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The *Chlamydomonas reinhardtii* proteins Ccp1 and Ccp2 are required for long-term growth, but are not necessary for efficient photosynthesis, in a low-CO₂ environment

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Abstract

The unicellular green alga *Chlamydomonas reinhardtii* acclimates to a low-CO₂ environment by modifying the expression of a number of messages. Many of the genes that increase in abundance during acclimation to low-CO₂ are under the control of the putative transcription factor Cia5. *C. reinhardtii* mutants null for *cia5* do not express several of the known low-CO₂ inducible genes and do not grow in a low-CO₂ environment. Two of the genes under the control of Cia5, *Ccp1* and *Ccp2*, encode polypeptides that are localized to the chloroplast envelope and have a high degree of similarity to members of the mitochondrial carrier family of proteins. Since their discovery, Ccp1/2 have been candidates for bicarbonate uptake proteins of the chloroplast envelope membrane. In this report, RNA interference was successful in dramatically decreasing the abundance of the mRNAs for *Ccp1* and *Ccp2*. The abundance of the Ccp1 and Ccp2 proteins were also reduced in the RNAi strains. The RNAi strains grew slower than WT in a low-CO₂ environment, but did not exhibit a mutant carbon concentrating phenotype as determined by the cells' apparent affinity for dissolved inorganic carbon. Possible explanations of this RNAi phenotype are discussed.

Introduction

When *Chlamydomonas reinhardtii* is grown in a low-CO₂ environment, it induces a carbon dioxide concentrating mechanism (CCM) (Moroney and Somanchi, 1999). The CCM allows cells to perform photosynthesis at low levels of CO₂ in the surrounding environment by increasing the concentration of CO₂ around Rubisco. This increase in CO₂ around Rubisco leads to an increased rate of photosynthesis and a decreased rate of photorespiration. When *C. reinhardtii* cells are shifted from a high- to low-CO₂, the abundance of several mRNA transcripts change as the organism adjusts

to its new surroundings (Burow *et al.*, 1996; Somanchi *et al.*, 1998; Im *et al.*, 2003).

The gene products for several of these messages that increase in abundance have been identified. The proteins of known identity include carbonic anhydrases, photorespiratory pathway enzymes, and light-harvesting complex components which change in abundance during acclimation to a low-CO₂ environment. However, many other low CO₂-inducible genes are predicted to encode proteins of unknown function. Two separate laboratories have independently isolated mutants of *Cia5* (Moroney *et al.*, 1989; Fukuzawa *et al.*, 1998). *C. reinhardtii* cells that are mutant for *Cia5* do not acclimate to a

low-CO₂ environment (Moroney *et al.*, 1989). The *cia5* mutant lacks the induction of dissolved inorganic carbon (DIC) transport, induction of *Cah1*, *Mca1* and *Mca2*, *Ccp1* and *Ccp2*, *Lci1*, or any of the low-CO₂ induced polypeptides. In addition, the up-regulation of phosphoglycolate phosphatase and glycolate dehydrogenase, and down-regulation of Rubisco biosynthesis does not occur in *cia5* (Moroney *et al.*, 1989; Marek and Spalding, 1991; Spalding *et al.*, 1991; Burow *et al.*, 1996). The *cia5* gene was recently cloned. It is constitutively expressed and encodes a protein of 76 kDa that contains a putative zinc-finger motif that is crucial to its regulatory function (Fukuzawa *et al.*, 2001; Xiang *et al.*, 2001).

Two low-CO₂ inducible proteins with a molecular mass of 36 kD were purified from membrane fractions of *C. reinhardtii* (Spalding and Jeffrey, 1989). These two peptides are immunologically distinct from the low-CO₂ inducible Cah1 protein which has a similar molecular weight (37 kDa) (Geraghty *et al.*, 1990). Using ³⁵S labeling of *C. reinhardtii* proteins, LIP-36, for low-CO₂ induced peptide, was localized to intact chloroplasts (Moroney and Mason, 1991) and then more specifically localized to chloroplast envelopes by Ramazanov *et al.* (1993). The LIP-36 protein was purified from 8 L of low-CO₂ adapted cells of *C. reinhardtii* and sequenced. Using degenerate PCR primers, the cloning and over-expression of two genes encoding LIP-36 was achieved (Chen *et al.*, 1997). The two genes were renamed to *Ccp1* and *Ccp2*, for *chloroplast carrier proteins 1* and *2*, and are predicted to encode proteins which are 95.7% identical, of 358 and 355 amino acids, respectively. Neither gene is expressed in the *cia5* mutant background. The two proteins are predicted to have six transmembrane domains and are similar to proteins of the mitochondrial carrier protein super family. Proteins belonging to this family transport a wide variety of metabolites and ions (Walker and Runswick, 1993). Ccp1/2 are most similar to the *Arabidopsis thaliana* protein Bou (36% identity). Bou is necessary for seedling development in the light and may be involved in a pathway that would be an alternative to the glyoxylate pathway (Lawand *et al.*, 2002). Bou is most similar to the mitochondrial carnitine acyl carriers proteins. In animals and yeast, these transmembrane proteins are involved in the transport of lipid-derived molecules across

mitochondrial membranes for energy and carbon supply.

The location of the Ccps in the chloroplast envelope, their induction in a low-CO₂ environment, and their similarity to the mitochondrial carrier protein family has lead to the hypothesis that the Ccps may be involved in the active accumulation of DIC into the chloroplast. RNA interference (RNAi) has recently been utilized to silence the expression of several proteins in *C. reinhardtii* (for examples, see Sineshchekov *et al.*, 2002; Koblenz *et al.*, 2003). This paper describes the growth and photosynthetic characteristics of RNAi strains of *C. reinhardtii* that lack *Ccp1/2* mRNA and protein in a low-CO₂ environment to test the hypothesis that the Ccp proteins are required for optimal rates of photosynthesis in a low-CO₂ environment.

Materials and methods

Algal strains and transformation conditions

Culture conditions were similar to those used previously (Rawat and Moroney, 1995). The strain D66 (*nit2*⁻, *cw15*, *mt*⁺) was obtained from Rogene Schnell, University of Arkansas-Little Rock. *rca-1* is a strain missing Rubisco activase that was generated by insertional mutagenesis as previously described (Pollock *et al.*, 2003). For transformation, D66 cells were first grown in 100 ml of TAP medium (Sueoka, 1960) for 2 days and then transferred to 500 ml of TAP medium 24 h before the experiment. Cells were harvested and resuspended at a density of 2×10^8 cells ml⁻¹ in TAP plus 60 mM sucrose (Shimogawara *et al.*, 1998). For electroporation, 1 µg of pSL72-LIP36G1 DNA was linearized with *StuI* and added to 250 µl of the resuspended cells in an electroporation cuvette (0.4 cm gap width BioRad). This mixture was held on ice for 15 min. The electroporation setting conditions were a capacitance of 25 µF, the pulse control set to ∞, and no shunt resistor. The voltage was set to 2000 V cm⁻¹ and the elapsed time was between 10 and 13 ms. A BioRad electroporator Gene Pulser II was used. After electroporation, the cells were allowed to recover overnight in 10 ml of TAP supplemented with 60 mM sucrose in 30 µM photons. Approximately 16–18 hours later, the cells were harvested and

resuspended in 100 μ l of TAP. 6×10^7 cells were plated onto TAP + paromomycin ($5\text{--}15 \mu\text{g ml}^{-1}$) plates. The cells were then allowed to grow in low-light conditions.

Cloning of the RNAi construct

Standard molecular biological techniques were utilized to clone the genomic DNA and the cDNA of *Ccp1* into the pSL72 plasmid (Figure 1). pSL72 is a pBS vector where the *psaD* promoter is cloned between *KpnI* and *XhoI*, and the *psaD* terminator between *XbaI* and *NotI*. This promoter drives the expression of the *AphVIII* coding sequence cloned between *NdeI* and *EcoRV*. The genomic DNA fragment was ligated to the inverted cDNA fragment at a *ScaI* site common to both fragments. This fragment was cloned into the *EcoRV*, alkaline phosphatase treated pSL72. The genomic DNA fragment contains exons and introns from exons 2 to exon 6. The cDNA fragment contains cDNA equivalent to the exons 2–6 in the genomic DNA fragment above.

Photosynthesis assays

Cultures were grown mixotrophically in 100 ml of TAP media on a shaker set at 180 rpm in moderate light. After 48 h, the cultures were transferred to minimal medium without a carbon source and bubbled with 5% CO_2 in air for 24 h. The cultures were then acclimated to low- CO_2 by bubbling with air for 16–20 hours. All conditions were carried out under continuous light ($80 \mu\text{E m}^2 \text{s}^{-1}$).

The affinity of the wild-type and RNAi strains for external inorganic carbon was estimated as described by Pollock and Colman (2001). The

photosynthetic rate of algal cells was measured with an oxygen electrode (Rank Brothers, Cambridge, UK). For the *rcal* related experiments, cells were transferred to the electrode chamber, where they were allowed to exhaust the medium (50 mM Na/K- PO_4 , pH 7.8) of endogenous DIC until net O_2 exchange was zero. For the pH-related experiments, the biological buffers MES-KOH, MOPS-KOH, and HEPES-KOH were used for pH 6.3, 7.3, and 8.3, respectively, were used at a concentration of 25 mM.

Results

Ccp1 and *Ccp2* RNAi strains of *C. reinhardtii* require an environment that is enriched in CO_2 to grow optimally

Figure 1 shows the RNAi construct for *Ccp1*. The pSL72 vector uses the *PsaD* promoter and terminator to express the *AphVIII* paromomycin resistance gene. An inverted repeat of the genomic region and cDNA of *Ccp1* containing exons 2–6 was cloned into the vector pSL72 between the *AphVIII* gene and the *PsaD* terminator (Figure 1). The strain D66 was transformed with *StuI* linearized pSL72–RNAi construct and 500 transformants were selected in media containing the drug paromomycin. The 500 transformants were then replica plated onto minimal media in high- CO_2 and low- CO_2 . Twenty-seven of the 500 (5.4%) paromomycin transformants grew slower than the parent D66 in a low- CO_2 environment, but grew normally in a high- CO_2 environment (Figure 2). The high- CO_2 requiring phenotypes were strongest within 2 months of generating the RNAi strains. The high- CO_2 growth requirement became less severe over a period of months, but this did not correlate with an increase in abundance of the Ccp proteins (data not shown).

Ccp1 and *Ccp2* RNAi strains of *C. reinhardtii* have dramatically decreased levels of Ccp mRNA and protein

From the pool of 27 transformants that grew slower in a low- CO_2 environment, three transformants were selected for further analysis. Two additional paromomycin resistant transformants that were healthy in a low- CO_2 environment were also

Figure 1. The *Ccp1*-RNAi construct. The inverted repeat was constructed by ligating a genomic DNA fragment (gDNA) to a complementary cDNA fragment in the opposite direction. The two fragments contain exons 2–5 and the first 35 bps of exon 6. The inverted repeat was cloned into the vector pSL72 between the *AphVIII* paromomycin resistance gene and the *PsaD* terminator.

Figure 2. The secondary screening of the 27 paromomycin resistant transformants that grew slower than the parent D66 in a low-CO₂ environment. The strains were grown in four environments. A: TAP, B: TAP plus paromomycin, C: minimal medium in 35 ppm CO₂, and D: minimal medium in high-CO₂. The two CCM mutants, *cia3* and *cia5*, are shown as examples of high-CO₂ requiring strains. The three RNAi strains, *i22*, *i24*, and *i26* were chosen for further analyses.

Figure 3. Northern blots measuring the abundance of the *Lci1*, *Ccp1*, and *Ccp2* mRNA, and Western blot to measure the abundance of the Ccp protein for the parent (D66), the mutant *cia5*, the three high-CO₂ requiring RNAi strains (*i22*, *i24*, and *i26*), and two of the healthy RNAi strains (*i10* and *i39*). All strains were adapted to a liquid low-CO₂ environment for 16–18 h.

selected. Figure 3 shows the abundance of the *Lci1*, *Ccp1*, and *Ccp2* mRNA and the Ccp protein for the parent (D66), the mutant *cia5*, the three sick RNAi

strains (*i22*, *i24*, and *i26*), and two of the healthy RNAi strains (*i10* and *i39*). Except for *cia5*, all of the analyzed strains expressed the low-CO₂ inducible gene *Lci1*. The *Ccp1* and *Ccp2* messages were absent in all three high-CO₂ requiring RNAi transformants and *cia5*, whereas reduced levels were present in the *i10* and *i39* transformants. Western blot analysis using polyclonal antibodies raised against the Ccp protein from *Chlamydomonas* showed that the Ccp proteins were dramatically reduced in abundance in the high-CO₂ requiring strains and *cia5* as compared to the wild-type parent D66 (Figure 3). A higher molecular weight cross-reacting protein of unknown identity can also be seen on the Western blots.

RNAi silenced strains exhibit wild-type photosynthetic kinetics

Rates of photosynthesis as a function of the DIC concentration were measured for D66 and the

RNAi strains acclimated to air levels of CO₂ for 18 h. When the RNAi strains were assayed at pH 6.3, 7.3, and 8.3, no difference in photosynthetic kinetics as compared to D66 WT were observed (Figure 4). In addition, the photosynthetic kinetics of the RNAi strains were compared to a WT-like paromomycin resistant strain and no differences were observed (data not shown).

The *Ccp1* RNAi construct was also transformed into the *rcal* strain. *rcal* contains a bleomycin resistance insert in the gene encoding Rubisco activase (Pollock *et al.*, 2003). Since *rcal* already grows poorly on low-CO₂, if the *Ccp1* RNAi did result in a decrease in inorganic carbon uptake the decrease would be easier to measure. In fact *rcal* cells expressing the *Ccp1* RNAi construct exhibited a sick on low-CO₂ phenotype that was

more severe than either the *rcal* mutant or the *Ccp1* RNAi strains. The strains quickly became bleached and little growth was observable on agar media in low-CO₂. However, these *rcal* cells expressing the *Ccp1* RNAi construct exhibited no significant changes in the apparent affinities of the strains for DIC (Figure 5). Thus, the apparent affinity of the cells for DIC was not affected by the absence of the Ccp proteins.

Discussion

The Ccp proteins were initially recognized and named LIP36G1 and LIP36G2, as potential components of the CCM in *C. reinhardtii* 15 years ago (Spalding and Jeffrey, 1989). This

Figure 4. The effect of the dissolved inorganic carbon concentration on the rate of photosynthesis. A: air-grown D66 (●), and the three RNAi strains *i22* (○), *i24* (▼), and *i26* (▽) tested at pH 8.3. B: air-grown D66 (●) and *i22* (○) at pH 6.3, and D66 (▼) and *i22* (▽) at pH 7.3. The inset graphs show the rates of oxygen evolution for the low concentrations of DIC. The chlorophyll concentrations ranged from 40 to 50 $\mu\text{g ml}^{-1}$. The light intensity was approximately 800 $\mu\text{E m}^{-2} \text{ s}^{-1}$.

Figure 5. The effect of the dissolved inorganic carbon concentration on the rate of photosynthesis of air-grown *rca1* (●) and *rca1* that lacks the Ccp1 and Ccp2 proteins (○). The error bars represent the standard deviation of the mean of three independent transformants.

report presents the first evidence that Ccp1/2 are not required for optimal rates of photosynthesis but are required for growth in a low-CO₂ environment. The use of an RNAi construct (Figure 1) to target the degradation of *Ccp1/2* mRNA was successful as measured by mRNA and protein abundance (Figure 3). Since there are two genes to target, *Ccp1* and *Ccp2*, it is unlikely that a chemical or random-insertional mutagenesis strategy would lead to the silencing of both of the *Ccp* genes. On the other hand, RNAi can be utilized to target similar transcripts, as is the case in gene families, and is the case with the almost identical open reading frames of *Ccp1* and *Ccp2*.

In the three analyzed RNAi-strains that showed efficient silencing of *Ccp1/2*, the low-CO₂ inducible transcript of *Lci1* was fully induced as compared to the wild-type parent D66, suggesting that the expression of other low-CO₂ inducible genes is unaffected by the absence of *Ccp* mRNA. These results suggest that the RNAi silencing mechanism is specifically affecting the transcript abundance of *Ccp1* and *Ccp2*. The absence of mRNA for *Ccp1* and *Ccp2* was mirrored by the reduction in the Ccp proteins detected using antibody raised against Ccp1, and the enhanced chemiluminescence detection method (Figure 3). Thus, the RNAi strategy was successful in targeting the *Ccp1/2* mRNA to effectively reduce the abundance of Ccp1/2 protein to undetectable levels.

Surprisingly, RNAi silenced strains exhibited normal rates of photosynthesis at all tested concentrations of DIC, and over a range of pHs (Figures 4 and 5). This was not expected because the RNAi strains exhibited slower growth under low CO₂ on solid media than wild-type cells (Figure 2). On the other hand, the RNAi strains grew at a rate similar to the wild-type parent in liquid cultures over a short time-course of 48 h in minimal media (data not shown). It is likely that the extremely low concentration of CO₂ used during screening on solid media (35 ppm CO₂ in air) was required to produce a long-term growth phenotype in the RNAi strains, whereas air-adapted liquid cultures were only maintained for a maximum of 72 h since liquid cultures of *C. reinhardtii* reach stationary phase within a few days depleting the media of essential nutrients and limiting the effective light intensity.

In an attempt to magnify any potential CCM mutant phenotype of the RNAi strains, the RNAi vector was transformed into the *C. reinhardtii* Rubisco activase (*rca1*) mutant. In previous work (Pollock *et al.*, 2003), the *rca1* mutant strain was shown to require a high-CO₂ environment to photosynthesize optimally. Of particular utility to this report, in the *rca1 cia5* double mutant, where the CCM is not functional, cells were incapable of performing photosynthesis at low concentrations of DIC. Thus, by expressing the *Ccp1* RNAi construct in the *rca1* mutant background, any disruption in the active accumulation of DIC should be readily apparent. No significant changes in the apparent affinities of the strains for DIC were observed (Figure 5). Therefore, Ccp1 and Ccp2 are required for growth in a low-CO₂ environment, but not for the efficient operation of the CCM.

Are the Ccp proteins an integral component of DIC transport into the chloroplast of C. reinhardtii?

By modifying the abundance of Ccp1 and Ccp2 several high-CO₂ requiring strains of *C. reinhardtii* were generated (Figure 2). In cyanobacteria, four distinct uptake systems are required for efficient uptake of DIC (Omata *et al.*, 2002; Shibata *et al.*, 2002). When one of the cyanobacterial DIC uptake systems is mutated, the remaining three systems compensate for its loss and no mutant phenotype is observed. It is likely that *C. rein-*

hardtii strains that lack the Ccp proteins adjust the global expression of several genes to compensate for the absence of the Ccp proteins. One possible explanation for these results is that the Ccps are involved in DIC uptake across the chloroplast envelope but additional yet undiscovered components of DIC uptake systems are present in *C. reinhardtii* that compensate for the loss of the Ccps in the chloroplast envelope. This may be the reason why several laboratories have not reported on high-CO₂ requiring strains of *C. reinhardtii* that have a mutation affecting a single component involved in the active uptake of DIC. Interestingly, one CCM mutant characterized in *C. reinhardtii* has a defect in the regulatory protein Cia5. *cia5* mutants grow slowly in a low-CO₂ environment and are unable to induce, or depress, the CCM in response to changes in CO₂ concentration. Since several dozen genes are under the control of Cia5, it is easy to speculate that a mutation in a single component of the DIC uptake mechanism will not lead to a CCM mutant phenotype in liquid culture, as is the case with the two Ccp proteins.

A second possible explanation for the growth characteristics of the Ccp1/2 RNAi strain is that the Ccp peptides are not DIC transporters but are required for some other aspect of growth in a low-CO₂ environment, for example as a transporter of metabolic intermediates involved in photorespiration. A third hypothesis is that Ccp1 and Ccp2 encode proteins similar to the *bou* protein described in *A. thaliana*. If this were the case, Ccp RNAi strains may be deficient in some general transport of metabolic intermediates such as acetate between the chloroplast and the cytosol. Any of these hypotheses could explain why long-term growth on solid media in a low-CO₂ environment leads to a slow-growth phenotype in the RNAi strains.

By silencing the expression of the Ccp proteins, this work provides evidence that Ccp is required for normal growth in a low-CO₂ environment, but not absolutely required for efficient photosynthesis at low concentrations of CO₂. Further biochemical analyses will be required to determine the role of the Ccps in a low-CO₂ environment in *C. reinhardtii*.

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