⁻¹. Transformation was started by mixing 0.3 mL cell suspension (3x10^8 cell·mL⁻¹), 300 mg sterilized glass beads, 5 μg DNA and 0.1 mL 20% PEG. The mixture was then agitated at top speed in a 15 mL tube for 15 seconds. After beads settled to the bottom, the cell suspension was plated onto minimal media plates and maintained in low CO₂ chamber (70ppm CO₂ in air). Colonies that were able to grow heterotrophically under low CO₂ conditions were examined using light microscopy to screen for the strains with a pyrenoid. RNA was then extracted from putative complemented strains to confirm the reappearance of the full length *CIA6* mRNA.
**Immunolocalization Studies Using Electron Microscopy**

Immunolocalization procedure was performed as described previously (Mitra et al., 2004). Briefly, about 2 mL cell suspension was fixed with an equal volume of 1% OsO₄, 2% formaldehyde, 0.5% glutaraldehyde, and 0.1 mM sodium cacodylate buffer (pH=7.2) for 30 minutes. The solution was then extracted into a 10mL syringe with a Swinney filter holder fitted with a 13 mm diameter 5 μm pore polycarbonate filter and fixed for another 30 min, followed by 15 min washes with 0.1 M cacodylate buffer containing 0.02 M glycine for 5 times. Materials were rinsed with distilled water and stained with 0.5% uranyl acetate in dark for 30 minutes. After this, excess cells were rinsed and the samples were dehydrated using ethyl alcohol series. Samples were then infiltrated and embedded in LR White resin (EMS, PA). Embedded samples were sectioned with a DuPont Sorvall microtome (Wilmington, DE) to 70 nm. TEM sections were mounted on collodion coated nickel grids.

The immunocytochemical procedure was similar to the method of (Borkhsenious et al., 1998) with some modifications. Sections were pretreated with 2% sodium-meta-periodate (Sigma, St. Louis, MO) for 15 minutes to remove any residual glutaraldehyde, then blocked two times for 30 minutes in blocking solution (2% BSA and 0.1% Tween-20 in PBS). The sections were incubated for 90 minutes with Rubisco antibody (1:50) or with the preimmune serum with the same dilution. The grids were transferred to 1:50 dilution of Protein-A gold labeled (20nm, Sigma, St. Louis, MO) for one hour. Antibodies were all diluted in blocking solution. Finally the sections were rinsed with distilled water and photographed under transmission electron microscopy.

The fraction of Rubisco in the pyrenoid and in the chloroplast stroma was measured as previously described (Borkhsenious et al., 1998). Here the pyrenoid area is excluded from the total chloroplast stroma. The immunogold particle density in the pyrenoid (Dₚ, #·μm⁻²) was first calculated by dividing the total number of immunogold particles in the pyrenoid by the area of pyrenoid. Similarly, the immunogold particle density in the chloroplast stroma (Dₛ, #·μm⁻²) was calculated by dividing the total number of immunogold particles in the chloroplast stroma by the total area of chloroplast stroma. The total immunogold particles in the pyrenoid or stroma were then calculated by multiplying the immunogold density (D) averaged from 25 TEM thin sections and the average volume of each compartment (Lacoste-Royal and Gibbs, 1987). The fraction of Rubisco in the pyrenoid (Fractionₚ) was further calculated by dividing the total particles in the
pyrenoid by the total number of particles from pyrenoid and chloroplast stroma. The final equation is shown below:

\[
\text{Fraction}_p = 100 \times \frac{[D_p \times 2.4]}{[D_p \times 2.4 + D_s \times 35.6]}; \text{ where } D = \frac{\text{(number of particles)}}{\text{(area)}}
\]

Other methods

The CO₂ concentration in the growth chambers was measured using an infrared gas analyzer (The Analytical Development Co. Ltd, Hoddesdon, England). Protein concentration was determined using Bradford 10x Dye reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. The growth curve experiments were standardized based on chlorophyll mass (1.9μg·mL⁻¹) and approximately the same cell density. Chlorophyll content was measured as the total content from chlorophyll a plus chlorophyll b (Arnon, 1949; Holden, 1976). Chlorophyll was extracted by 100% methanol and was quantified spectrophotometrically (Arnon, 1949) and calculated according to Holden (1976). Cell density values were determined by direct counting in a hemacytometer chamber (Hausser Scientific, Horsham, PA). For pyrenoid staining and observation using light microscopy, *C. reinhardtii* cells were stained with 0.05% bromophenol blue (BPB) in 0.1% HgCl₂ (Kuchitsu et al., 1988).

**FIGURE LEGENDS**

**Figure 1.** The mutant *cia6* demonstrated a typical CCM deficient phenotype. The ability of *cia6* to grow photoautotrophically was tested by comparing its growth on minimum plates under high CO₂ (5% CO₂ [v/v], panel A) and very low CO₂ (0.01% CO₂ [v/v], panel B) conditions. A, *cia6* grew well in high CO₂ compared to its parental strain D66. B, *cia6* grew poorly under low CO₂ condition, similar to the CCM deficient mutant *cia5*. The complemented *cia6*, *cia6*-comp, in which the wild-type *CIA6* gene was put back into *cia6*, exhibited a phenotype similar to that of the wild-type D66.

**Figure 2.** The mutant *cia6* showed a reduced affinity for C₅. A and B, The rates of photosynthesis of D66 ( ■ ), *cia6* ( ● ), and *cia5* ( ♦ ) as a function of the dissolved inorganic carbon concentration (DIC) for high CO₂ grown (A) and low CO₂ grown (B) cells were measured. C, The C₅ accumulation in low CO₂ acclimated D66 ( ■ ) and *cia6* ( ● ) was measured during the time
course of $^{14}$C-DIC accumulation. Each point represents the mean and standard error of three separate experiments.

**Figure 3.** The mutant *cia6* has an insertion in the *CIA6* locus. A, Southern blot analysis using the bleomycin resistance gene as a probe. D66 (1) and *cia6* (2) genomic DNA was digested with NcoI (Nc), NotI (No), PstI (P), and SacI (S) and blotted onto a nylon membrane. The membrane was then probed with a $^{32}$P-labeled pSP124s specific fragment containing *Ble* DNA and the *RBCS2* intron. The weak bands present in both D66 and *cia6* correspond to the *RbcS2* intron, which is present in both the *Ble* mutant as well as the endogenous *RbcS2* gene. B, Genomic structure of the *CIA6* locus in wild-type and *cia6* showing the introns and exons, the site of the pSP124s insertion in *cia6*, and the cDNA primer sites (arrows) used for the RT-PCR analysis in figure 3C. C, RT-PCR analysis of the D66 and *cia6* strains using polyA RNA from high and low CO$_2$ grown cells as templates for reverse transcription. The primer-binding sites for *CIA6* are shown in figure 3B and amplify an 1100 bp product from cDNA. The internal control fragment (730 bp) is the product of primers designed to amplify Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The last lane contains the NEB 2-LOG ladder.

**Figure 4.** Electron micrographs (A-D, F and H) and light micrographs (E and G) of *C. reinhardtii* cells grown in minimum medium in high (A and C) and low CO$_2$ (B and D, E-H) conditions. A, Wild-type D66 grown under high CO$_2$ conditions. B, Wild-type D66 grown under low CO$_2$ conditions. C, Mutant *cia6* grown under high CO$_2$ conditions. D, Mutant *cia6* grown under low CO$_2$ conditions. E, Light microscopes of *cia6* cells showing multiple pyrenoid or pyrenoid-like structure (labeled as “*””) in the *cia6* observed under light microscope. F, Electron micrographs showing multiple pyrenoid or pyrenoid-like structures (labeled as “*””) in the *cia6* observed under electron microscope. G, Pyrenoid (labeled as “P”) and nucleus (labeled as “N”) is observed as dark stainings in the complemented *cia6* grown under low CO$_2$ conditions. H, A positive pyrenoid in the complemented *cia6* grown under low CO$_2$ conditions. P, pyrenoid; N, nucleus; s, starch; *, indicates the location of pyrenoid like bodies in mutant cells.

**Figure 5.** Complementation rescued the CCM deficiency in *cia6*. By transforming genomic DNA containing the wild type *CIA6* gene into the *cia6* mutant, the resultant complemented strain
cia6-comp exhibited a growth phenotype similar to the wild-type (See Figure 1). A, The rate of photosynthesis as a function of the dissolved inorganic carbon was measured using low CO$_2$ grown D66 (●), cia6 (▼), cia5 (▲) and the complemented strain cia6-comp (■). B, RT-PCR analysis of the D66, cia6, cia5 and the complemented cia6 (cia6-comp) using RNA isolated from low CO$_2$ grown cells as templates for reverse transcription. Primers were designed to amplify the entire ORF. The reappearance of CIA6 full length ORF in the complemented strain (cia6-comp) was observed on a DNA agarose gel. The last lane contained the no RNA control.

Figure 6. Evolutionary relationships of CIA6 amongst 13 taxa. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Each protein name begins with the abbreviation of its scientific name, and followed by its NCBI accession number. The species are: Volvox carteri f. nagariensis; Micromonas pusilla CCMP1545; Physcomitrella patens subsp. Patens; Vitis vinifera; Ricinus communis; Selaginella moellendorffii; Micromonas sp. RCC299; Selaginella moellendorffii; Populus trichocarpa; Arabidopsis thaliana; Arabidopsis lyrata subsp. lyrata; Sorghum bicolor; Oryza sativa.

Figure 7. Rubisco content in the wild-type D66 and mutant cia6 cells were investigated using western blot (A) and immunoglod labeling (B-I) against anti-Rubisco antibody. A, Total protein isolated from D66 and cia6 grown under high and low-CO$_2$ conditions for 12 hours and 24 hours. Using western blot, the amount of dissociated Rubisco large and small subunits (RbcL & RbcS) were estimated to be normal in the mutant compared to the wild-type. B and C, High-CO$_2$-grown wild-type cells D66 grown on minimal medium probed with an antibody raised against Rubisco. D and E, Low-CO$_2$-grown wild-type cells D66 grown on minimal medium probed with an antibody raised against Rubisco. F and G, High-CO$_2$-grown cia6 grown on minimal medium probed with an antibody raised against Rubisco. H and I, Low-CO$_2$-grown cia6 grown on minimal medium probed with an antibody raised against Rubisco.

Bars indicate 1 µm. P, Pyrenoid; N, nucleus; Th, thylakoid; *, Pyrenoid-like structures in cia6

Figure 8. A higher chlorophyll content per cell was observed in the mutant cia6. Cultures of D66 (●), cia6 (▼), cia5 (▲) and cia6-comp (■) were started at the same chlorophyll
concentration, and subjected to low CO$_2$ stress. For the subsequent 6 days, samples were collected every 24 hours and chlorophyll concentration (A) and cell density (B) were measured, and the chlorophyll concentration per cell were calculated (C). A, Cell density was determined by direct counting using a hemocytometer. B, Chlorophyll concentration was measured and the mutant $cia6$ had the highest chlorophyll concentration at the end of the time course. C, Chlorophyll content per cell values were plotted so that it was clear that the mutant $cia6$ had an increased chlorophyll content per cell compared to the other tested strains. Each point represents the mean and standard deviation of three separate experiments.

**Figure 9.** Time course of $LCIB$, $CAH4$ and $CCP1$ levels during low CO$_2$ induction process as measured by Q-RT PCR (A and B) and western blotting (C). A, Wild-type D66 cells subjected to low CO$_2$ induction were sampled at the time points as indicated in the figure. $CBLP$ gene was used as the internal control. (B) Mutant $cia6$ cells subjected to low CO$_2$ induction were sampled at time points as indicated in the figure. $CBLP$ gene was used as the internal control. (C) Wild-type D66 and mutant $cia6$ protein samples were collected at the time points as indicated in the figure, and were then subjected to Western blot analysis using antibody raised against CCP1, LCIB, and CAH4. Anti-$\alpha$-Tubulin antibody was used as a loading control.

**Figure S1.** A higher chlorophyll content per cell was observed in the mutant $cia6$. Cultures of D66 (●), $cia6$ (▼), $cia5$ (▲) and $cia6$-comp (■) were initially started in TAP medium, and then switched to minimal or TAP medium at the same chlorophyll concentration of 1.9 $\mu$g·mL$^{-1}$ till the stationary phase is reached. Samples were collected every 24 hours and chlorophyll concentration and cell density were measured, and the chlorophyll concentration per cell were calculated. Each point represents the mean and standard error of three separate experiments.
References


McKay RML, Gibbs SP, Vaughn KC (1991) RuBisCo activase is present in the pyrenoid of green algae. Protoplasma 162: 38-45


Moroney JV, Tolbert NE, Sears BB (1986) Complementation analysis of the inorganic carbon concentrating mechanism of Chlamydomonas reinhardtii. Molecular and General Genetics MGG 204: 199-203


Pronina NA, Semenenko VE (1992) Carbonic Anhydrase Activity and Fatty-Acid Composition of Photosystem Deficient and High CO₂ Required Mutants of Chlamydomonas reinhardtii. Photosynthesis Research 34: 201-201


Ramazanov Z, Rawat M, Henk MC, Mason CB, Matthews SW, Moroney JV (1994) The induction of the CO₂-concentrating mechanism is correlated with the formation of the starch sheath around the pyrenoid of Chlamydomonas reinhardtii. Planta 195: 210-216


Vaughn KC, Campbell EO, Hasegawa J, Owen HA, Renzaglia KS (1990) The pyrenoid is the site of ribulose 1,5-bisphosphate carboxylase/oxygenase accumulation in the hornwort (Bryophyta: Anthocerotae) chloroplast. Protoplasma 156: 117-129


Table 1. The $K_{1/2}$ (DIC) value of cia6-comp was recovered to the wild-type (D66) level.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Strains</th>
<th>D66</th>
<th>cia6</th>
<th>cia5</th>
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<td>High CO₂</td>
<td></td>
<td>179±23</td>
<td>296±24</td>
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<td>Low CO₂</td>
<td></td>
<td>29±7</td>
<td>106±17</td>
<td>138±26</td>
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$K_{1/2}$(DIC) value is calculated by regression analysis from three individual experiments using Michaelis–Menten kinetics equation: $V = V_{max}[S]/(K_m+[S])$, in which [S] equals to the bicarbonate concentration, $V$ equals to the oxygen evolution rate at each bicarbonate concentration, $V_{max}$ equals to the maximum oxygen evolution rate when bicarbonate concentration is saturated, $K_m$ equals to $K_{1/2}$(DIC).
Table 2. Comparison of the Rubisco fraction found in the pyrenoid and in the chloroplast stroma between wild-type and *cia6*. The data shown are the averages of 25 *C. reinhardtii* EM thin sections and a total of 300 particles counted.

<table>
<thead>
<tr>
<th><em>C. reinhardtii</em> strain</th>
<th>Immunogold density (particles/μm²)</th>
<th>% Rubisco in the pyrenoid</th>
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<tr>
<td><em>cia6</em></td>
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Figure 1. The mutant cia6 demonstrated a typical CCM deficient phenotype. The ability of cia6 to grow photoautotrophically was tested by comparing its growth on minimum plates under high CO₂ (5% CO₂ [v/v], panel A) and very low CO₂ (0.01% CO₂ [v/v], panel B) conditions. A, cia6 grew well in high CO₂ compared to its parental strain D66. B, cia6 grew poorly under low CO₂ condition, similar to the CCM deficient mutant cia5. The complemented cia6, cia6-comp, in which the wild-type CIA6 gene was put back into cia6, exhibited a phenotype similar to that of the wild-type D66.
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FIGURE 3

A

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← 10 Kb
← 3 Kb
← 1 Kb

B

CIA6

Cia6

pSP124s

500bp

C

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<tr>
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CIA6

GAPHD
**Figure 3.** The mutant *cia6* has an insertion in the *CIA6* locus. A, Southern blot analysis using the bleomycin resistance gene as a probe. D66 (1) and *cia6* (2) genomic DNA was digested with NcoI (Nc), NotI (No), PstI (P), and SacI (S) and blotted onto a nylon membrane. The membrane was then probed with a 32P-labeled pSP124s specific fragment containing *BleR* DNA and the *RBCS2* intron. The weak bands present in both D66 and *cia6* correspond to the *RbcS2* intron, which is present in both the BleR mutant as well as the endogenous *RbcS2* gene. B, Genomic structure of the *CIA6* locus in wild-type and *cia6* showing the introns and exons, the site of the pSP124s insertion in *cia6*, and the cDNA primer sites (arrows) used for the RT-PCR analysis in figure 3C. C, RT-PCR analysis of the D66 and *cia6* strains using polyA RNA from high and low CO2 grown cells as templates for reverse transcription. The primer-binding sites for *CIA6* are shown in figure 3B and amplify an 1100 bp product from cDNA. The internal control fragment (730 bp) is the product of primers designed to amplify Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The last lane contains the NEB 2-LOG ladder.
FIGURE 4

A

D66 High CO₂

B

D66 Low CO₂

C

cia6 High CO₂

D

cia6 Low CO₂

E

cia6 Low CO₂

F

nia6 Low CO₂

G

cia6-comp Low CO₂

H

cia6-comp Low CO₂
Figure 4. Electron micrographs (A-D, F and H) and light micrographs (E and G) of *C. reinhardtii* cells grown in minimum medium in high (A and C) and low CO$_2$ (B and D, E-H) conditions. A, Wild-type D66 grown under high CO$_2$ conditions. B, Wild-type D66 grown under low CO$_2$ conditions. C, Mutant *cia6* grown under high CO$_2$ conditions. D, Mutant *cia6* grown under low CO$_2$ conditions. E, Light microscopes of *cia6* cells showing multiple pyrenoid or pyrenoid-like structure (labeled as “*”*) in the *cia6* observed under light microscope. F, Electron micrographs showing multiple pyrenoid or pyrenoid-like structures (labeled as “*”*) in the *cia6* observed under electron microscope. G, Pyrenoid (labeled as “P”) and nucleus (labeled as “N”) is observed as dark stainings in the complemented *cia6* grown under low CO$_2$ conditions. H, A positive pyrenoid in the complemented *cia6* grown under low CO$_2$ conditions. P, pyrenoid; N, nucleus; s, starch; *, indicates the location of pyrenoid like bodies in mutant cells.
Figure 5. Complementation rescued the CCM deficiency in cia6. By transforming genomic DNA containing the wild type CIA6 gene into the cia6 mutant, the resultant complemented strain cia6-comp exhibited a growth phenotype similar to the wild-type (See Figure 1). A, The rate of photosynthesis as a function of the dissolved inorganic carbon was measured using low CO2 grown D66 (●), cia6 (▲), cia5 (▲) and the complemented strain cia6-comp (■). B, RT-PCR analysis of the D66, cia6, cia5 and the complemented cia6 (cia6-comp) using RNA isolated from low CO2 grown cells as templates for reverse transcription. Primers were designed to amplify the entire ORF. The reappearance of CIA6 full length ORF in the complemented strain (cia6-comp) was observed on a DNA agarose gel. The last lane contained the no RNA control.
**FIGURE 6**

Figure 6. Evolutionary relationships of CIA6 amongst 13 taxa. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Each protein name begins with the abbreviation of its scientific name, and followed by its NCBI accession number. The species are: *Volvox carteri f. nagariensis*; *Micromonas pusilla* CCMP1545; *Physcomitrella patens* subsp. *Patens*; *Vitis vinifera*; *Ricinus communis*; *Selaginella moellendorffii*; *Micromonas sp. RCC299*; *Selaginella moellendorffii*; *Populus trichocarpa*; *Arabidopsis thaliana*; *Arabidopsis lyrata* subsp. *lyrata*; *Sorghum bicolor*; *Oryza sativa*. 
**FIGURE 7**

A diagram showing the expression of RbcL and RbcS proteins under different conditions. The image includes a table with 12 hr and 24 hr time points, and strains D66 and cia6 for both RbcL and RbcS proteins.
**Figure 7.** Rubisco content in the wild-type D66 and mutant *cia6* cells were investigated using western blot (A) and immunoglod labeling (B-I) against anti-Rubisco antibody. A, Total protein isolated from D66 and *cia6* grown under high and low-CO$_2$ conditions for 12 hours and 24 hours. Using western blot, the amount of dissociated Rubisco large and small subunits (RbcL & RbcS) were estimated to be normal in the mutant compared to the wild-type. B and C, High-CO$_2$-grown wild-type cells D66 grown on minimal medium probed with an antibody raised against Rubisco. D and E, Low-CO$_2$-grown wild-type cells D66 grown on minimal medium probed with an antibody raised against Rubisco. F and G, High-CO$_2$-grown *cia6* grown on minimal medium probed with an antibody raised against Rubisco. H and I, Low-CO$_2$-grown *cia6* grown on minimal medium probed with an antibody raised against Rubisco.

Bars indicate 1 µm. P, Pyrenoid; N, nucleus; Th, thylakoid; *, Pyrenoid-like structures in *cia6*.
Figure 8. A higher chlorophyll content per cell was observed in the mutant *cia6*. Cultures of D66 (●), *cia6* (▲), *cia5* (▼) and *cia6*-comp (■) were started at the same chlorophyll concentration, and subjected to low CO₂ stress. For the subsequent 6 days, samples were collected every 24 hours and chlorophyll concentration (A) and cell density (B) were measured, and the chlorophyll concentration per cell were calculated (C). A, Cell density was determined by direct counting using a hemocytometer. B, Chlorophyll concentration was measured and the mutant *cia6* had the highest chlorophyll concentration at the end of the time course. C, Chlorophyll content per cell values were plotted so that it was clear that the mutant *cia6* had an increased chlorophyll content per cell compared to the other tested strains. Each point represents the mean and standard deviation of three separate experiments.
**Figure 9.** Time course of *LCIB*, *CAH4* and *CCP1* levels during low CO₂ induction process as measured by Q-RT PCR (A and B) and western blotting (C). (A) Wild-type D66 cells subjected to low CO₂ induction were sampled at the time points as indicated in the figure. *CBLP* gene was used as the internal control. (B) Mutant *cia6* cells subjected to low CO₂ induction were sampled at time points as indicated in the figure. *CBLP* gene was used as the internal control. (C) Wild-type D66 and mutant *cia6* protein samples were collected at the time points as indicated in the figure, and were then subjected to Western blot analysis using antibody raised against CCP1, LCIB, and CAH4. Anti-α-Tubulin antibody was used as a loading control.