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Research Article

# Mitochondrial carbonic anhydrases are needed for optimal photosynthesis at low $CO_2$ levels in *Chlamydomonas*

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is James Moroney (btmoro@lsu.edu).

## Abstract

*Chlamydomonas reinhardtii* can grow photosynthetically using $CO_2$ or in the dark using acetate as the carbon source. In the light in air, the $CO_2$ concentrating mechanism (CCM) *of C. reinhardtii* accumulates $CO_2$, enhancing photosynthesis. A combination of carbonic anhydrases (CAs) and bicarbonate transporters in the CCM of *C. reinhardtii* increases the $CO_2$ concentration at Ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) in the chloroplast pyrenoid. Previously, CAs important to the CCM have been found in the periplasmic space, surrounding the pyrenoid and inside the thylakoid lumen. Two almost identical mitochondrial CAs, CAH4 and CAH5, are also highly expressed when the CCM is made, but their role in the CCM is not understood. Here, we adopted an RNAi approach to reduce the expression of CAH4 and CAH5 to study their possible physiological functions. RNAi mutants with low expression of CAH4 and CAH5 had impaired rates of photosynthesis under ambient levels of $CO_2$ (0.04% $CO_2$ [v/v] in air). These strains were not able to grow at very low $CO_2$ (<0.02% $CO_2$ [v/v] in air), and their ability to accumulate inorganic carbon ($C_i = CO_2 + HCO_3^-$) was reduced. At low $CO_2$ concentrations, the CCM is needed to both deliver $C_i$ to Rubisco and to minimize the leak of $CO_2$ generated by respiration and photorespiration. We hypothesize that CAH4 and CAH5 in the mitochondria convert the $CO_2$ released from respiration and photorespiration as well as the $CO_2$ leaked from the chloroplast to $HCO_3^-$ thus "recapturing" this potentially lost $CO_2$.

## Introduction

Aquatic photosynthetic organisms face several challenges obtaining $CO_2$ from the environment including slow diffusion of gases in water, pH fluctuations, and the slow interconversion of inorganic carbon ($C_i$) forms. Aquatic photosynthetic organisms have adapted to these changing conditions by developing a carbon dioxide concentrating mechanism (CCM). In *Chlamydomonas reinhardtii* (referred to as *Chlamydomonas* hereafter), the CCM occurs only when it is grown in a low $CO_2$ environment (Moroney and Somanchi, 1999; Spalding, 2008). The CCM increases the $CO_2$ around Ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) enhancing its carboxylase activity (Moroney and Ynalvez, 2007). Oxygen competes with $CO_2$ for the active site of Rubisco, hence the CCM favors the carboxylase activity of Rubisco at the same time as reducing the

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oxygenation process. In low $CO_2$ conditions, Rubisco is packaged inside a structure called the pyrenoid, and is surrounded by a starch sheath (Ramazanov et al., 1994; Freeman Rosenzweig et al., 2017; Itakura et al., 2019). *Chlamydomonas* acclimates to low $CO_2$ by maintaining a CCM, which includes proteins that aid in the delivery of $C_i$ ($C_i = CO_2 + HCO_3^-$) to Rubisco in the pyrenoid.

Two major components of the CCM are $C_i$ transporters and carbonic anhydrases (CAs). The known $C_i$ transporters are low $CO_2$-induced 1 (LCI1), high-light activated 3 located on the plasma membrane, LCI A (LCIA) on the chloroplast envelope, and three bestrophin-like proteins (BST1, BST2, and BST3) on the thylakoid membrane (Ohnishi et al., 2010; Yamano et al., 2015; Mukherjee et al., 2019; Kono et al., 2020). These transporters increase the bicarbonate concentration inside the chloroplast relative to the external $HCO_3^-$ concentration. *Chlamydomonas* has several CAs and some of these are important in the functioning of the CCM (Moroney et al., 2011). One of them is CA 3 (CAH3), an α-type CA located inside the thylakoid lumen (Karlsson et al., 1998). CAH3 is essential for maintaining high $CO_2$ inside the chloroplast by converting $HCO_3^-$ to $CO_2$ in the acidic thylakoid lumen (Moroney and Ynalvez, 2007; Spalding, 2008). CAs 1 and 2 (CAH1 and CAH2) are two other α-type CAs that are present in the periplasmic space (Fujiwara et al., 1990; Rawat and Moroney, 1991). CAH1 is highly upregulated when *Chlamydomonas* is grown in low $CO_2$, as are all the $C_i$ transporters. In addition, the expression of CAH1 and the $C_i$ transporters require the transcription activator Ci accumulation deficient 5 (CIA5), as cells with mutations in *CIA5* fail to express these proteins. The CCM components are under regulation by CIA5 and they show high expression in low $CO_2$ conditions (Moroney et al., 1989; Fang et al., 2012).

CAs 4 and 5 (CAH4 and CAH5) were the first β-type CAs to be discovered in *Chlamydomonas* and they are present in mitochondria (Eriksson et al., 1995). CAH4 and CAH5 are almost identical, and the genes encoding these proteins are present as an inverted repeat on chromosome 5 (Eriksson et al., 1996). Since CAH4 and CAH5 are nearly identical, they will be referred to a CAH4/5 for the remainder of this manuscript. *CAH4/5* are among the most highly upregulated genes when *Chlamydomonas* is grown in low $CO_2$ conditions (Fang et al., 2012). An immunogold labeling experiment confirmed that CAH4/5 are present in mitochondria in cells grown under low $CO_2$ conditions and are undetectable in high $CO_2$ conditions (Moroney et al., 2011). The expression pattern of CAH4/5 strongly resembles that of a CCM component, which is surprising since they are in the mitochondria.

The physiological role of CAH4/5 is still not clear although a number of hypotheses have been proposed about their function in *Chlamydomonas*. One suggested role of CAH4/5 is to maintain the pH of the mitochondrial matrix by generating $H^+$ ions during hydration of $CO_2$. These $H^+$ ions would balance the pH change caused by the production of $NH_3$ by glycine decarboxylation (Eriksson et al., 1998). Another idea by Raven (2001) proposes CAH4/5 is needed to retain $CO_2$ generated by the mitochondria. In another study, CAH4/5 was hypothesized to be required for anaplerotic reactions (Giordano et al., 2003). Giordano et al. (2003) showed that the levels of CAH4/5 decrease with increasing ammonium concentration in growth media as $HCO_3^-$ produced by CAH4/5 is used by PEP carboxylase for $NH_4^+$ assimilation. However, until now, no mutant for CAH4/5 protein has been reported, so there is little experimental evidence testing these hypotheses.

In this report, we sought to elucidate the function of CAH4/5 in *Chlamydomonas* by using an RNAi strategy where we screened for mutants exhibiting low mRNA and protein expression levels of CAH4/5. Here, we describe the growth and physiological properties of *cah4/5* RNAi mutants under a variety of autotrophic conditions. We found that wild-type (WT) levels of expression of CAH4/5 are required to maintain optimal rates of photoautotrophic growth on ambient levels of $CO_2$.

## Results

### CAH4/5 expression is strongly affected by $CO_2$ levels and is under the control of CIA5

CAH4/5 expression was measured in photoautotrophically grown cells using a CAH4/5 specific antibody (Figure 1). In the light, CAH4/5 was nearly absent in cells maintained on high $CO_2$ (5% $CO_2$ [v/v] in air) conditions but was strongly expressed when cells were transferred to ambient $CO_2$ (0.04% $CO_2$ [v/v] in air; Figure 1). The results agree with early reports by Eriksson et al. (1998) and Giordano et al. (2003). In addition, CAH4/5 is under the control of CIA5 (Figure 2). CAH4/5 expression was not visible in *cia5* cells in light conditions at ambient $CO_2$ or high $CO_2$ concentrations (Figure 2). Thus, the expression of *CAH4/5* closely matches the expression of proteins involved in the CCM.

### Identification of *cah4/5* RNAi lines with reduced levels of CAH4/5

CAH4 and CAH5 proteins differ by only one amino acid (Supplemental Figure S1; Eriksson et al. 1995), so they likely have similar functions in vivo. Therefore, the expression of both genes was reduced to help elucidate their physiological functions. To this end, an RNAi construct that targets a common region of the CAH4 and CAH5 genes was designed (Supplemental Table S1). This construct was used to transform strain D66 (the WT or WT strain), and transformants were selected on paromomycin and kept at high $CO_2$ levels. The resultant transformants were then screened by growing them on both high and very low $CO_2$ (<0.02% $CO_2$ [v/v] in air). Colonies that grew well on high $CO_2$ but poorly on very low $CO_2$ were selected, and their expression of CAH4/5 was examined. Two independent colonies were selected that showed reduced expression of *CAH4/5* transcript and

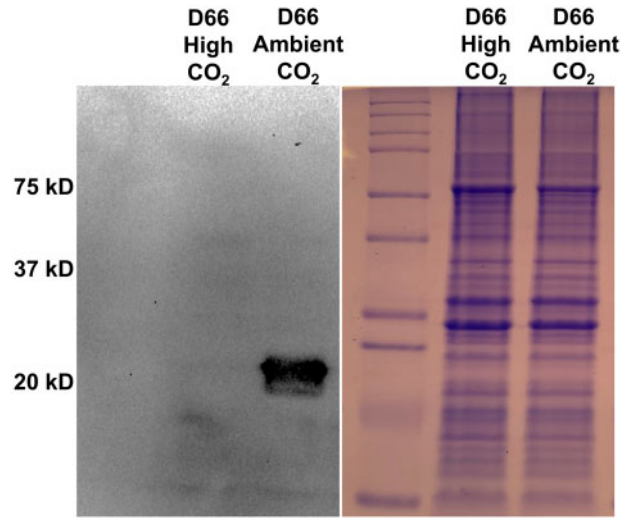


**Figure 1** CAH4/5 protein levels are strongly affected by $CO_2$ levels. The left panel is an immunoblot probed with anti-CAH4 antibodies showing the CAH4/5 levels in D66 grown on minimal media in light at high $CO_2$ or ambient $CO_2$. Cells were grown in MIN media for 72 h in high $CO_2$ conditions before incubating them under the respective $CO_2$ conditions. The right panel is an SDS–PAGE gel of the same samples stained with Coomassie Blue.

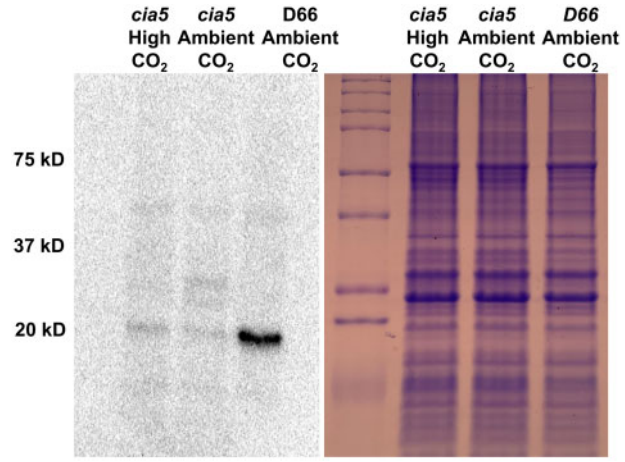


**Figure 2** CAH4/5 protein expression is under the control of CIA5. The left panel is an immunoblot probed with anti-CAH4 antibodies showing CAH4/5 levels in *cia5* grown on MIN media in light at high $CO_2$ or ambient $CO_2$ compared to D66 grown at ambient $CO_2$. Cells were grown in MIN media for 72 h in high $CO_2$ conditions before incubating them under the respective $CO_2$ conditions. The right panel is an SDS–PAGE gel of the same samples stained with Coomassie Blue.

protein. These strains were designated as *cah4/5-1* and *cah4/5-2*. Figure 3A shows the *CAH4/5* mRNA levels in the two *cah4/5* RNAi strains as well as WT cells after cells were switched from 5% $CO_2$ in air to ambient $CO_2$ levels for 12 h. Both RNAi strains had less *CAH4/5* mRNA than WT cells but *cah4/5-2* consistently had less mRNA expression than *cah4/5-1* (Figure 3A). Immunoblot analysis using polyclonal antibodies raised against the CAH4/5 protein from *Chlamydomonas* showed a corresponding decrease in CAH4/5 proteins as compared to the WT D66 (Figure 3B) with both strains having less than 20% of the WT amount of CAH4/5. In WT cells, the CAH4/5 protein is highly expressed when cells are grown in ambient $CO_2$ conditions but this expression is reduced drastically in high $CO_2$ conditions (Figure 3B, lanes 1 and 2). Neither RNAi strain had elevated levels of CAH4/5 even when grown in ambient $CO_2$. In agreement with the mRNA results, the amount of CAH4/5 protein was reduced in both RNAi lines (Figure 3B).

## Growth analysis of CAH4/5 RNAi mutants

The growth of *cah4/5-1* and *cah4/5-2* was tested on high, ambient, and very low $CO_2$ at different pH levels. For this experiment, the strains *cia3* and *cia5* were used as controls. The first control, *cia3*, is a knockout of *CAH3*, which is responsible for encoding the thylakoid lumen CA. The second control, *cia5*, is a knockout of gene *CIA5*, which is a transcription factor controlling the expression of many CCM genes (Moroney et al., 1989; Fukuzawa et al., 2001; Xiang et al., 2001). *CAH4/5* mutants showed reduced growth at all three pH levels tested (7.2, 7.8, 8.4) in ambient and very low $CO_2$ conditions (Figure 4A). The poor growth phenotype was more severe at pH 7.8 and 8.4 and worse under very low $CO_2$ conditions (Figure 4A). However, at high $CO_2$, the growth of the *cah4/5* RNAi mutants was similar to the WT at all pH. Therefore, the *cah4/5* RNAi lines show a classic CCM phenotype, growing well on high $CO_2$ but poorly under ambient or very low $CO_2$ concentrations. In addition, growth was worse at high pH where most of the $C_i$ is in the form of $HCO_3^-$. Therefore, CAH4/5 is required for optimal photoautotrophic growth of *Chlamydomonas* under ambient or very low $CO_2$ conditions.

To confirm the reduction of CAH4/5 is the reason for the phenotype, a reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiment was performed to determine whether the expression of other CCM genes was affected by the reduction in the CAH4/5 transcript. All the CCM genes in the RNAi strains were expressed at levels equal to or slightly greater than seen in WT cells (Supplemental Figure S2). As an additional confirmation that the reduction in the CAH4/5 transcripts was the cause of the poor growth phenotype, CAH5 was transformed back into the *cah4/5-2* RNAi line. The complementation of *cah4/5-2* was achieved by expressing CAH5 CDS (804 bp) without including 5′ UTR and 3′ UTR in the invitrogen pChlamy _4 vector under control of Hsp70A-Rbc S2 hybrid promoter. Results of growth experiments show that the WT phenotype in low $CO_2$ conditions was restored in complemented lines (referred to as com1, com2, and com 3; Supplemental Figure S3).

## Reduction in CAH4/5 expression decreases the cells' affinity for $C_i$

The ability of algal cells to accumulate $C_i$ increases when the CCM is induced and cells with an active CCM have very high affinities for $C_i$. The CCM allows cells to accumulate $C_i$ to higher levels than that can be attained by diffusion. Both *cah4/5-1* and *cah4/5-2* show a severe reduction in $C_i$ affinity

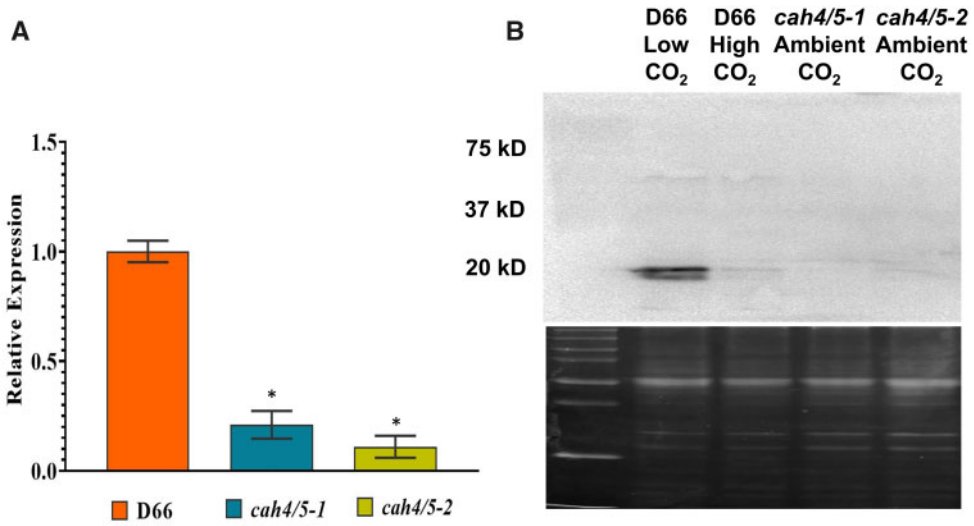


**Figure 3** Relative mRNA and protein expression of CAH4/5 in D66 and the knockdown mutants. A, RT-qPCR shows expression of CAH4/5 genes in knockdown lines *cah4/5-1* and *cah4/5-2* and in D66. Cells were grown in MIN media for 48 h in high $CO_2$ conditions before transferring them to ambient $CO_2$ for 12 h before harvesting for RNA. Error bars represent standard deviation from three biological replicates. Transcript levels were calculated using $2^{-\Delta\Delta CT}$ relative to the reference gene *CBLP* and reported relative to the corresponding WT D66 cells. The asterisk indicates the value is significantly different from the control (*$P < 0.05$ by Student's *t* test). B, Western blot showing protein levels of CAH4/5 in knockdown lines *cah4/5-1* and *cah4/5-2* and D66 grown in ambient $CO_2$ (0.04% $CO_2$ (v/v) in air. Cells were initially grown in MIN media in the light for 48 h in high $CO_2$ conditions before incubating them for 12 h at ambient $CO_2$. The top panel is an immunoblot using an antibody raised against CAH4; the bottom panel is SDS–PAGE of the samples stained with Coomassie Blue.

at pH 7.8 and 8.4 when grown under low $CO_2$ conditions (Figure 5). The higher $K_{0.5}(C_i)$ observed in the mutants indicates they have a lower $C_i$ affinity as compared to the WT, D66. In addition, the reduced affinity for $C_i$, as indicated by the higher $K_{0.5}(C_i)$, becomes more severe at higher pH as shown in Figure 5, B and C.

At pH 8.4, the $K_{0.5}(C_i)$ for *cah4/5-1* is 300 μM, and *cah4/5-2* is 225 μM, as compared to a $K_{0.5}(C_i)$ of 75 μM in D66. Similarly, the $K_{0.5}(C_i)$ of *cah4/5-1*, *cah4/5-2* and D66 at pH 7.8 are 150, 98, and 55 μM, respectively. These data, like the growth data, show that the *cah4/5* RNAi strains have a reduced ability to use $C_i$ especially at higher pH.

## RNAi silenced strains exhibit reduced $C_i$ accumulation

$C_i$ accumulation was measured in WT (D66) and the *cah4/5* RNAi strains at low $C_i$ conditions to assess the importance of CAH4 and CAH5 in accumulation and fixation of $C_i$. At pH 7.8, there is a substanstial decrease in $C_i$ fixation and $C_i$ accumulation for the *cah4/5* mutants as compared to D66 (Figure 6, A and B). $C_i$ accumulation and fixation is further reduced in both the RNAi lines when pH is increased from 7.8 to 8.4 (Figure 7, A and B). The difference in accumulation was visible from earliest time point, that is, 15 s up to and including 90 s. In both mutants, $C_i$ accumulation is only 30%–40% of the levels observed in WT D66 cells. This clearly indicates that CAH4 and CAH5 in the mitochondria play an important role in $C_i$ accumulation and retention in ambient $CO_2$ conditions in *Chlamydomonas*.

## Mitochondria and CAH4/5 are located to the cell periphery in cells growing under low $CO_2$ conditions

When observed using transmission electron microscopy (TEM) the number and localization of *Chlamydomonas* mitochondria varies with the cells growth conditions (Geraghty and Spalding, 1996). When grown in high $CO_2$, D66 cells had fewer mitochondria as compared to D66 cells grown in ambient $CO_2$ (Figure 8, A and B). However, the cross sections of the mitochondria seen in cells growing at high $CO_2$ were larger than those observed in cells grown under ambient $CO_2$ (Figure 8B). In addition, the distribution of the mitochondria was different. In cells grown in high $CO_2$, the mitochondria were distributed throughout the cells (Figure 8A). However, in cells grown under ambient $CO_2$, the mitochondria can be clearly seen distributed near plasma membrane (Figure 8B). These observations are similar to those reported by Geraghty and Spalding (1996). This observation can also be seen in Supplemental Figure S4, where TEM images of multiple cells are shown. The mitochondrial location was confirmed using cells containing CAH5 tagged with Venus. In those cells expressing the CAH5-venus protein, in high $CO_2$ grown cells CAH5 is localized mostly in the center of the cell, whereas in all cells acclimated to ambient $CO_2$, CAH5 was localized to the periphery of the cell (Figure 9; Supplemental Figure S5).

## Discussion

CAH4 and CAH5 were initially discovered in *Chlamydomonas* in 1996 (Eriksson et al, 1996). The genes encoding CAH4 and CAH5 are arranged as an inverted

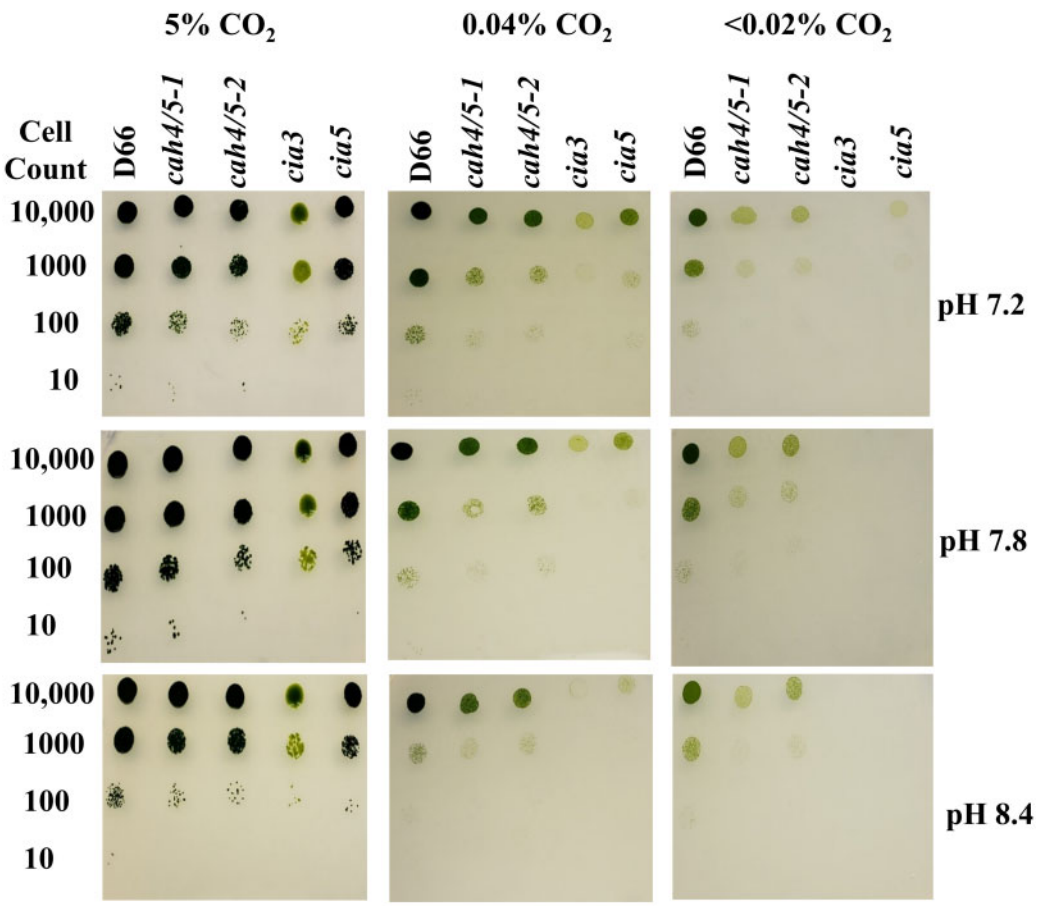


**Figure 4** Growth of mutants *cah4/5-1* and *cah4/5-2* at different $CO_2$ levels. Growth analysis showing D66, *cah4/5-1*, *cah4/5-2*, *cia3*, and *cia5*. Cells were diluted to $6.6 \times 10^6$ cells $mL^{-1}$, followed by 1:10 serial dilution three times at very low $CO_2$, ambient $CO_2$, and high $CO_2$ at pH 7.2, pH 7.8, and pH 8.4, respectively. Cells were grown for 6 d. The *cia3* and cia5 mutants were included as a CCM-deficient control. Cells were initially grown in TAP media at ambient $CO_2$ in the light before spotting them onto plates.

repeat on chromosome 5 and are controlled by a single promoter (Eriksson et al., 1996). The expression of CAH4/5 increases dramatically when cells are shifted from 5% $CO_2$ conditions to ambient levels of $CO_2$ or lower in the light (Eriksson et al., 1998; Giordano et al., 2003; Fang et al., 2012). Immunogold labeling and Venus tagging experiments both show that CAH4/5 is localized to the mitochondrial matrix (Geraghty and Spalding, 1996; Moroney et al., 2011; Tirumani et al., 2014; Mackinder et al., 2017). This report presents evidence that CAH4 and CAH5 are required for autotrophic growth in the light when the $CO_2$ level is low. Since *CAH4* and *CAH5* have almost identical open reading frames, we used an RNAi approach to target both genes simultaneously. A similar approach was recently used to reduce the expression of three similar bestrophin genes in *Chlamydomonas* (Mukherjee et al., 2019). The RNAi construct successfully reduced the amount of *CAH4/5* mRNA (Figure 3A). The reduction of *CAH4/5* mRNA expression in the RNAi mutants was mirrored by a similar reduction in protein abundance as estimated by immunoblots (Figure 3B). The expression of other CCM genes was also tested and they were expressed in the RNAi strains at levels equal to or slightly higher than in the WT D66 (Supplemental Figure S2). In complementation experiments, the expression of the CAH5 coding region from the WT gene was able to restore the normal growth phenotype (Supplemental Figure S3). These results suggest that the RNAi construct is specifically affecting the transcript abundance of CAH4 and CAH5.

The *cah4/5* RNAi silenced strains exhibited reduced rates of photosynthesis at low concentrations of dissolved $C_i$ (DIC), and over a range of pH levels (Figure 5). The RNAi strains also exhibited slower growth under ambient $CO_2$ and very low $CO_2$ when compared to WT cells (Figure 4). On the other hand, the RNAi strains grew at a rate similar to the WT parent in high $CO_2$ conditions (Figure 4). It is likely that at high $CO_2$ levels there is no need for a CCM in *C. reinhardtii* cells.

The slow growth observed in low $CO_2$ (400 ppm $CO_2$ or lower; Figure 4) is likely due to the reduced ability of the RNAi mutants to accumulate and retain $C_i$. The RNAi lines had a lower apparent affinity for $C_i$ (Figure 5) and a reduced ability to accumulate added $^{14}C_i$ (Figures 6 and 7). Algae with a CCM must not only be able to accumulate $C_i$ for photosynthesis, but they also must reduce $CO_2$ leakage. An important aspect of preventing the leakage is the $pK_a$ of the interconversion of $HCO_3^-$ and $CO_2$, which is about 6.4. $CO_2$

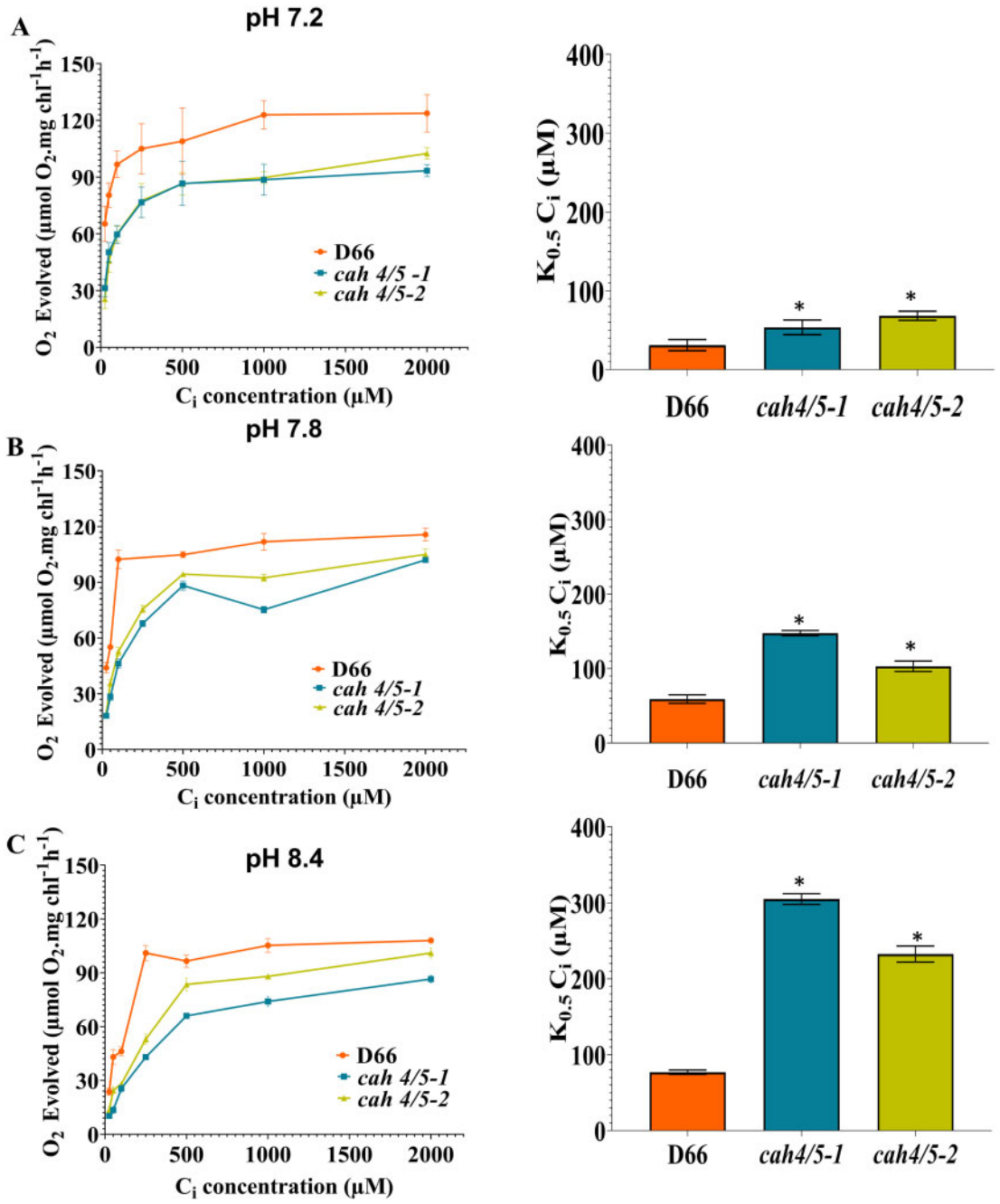


**Figure 5** Photosynthetic oxygen evolution of the *cah4/5* knockdown RNAi lines and D66. $C_i$ affinity and $K_{0.5}(C_i)$ were estimated for *cah4/5-1*, *cah4/5-2*, and D66 acclimated to ambient $CO_2$ for 12 h at (A) pH 7.2, (B) pH 7.8, and (C) pH 8.4. $K_{0.5}$ ($C_i$) values ($C_i$ concentration needed for half maximum oxygen evolution) were calculated from the $O_2$ evolution versus $C_i$ curves. Asterisk indicates the value is significantly different from the control ($^*P < 0.05$ by Student's *t* test). Cells were grown in MIN media for 48 h in high $CO_2$ conditions before incubating them for 12 h at ambient $CO_2$ at the indicated pH. Each point in $O_2$ evolution versus $C_i$ curves represents the mean and SD of three technical replicates from a representative experiment. Error bars in $K_{0.5}$ ($C_i$) values indicate SD.

is thought to be able to readily cross cell membranes while $HCO_3^-$ is about 100-fold slower in crossing membranes (Gutknecht et al., 1977; Tolleter et al., 2017). The chloroplast stroma has a pH close to 8 in the light while the thylakoid lumen has a pH close to 5.5. The acidic environment inside the thylakoid lumen favors the conversion of $HCO_3^-$ to $CO_2$, a reaction catalyzed by the luminal CA, CAH3. This increases the $CO_2$ concentration around Rubisco, but the resulting high $CO_2$ concentration also leads to $CO_2$ leakage problems (Karlsson et al., 1998; Moroney and Ynalvez, 2007; Spalding, 2008). The $CO_2$ gradient from inside the pyrenoid to the outside of the cell could lead to a significant loss of $CO_2$ by diffusion (Raven, 2001). The LCIB/C complex surrounding the pyrenoid is an important component of *Chlamydomonas* CCM. It has been proposed that this complex may reduce $CO_2$ leakage from the pyrenoid by converting

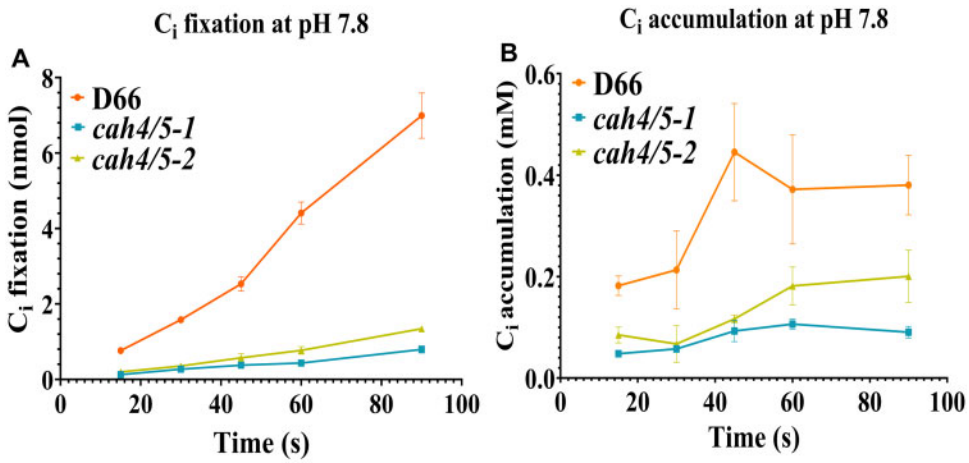


**Figure 6** $C_i$ uptake of D66 and *cah4/5* RNAi knockdown lines at pH 7.8. The silicone oil method was used to estimate $C_i$ fixation and $C_i$ accumulation (see "Materials and methods"). Cells were grown in elevated $CO_2$ and then acclimated to ambient $CO_2$ for 12 h prior to the assays. Cells were depleted of endogenous $C_i$ in a 6-mL chamber with a Clark electrode before performing the assays. Time courses of $CO_2$ fixation (A) and $C_i$ accumulation (B) are shown at pH 7.8. Each point represents the mean and SD of three technical replicates from a representative experiment.

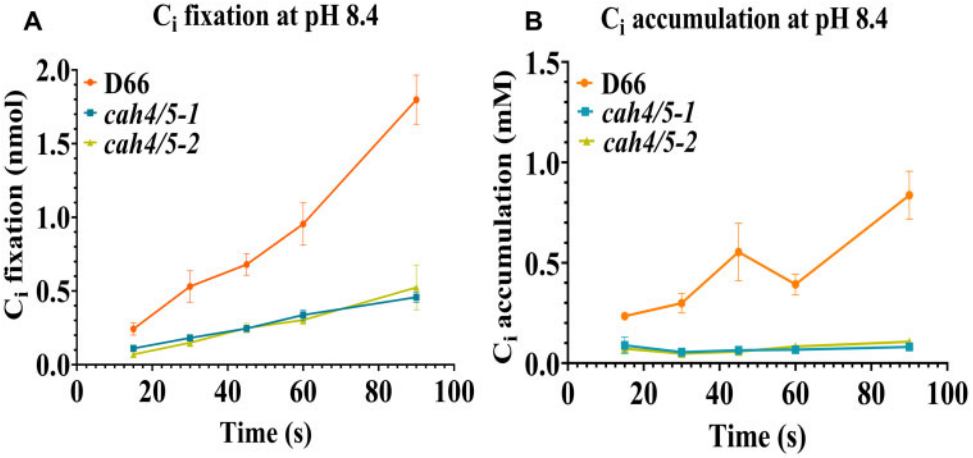


**Figure 7** $C_i$ uptake of D66 and *cah4/5* RNAi knockdown lines at pH 8.4. The silicone oil method was used to estimate $C_i$ fixation and $C_i$ accumulation (see "Materials and methods" section). Cells were grown in elevated $CO_2$ and then acclimated to ambient $CO_2$ for 12 h prior to the assays. Cells were depleted of endogenous $C_i$ in a 6-mL chamber with a Clark electrode before running the assays. Time courses of $CO_2$ fixation (A) and $C_i$ accumulation (B) are shown at pH 8.4. Each point represents the mean and SD of three technical replicates from a representative experiment.

that $CO_2$ to $HCO_3^-$ (Yamano et al., 2010; Wang et al., 2015; Mackinder et al., 2017). By converting the $CO_2$ to $HCO_3^-$, a reaction favored by the stromal pH, the loss of $C_i$ is less severe since $HCO_3^-$ crosses membranes more slowly.

We propose that CAH4 and CAH5 can reduce $CO_2$ leakage from the cell in two ways. First, the mitochondria produce $CO_2$ through respiration and photorespiration. It has been estimated that mitochondrial respiration in the light is about 10% the rate of $CO_2$ fixation (Raven, 1984). In addition, even though the rate of photorespiration is reduced by the CCM, it has been measured to be between 2% and 5% of the rate of $CO_2$ assimilation (Moroney et al., 1986). The $CO_2$ produced by these processes has the potential to be lost from the cell, and cells acclimated to low $CO_2$ cannot afford to lose this carbon. We propose that CAH4/5 is produced at high levels when *Chlamydomonas* is grown in the light on low $CO_2$ to reduce $CO_2$ leakage. As shown in Figure 10, the $CO_2$ generated in the mitochondria matrix is converted to $HCO_3^-$ by CAH4/5, and using a possible bicarbonate transporter (Pollock et al., 2004) the $HCO_3^-$ is brought back to the cytosol. Since no CA has been found in the cytoplasm, the rate of conversion of this $HCO_3^-$ back to $CO_2$ in the cytosol should be minimal. Thus, the $HCO_3^-$ can be then transported back to the thylakoid lumen using bicarbonate transporters present on the chloroplast inner membrane and thylakoid membrane (Mukherjee et al., 2019; Figure 10). It is also likely that some of the bicarbonate generated in the mitochondria is used in the anaplerotic reactions (Giordano et al., 2003).

A second way CAH4/5 can help in the accumulation of $C_i$ is by helping LCIB/C in recapturing $CO_2$ leaking from the pyrenoid. The positioning of the mitochondria to the

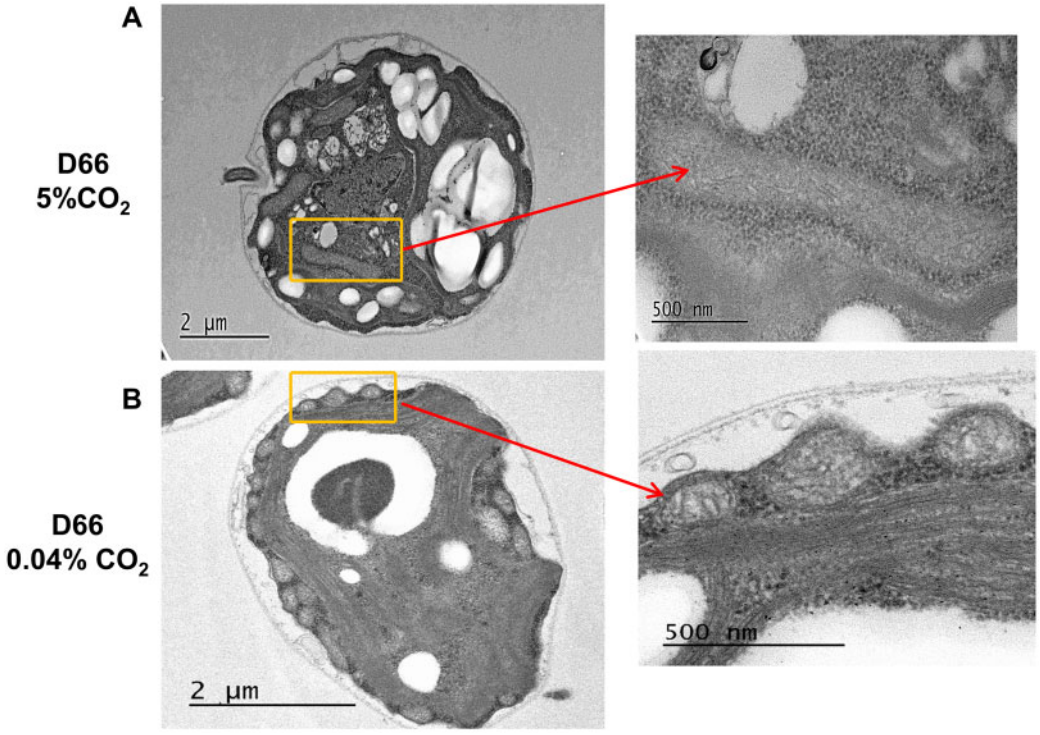


**Figure 8** Mitochondrial localization in WT D66 cells with change in $CO_2$ levels. TEM of sectioned *Chlamydomonas* WT cells at (A) high $CO_2$ and (B) ambient $CO_2$ levels. WT cells were grown in MIN media for 48 h in high $CO_2$ before incubating them for 12 h at their respective conditions. Areas shown by the rectangles are enlarged (right) to reveal mitochondrial structures. Scale bar, 2 µm (A), 2 µm (B), and 500 nm (enlargements).

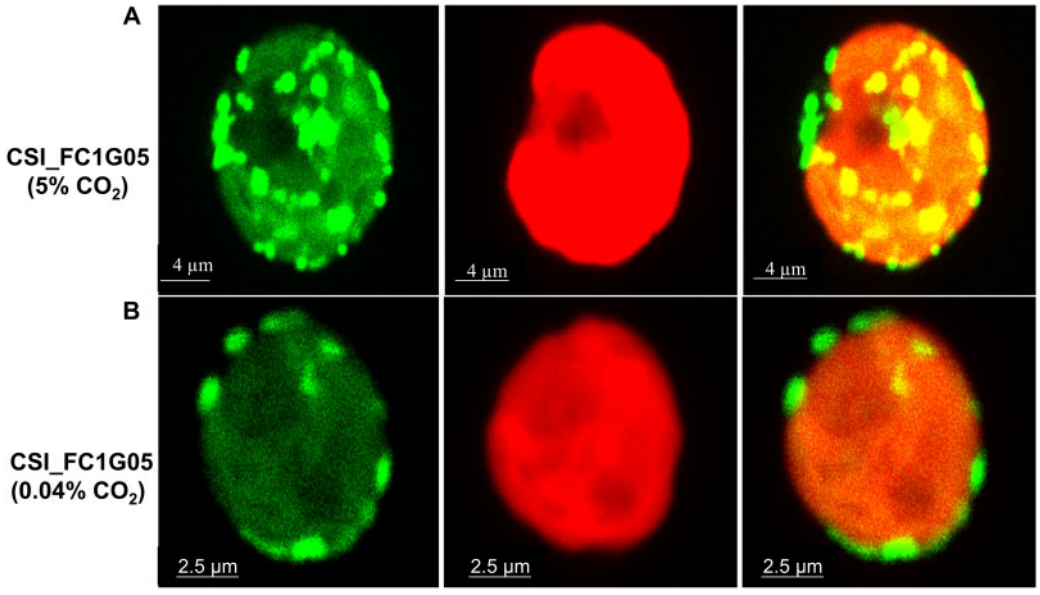


**Figure 9** CAH5 protein localization in WT cells with changes depending on the $CO_2$ levels during growth. Confocal images were taken of CSI_FC1G05 cells expressing pLM005-*CAH5*-Venus-3xFLAG at (A) high $CO_2$ and (B) ambient $CO_2$ levels. WT cells were grown in MIN media for 48 h in high $CO_2$ before incubating them for 12 h at their respective conditions. Scale bar, 4.2 µm (A), 2.5 µm (B).

periphery of the cell (Figures 8 and 9;Tirumani et al., 2014), allows CAH4/5 to become a second layer of CA $CO_2$ must cross before exiting the cell.

As suggested by Geraghty and Spalding (1996), the positioning of the mitochondria may also be important in intercepting glycolate coming from the chloroplast. *Chlamydomonas* cells grown under 5% $CO_2$ in air will excrete large amounts of glycolate into the medium (Hess and Tolbert, 1967) if placed in a low $CO_2$ environment in the light. They will continue to excrete glycolate until the CCM is induced in about 4 h. In addition to upregulating the expression of photorespiratory genes (Chen et al., 1996; Tural

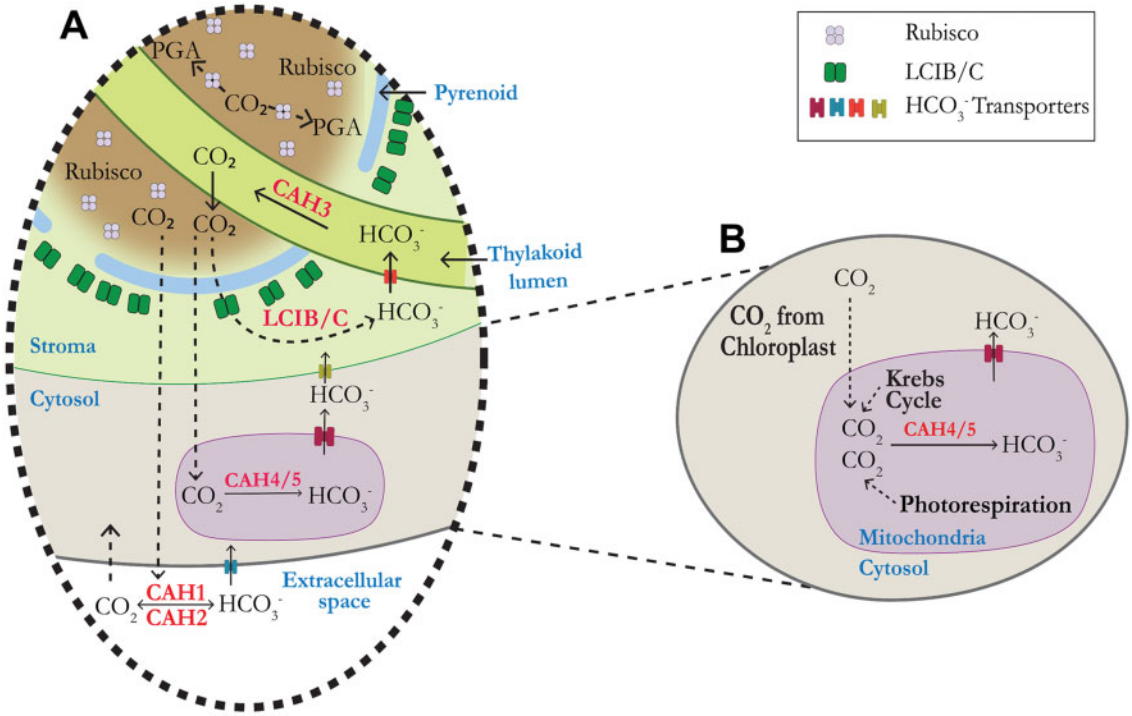


**Figure 10** Model showing the proposed physiological role of mitochondrial CAH4/5 in the CCM of *Chlamydomonas*. In (A) known CAs (CAH1, CAH2, CAH3, and LCIB) are indicated in the periplasmic space, the chloroplast stroma, and the thylakoid lumen, respectively. Dotted lines indicate the leakage of $CO_2$ from the pyrenoid and how it is recaptured by different layers of CAs. PGA, phosphoglyceric acid. B, The proposed recapturing of $CO_2$ in mitochondria. The $CO_2$ leakage arises from the chloroplast, mitochondrial respiration, and photorespiration.

and Moroney, 2005) the repositioning of the mitochondria would favor the recapture of glycolate with CAH4/5 converting the $CO_2$ released by glycine decarboxylase to $HCO_3^-$. Thus, the upregulation of CAH4/5 in conjunction with repositioning of mitochondria helps in retaining the $CO_2$ generated by the mitochondria as well as helps LCIB/C reduce $CO_2$ leakage from the chloroplast.

## Materials and methods

### Cell culture and growth

*Chlamydomonas reinhardtii* culture conditions were similar to those described previously (Ma et al., 2011). The D66 strain (*nit2*$^-$, *cw15*, *mt*$^+$) was obtained from the Chlamydomonas Resource Center (https://www.chlamycollection.org; Zhang et al., 2014). Tris-Acetate-Phosphate (TAP) and Minimal (MIN) media (acetate free) were prepared according to Sueoka (1960). TAP and MIN plates for growth included 1.5% (w/v) agar. The colonies from TAP plate are used to start a mixotrophic culture using 100-mL TAP liquid media. The cells were cultured in TAP media for 48 h at ambient $CO_2$ (0.04% $CO_2$ [v/v] in air) under continuous light illumination of 100 μE $m^{-2}$ $s^{-1}$. Cells in early log phase were washed and harvested in MIN media and bubbled with high $CO_2$ (5% $CO_2$ [v/v] in air) for 48 h. The CCM was induced by transferring the cells to low $CO_2$ 0.04% $CO_2$ (v/v) in air) bubbling for 12 h. High $CO_2$ was generated by mixing $CO_2$ with air such that final $CO_2$ concentration was (5% $CO_2$ [v/v] in air). For ambient $CO_2$ levels building air measured at (0.04% $CO_2$ [v/v] in air) was used. For very low $CO_2$ ambient air was mixed with the $CO_2$-free air to bring the $CO_2$ level to less than 0.02% v/v $CO_2$.

### Generation of RNAi construct and algal transformations

Artificial microRNA constructs for the knockdown of the CAH4 and CAH5 proteins were made using the RNAi protocol of (Molnar et al., 2009). The target sequences aligning to the "common region" of CAH4 and CAH5 were designed using Web MicroRNA Designer website (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The Web Micro RNA designer suggests suitable amiRNA based on optimal hybridization properties and it avoids the off-targets to other genes in *Chlamydomonas* genome. No obvious off targets were identified by the program and BlastN search of the *Chlamydomonas* genome indicated no other matches to the region of the RNAi target besides these two CA genes. The pChlamyRNA3int plasmid was obtained from the Chlamydomonas Resource Center and the oligos were annealed to it using the SpeI restriction site. Supplemental Table S1 details the oligos used for the miRNA construct. For creation of the RNAi strains, D66 cells were first grown in TAP in the light. When the cells reached an $OD_{730}$ between 0.2 and 0.3 (~2–3 × $10^6$ cells $mL^{-1}$) they were washed and resuspended in GeneArt MAX Efficiency Transformation Reagent for Algae at a density of ~2–3 × $10^6$ cells $mL^{-1}$. For electroporation, 2 μg of pChlamyRNA3int plasmid was

added to 250 μL of the D66 resuspended cells in an electroporation cuvette (0.4-cm gap width, BioRad). The protocol from Invitrogen was followed (https://www.thermofisher.com/order/catalog/product/A24229#/A24229).

## Screening of RNAi mutants

TAP plates containing antibiotic paromomycin (4 μg $mL^{-1}$, Invitrogen) were used for selecting transformed colonies and TAP paromomycin plates were maintained in high $CO_2$. Around 600 paromomycin resistant transformed colonies were selected by replica plating on MIN media plates and screening them in a high $CO_2$ chamber and a very low $CO_2$ chamber with continuous illumination (100 μmol photons $m^{-2}{\cdot}s^{-1}$) for 4 d. The colonies growing poorly on very low $CO_2$ were selected for spot tests. D66 and cia3 (Moroney et al., 1985) were the control strains used for the initial screening. Selected colonies for spot test were grown to log phase in TAP media and then resuspended in MIN media to a final concentration of $6.6 \times 10^5$ cells $mL^{-1}$. Spot tests were done by spotting 15 μL of sample (10,000 cells) followed by three 1:10 serial dilutions. These MIN media plates were screened by keeping them in high, ambient and very low $CO_2$ chambers under continuous illumination at 100 μmol photons $m^{-2}$ $s^{-1}$ for 7 d. The level of $CO_2$ was monitored using an Environmental Gas Monitor (EGM-4, PP systems, Massachusetts, USA).

## Gene expression and immunoblot analysis

Trizol reagent was used to extract RNA using a protocol from Invitrogen. ProtoScript First Strand cDNA Synthesis Kit (NEB) was used to make cDNA using 1 μg RNA per sample. As per manufacturer's instructions (Luna Universal One-Step RT-qPCR Kit) 100 ng RNA per sample was used to perform RT-qPCR using QuantStudio 6. G protein beta subunit-like polypeptide (CBLP) was used as a reference gene for all RT-qPCR. A list of primers is provided in Supplemental Table S1. For the immunoblot analysis, cell cultures were grown for 72 h in high $CO_2$ conditions in MIN media and then incubated at ambient $CO_2$ (~0.04% $CO_2$) for 12 h. Cells were collected by centrifugation and washed with 25-mM Hepes (pH 7.2). Samples were then normalized to 10-μg protein·$\mu L^{-1}$ for each sample. Five micrograms of protein was boiled with 2X Laemmli sample buffer and β-mercaptoethanol for 10 min at 80°C prior to analysis. Samples were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% (w/v) polyacrylamide gels (Mini-PROTEAN TGX, Bio-Rad Laboratories). Proteins were transferred to a PVDF-FL membrane using a Bio-Rad semidry blotting system. The membrane was blocked in 1% (w/v) bovine serum albumin and TTBS (TBS containing 0.1% Tween) for 1 h at 4°C. CAH4/5 primary antibody (Agrisera) was used with a dilution of 1:10,000 for 1 h at room temperature. Anti-rabbit secondary antibody diluted in 1% Bovine serum albumin and TTBS was used with a dilution of 1:4,000 at room temperature. A BioRad chemiluminescence instrument was used to observe the CAH4/5 protein bands for the cells.

## Photosynthetic assay

*Chlamydomonas* cultures were grown in TAP medium (100 μmol photons $m^{-2}$ $s^{-1}$) to logarithmic phase. Cells were washed in MIN media and then bubbled with high $CO_2$ for 24 h in MIN media. The cells were then bubbled with ambient $CO_2$ in the light for 12 h to induce the CCM. Cells normalized to 100-μg chlorophyll were suspended in 25-mM HEPES-NaOH buffer (pH 7.84) or 25-mM EPPS-NaOH buffer (pH 8.4) that had been bubbled with nitrogen gas. A Clark $O_2$ electrode chamber (Rank Brothers, Cambridge, UK) illuminated at 300 μmol photons $m^{-2}{\cdot}s^{-1}$ was used to deplete $C_i$. Then different concentrations of $NaHCO^-_3$ were injected into the depleted cells and the slope was calculated for each point. DIC concentration needed for half maximal rate of oxygen evolution was calculated as $K_{1/2}$ ($C_i$).

## Ci uptake

Silicone oil centrifugation was used to estimate intercellular concentration of DIC as in Moroney et al. (1985). Cells were initially grown in TAP media and then transferred to high $CO_2$ for 24 h in MIN media. The cells were centrifuged and normalized to a concentration of 25-μg chlorophyll $mL^{-1}$. Cells (4 mL) were illuminated in a Clark electrode chamber in 25-mM EPPS-NaOH buffer (pH 7.8 or 8.4) until the $C_i$ was depleted to a net $O_2$ evolution rate of zero. Cells were maintained in the light until used. 300 μL of $C_i$ depleted cells were layered into centrifuge tubes containing 25 μL of 1 M glycine (pH 10) with 0.75% (w/v) SDS overlaid with 75 μL of Dow Corning AR200/AR 20 (3:1 [v/v]) silicone oil. Assays were performed at 25°C in 200 μmol photons $m^{-2}{\cdot}s^{-1}$ light in a Beckman Microfuge B. $C_i$ uptake was initiated by adding 3 μL of 2.5-mM $NaH^{14}CO_3$ to a final concentration of 25 μM, followed by the indicated time of illumination (between 15 and 90 s at 150 μmol photons $m^{-2}$ $s^{-1}$). The reaction was terminated by a 15-s centrifugation in a Microfuge B (Beckman). Internal $C_i$ was calculated using the difference between total and acid stable $^{14}C$ in the pellet and corrected for cell volume (Machingura et al., 2017).

## Transmission electron microscopy

For TEM, *Chlamydomonas* cells were grown in TAP medium until the $OD_{730}$ of the cells reached between 0.2 and 0.3. Then, the cells were washed and bubbled with high $CO_2$ for 24 h. After that, cells were incubated at high $CO_2$ or ambient $CO_2$ for 12 h. The cells were processed for TEM as described previously (Mitra et al., 2004). The cells were washed in MIN media and fixed in growth medium containing equal volume of 1% $OsO_4$, 2% formaldehyde, 0.5% glutaraldehyde, and 0.1-mM sodium cacodylate buffer (pH 7.2) for 30 min. Cells were then washed and negatively stained with 0.5% uranyl acetate for 30 min in dark, dehydrated in ethanol and embedded in London resin white. Ultrathin sections (~70 nm) were mounted onto collodion-coated nickel grids, and micrographs were obtained using a JEOL JEM-1400 TEM.

### Visualization of CAH5 protein

Venus fluorescence was visualized using a *Chlamydomonas* strain cMJ030 expressing pLM005-CAH5-Venus-3xFLAG (obtained from the Chlamydomonas Resource Center). *Chlamydomonas* cells overexpressing CAH5 were grown in TAP medium until the $OD_{730}$ of the cells reached between 0.2 and 0.3. Then, the cells were washed in MIN media and bubbled with high $CO_2$ for 24 h in MIN media. Cells were then incubated at high $CO_2$ and ambient $CO_2$ for 12 h. The Venus fluorescence was observed from the cells with a Leica SP8 confocal microscope using a 63× water-emersion lens. The white light laser was used with 5% laser power and smart gain was adjusted to 100%. Two fluorescence detection channels were used, the green channel for Venus signals (excited at 514 nm) and red channel for chlorophyll autofluorescence (excited at 488 nm). The emission wavelength of the fluorescence filter for Venus and chlorophyll was 520–560 and 640–721 nm, respectively. The images received from the Leica LAS X software was processed through standard deconvolution using Huygens Essential software.

### Complementation of *cah4/5 RNAi* mutant

Complementation of the *cah4/5-2* RNAi mutant was achieved by transformation of *cah4/5-2* RNAi mutant cells with invitrogen pChlamy _4 vector containing a CDS of *CAH5* driven by the Hsp70A-Rbc S2 hybrid promoter. The construct consists of no untranslated regions of the original CAH5 gene as the area targeted by the RNAi was in the 3′ UTR of the *CAH4* and *CAH5* genes. The electroporation method was used for cell transformation, and strains were selected for zeocin resistance. The presence of complemented DNA in selected *Chlamydomonas* strains was confirmed by PCR. The growth of the complemented line was then compared to the growth of the RNAi lines and D66 as described in the screening RNAi lines section.

### Quantification and statistical analysis

Statistical analyses were performed using Student's *t* test in GraphPad Prism 8. In all cases, at least at least three separate experiments were performed that showed similar trends. The data in the figures are the means and standard deviations for three technical replicates from one of the experiments. Statistical significance is defined as $P < 0.05$. Details are in the figure legends.

## Accession numbers

*CAH4* and *CAH5* can be found in the Phytozome under the accession numbers Cre05.g248400.t1.2 and Cre05.g248450.t1.1

## Supplemental data

The following materials are available in the online version of this article.

**Supplemental Figure S1**. Multiple sequence alignment of the CAH4 and CAH5 protein sequences.

**Supplemental Figure S2**. RT-qPCR of other CCM genes.

**Supplemental Figure S3**. Growth of strains complementing the *cah4/5* RNAi knockdown line at different $CO_2$ levels.

**Supplemental Figure S4**. Additional TEM images of whole cells of the D66 strain.

**Supplemental Figure S5**. Additional confocal images of whole cells of the CSI_FC1G05 expressing pLM005-*CAH5*-Venus-3xFLAG.

**Supplemental Table S1**. List of primers used in this study.

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*Conflict of interest statement*. None declared.

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