

Dynamics of Carbon-Concentrating Mechanism Induction and Protein Relocalization during the Dark-to-Light Transition in Synchronized *Chlamydomonas reinhardtii*¹^[W]^[OPEN]

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In the model green alga *Chlamydomonas reinhardtii*, a carbon-concentrating mechanism (CCM) is induced under low CO₂ in the light and comprises active inorganic carbon transport components, carbonic anhydrases, and aggregation of Rubisco in the chloroplast pyrenoid. Previous studies have focused predominantly on asynchronous cultures of cells grown under low versus high CO₂. Here, we have investigated the dynamics of CCM activation in synchronized cells grown in dark/light cycles compared with induction under low CO₂. The specific focus was to undertake detailed time course experiments comparing physiology and gene expression during the dark-to-light transition. First, the CCM could be fully induced 1 h before dawn, as measured by the photosynthetic affinity for inorganic carbon. This occurred in advance of maximum gene transcription and protein accumulation and contrasted with the coordinated induction observed under low CO₂. Between 2 and 1 h before dawn, the proportion of Rubisco and the thylakoid lumen carbonic anhydrase in the pyrenoid rose substantially, coincident with increased CCM activity. Thus, other mechanisms are likely to activate the CCM before dawn, independent of gene transcription of known CCM components. Furthermore, this study highlights the value of using synchronized cells during the dark-to-light transition as an alternative means of investigating CCM induction.

Carbon-concentrating mechanisms (CCMs) have evolved in most unicellular aquatic photosynthetic organisms to compensate for the kinetic constraints of the primary carboxylase Rubisco (Spreitzer and Salvucci, 2002) and limited availability of dissolved carbon dioxide (CO₂). In eukaryotic algae (Giordano et al., 2005; Moroney and Ynalvez, 2007; Wang et al., 2011) and cyanobacteria (Price et al., 2008), these CCMs are biophysical means of concentrating CO₂ around Rubisco, thereby favoring the carboxylation reaction and improving growth in CO₂-limited environments.

In the model green alga *Chlamydomonas reinhardtii*, three elements are important for CCM activity: first, inorganic carbon (Ci) transporters at the plasma membrane and chloroplast envelope (Spalding, 2008); second, carbonic anhydrases, which facilitate the interconversion of CO₂ and HCO₃⁻ and operate in parallel within each cellular compartment (Moroney et al., 2011); and third, the localization of Rubisco to a chloroplast micro-compartment called the pyrenoid to minimize CO₂

leakage (Ma et al., 2011; Meyer et al., 2012). The CCM is induced at low (air level) CO₂ concentrations, resulting in an increased whole-cell affinity for Ci and internal Ci accumulation (Badger et al., 1980), coincident with the de novo synthesis of several proteins (Manuel and Moroney, 1988).

Further candidate CCM genes have been identified as low CO₂-induced through microarray and transcriptomic studies (Miura et al., 2004; Yamano et al., 2008; Brueggeman et al., 2012; Fang et al., 2012) or by isolating mutants with reduced growth or Ci accumulation at low CO₂ (Spalding et al., 1983; Colombo et al., 2002; Thyssen et al., 2003). Low CO₂-induced genes investigated in this study include several Ci transporter candidates, some of which are among the most highly induced genes under low CO₂ (Brueggeman et al., 2012). *High Light-Activated3 (HLA3)* encodes a plasma membrane-localized ABC-type transporter (Duanmu et al., 2009), *Low CO₂-Inducible1 (LCII)* encodes another plasma membrane transporter (Ohnishi et al., 2010), and *LCIA, Chloroplast Carrier Protein1 (CCP1)*, and *CCP2* all encode proteins putatively involved in Ci transport and associated with the chloroplast envelope (Wang et al., 2011).

Two carbonic anhydrases were also of interest. The *Carbonic Anhydrase1 (CAH1)* gene encodes a periplasmic carbonic anhydrase and is highly CO₂ responsive, although CAH1-deficient mutants retain a functional CCM (Van and Spalding, 1999). *CAH3* encodes a thylakoid-lumenal carbonic anhydrase essential for growth at ambient CO₂, although the gene is minimally CO₂

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responsive and the protein is expressed equally under high and low CO₂ (Karlsson et al., 1998).

Expression of other CCM-related genes was also determined, including *Low-CO₂ Stress Response1 (LCR1)*, which encodes a MYB-domain transcription factor upstream of *CAH1* and *LC11* and downstream of CCM master regulator Inorganic Carbon Accumulation5 (CIA5 [also known as CCM1]; Yoshioka et al., 2004). LCIB and LCIC, which form a periphyrenoidal complex associated with carbon recapture (Wang and Spalding, 2014), were also included in this study, as were *RBCS* and *rbcl*, which encode the small and large subunits of Rubisco, the major pyrenoid component (Borkhsenius et al., 1998).

In addition to CO₂ responsiveness, CCM activity in *C. reinhardtii* is associated with light and photosynthesis. In particular, accumulation of Ci in *C. reinhardtii* cells and chloroplasts is light dependent (Spalding and Ogren, 1982) and may be driven by ATP from cyclic electron transport (Spalding et al., 1984). HLA3 is a member of the ATP-binding cassette superfamily, which suggests light dependency, and *HLA3* expression is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and in PSI and PSII mutants (Im and Grossman, 2002). Although originally identified as a high light-induced gene, *HLA3* may in fact be responding to low CO₂ levels brought about by increased photosynthesis at higher light intensities, consistent with the induction of other CCM genes under these conditions (Im and Grossman, 2002). Similarly, *CAH1* transcription requires light, low CO₂, and photosynthetic electron flow (Dionisio-Sese et al., 1990). At the protein level, the LCIB-LCIC complex relocates from the stroma to the area around the pyrenoid both in response to light and to CO₂ levels (Yamano et al., 2010). However, cells acclimating to low CO₂ induce external carbonic anhydrase activity and active HCO₃⁻ transport even in the dark, although induction is delayed compared with cells switched to low CO₂ in the light (Bozzo and Colman, 2000). This suggests that light, while an important regulator of CCM activity, is not an absolute requirement for expression of CCM components. In synchronized cells grown at ambient CO₂, genes encoding putative Ci transporters and mitochondrial carbonic anhydrases are transcriptionally up-regulated in the light, whereas other key CCM gene transcripts (*CAH3*, stromal carbonic anhydrase *CAH6*, and *LCIB*) reach maximum levels during the last half of the dark period (Tirumani et al., 2014).

While the majority of experiments investigating CCM induction have used cells in asynchronous cultures grown in continuous light, the aim of this study was to investigate the dynamics of CCM induction and molecular regulation in synchronized cells grown in dark/light cycles, focusing on a detailed time course during the dark-to-light period transition. Given the apparent down-regulation of the CCM in the dark (Marcus et al., 1986), we hypothesized that induction of the CCM during the dark-to-light transition might involve nontranscriptional/translational mechanisms regulating existing CCM components, unlike the CO₂ response, which requires strong

transcriptional up-regulation and de novo protein synthesis.

To investigate possible mechanisms of CCM regulation in synchronized cells, whole cell affinity for Ci ($K_{0.5}$; the concentration of Ci required for half-maximal photosynthetic rates) as well as the relative abundance of known CCM (CO₂-responsive) gene transcripts and proteins were measured across the dark-to-light transition, and unsynchronized cultures adapting to low CO₂ were used as a control. We also set out to determine the extent that the thylakoid luminal CAH3 and Rubisco relocated from the stroma to the pyrenoid during the dark-to-light transition, because this preferential localization appears to be required for a fully functioning CCM (Blanco-Rivero et al., 2012; Meyer et al., 2012).

Unsynchronized cells adapting to low CO₂ showed rapid increases in mRNA levels of CCM genes, followed by increased protein abundance and then CCM activity. In synchronized cells, CCM activity was initially down-regulated in the dark, but full CCM activity was inducible before dawn and also prior to maximum mRNA and protein accumulation. Rubisco and CAH3 were both found to have relocated to the pyrenoid, coincident with increased CCM activity.

RESULTS

The CCM Is Partially Repressed in the Dark But Can Be Induced 1 h before Dawn

CCM induction during the dark-to-light transition was measured at the whole-cell and molecular level to identify factors affecting CCM regulation in synchronized cultures. Whole-cell affinity for Ci ($K_{0.5}$) was derived using an oxygen electrode and HCO₃⁻ addition responses, following a light pretreatment used to deplete residual Ci supplies. In synchronized cells, $K_{0.5}$ for Ci was measured from 2 h before dawn to 6 h after dawn (Fig. 1A). A relatively high $K_{0.5}$ value (60 μM Ci) was found toward the end of the dark period (-2 h), indicating that the CCM was at least partially repressed in the dark, even after the short light pretreatment. From this time point, CCM inducibility rapidly increased, so that 1 h before dawn, cells were able to express a fully functioning CCM (as indicated by a low $K_{0.5}$ of 11 μM Ci). This high affinity for Ci was maintained through dawn and the first 6 h of the light period (mean $K_{0.5}$ of 11 to 19 μM Ci).

To provide a direct comparison for dark-to-light time courses, CCM induction in asynchronous cultures grown in continuous light and switched to low CO₂ was also investigated. The very high $K_{0.5}$ value (240 μM Ci) of high CO₂-adapted cells indicated that the CCM was fully repressed at the beginning of the time course (Fig. 1B). Affinity for Ci rapidly increased following the transfer to low CO₂, with the measured $K_{0.5}$ reduced to 105 μM Ci within 1 h of the shift to low CO₂. Cells showed maximum CCM induction (highest affinity for Ci) after 4 to 6 h at low CO₂, as indicated by low $K_{0.5}$

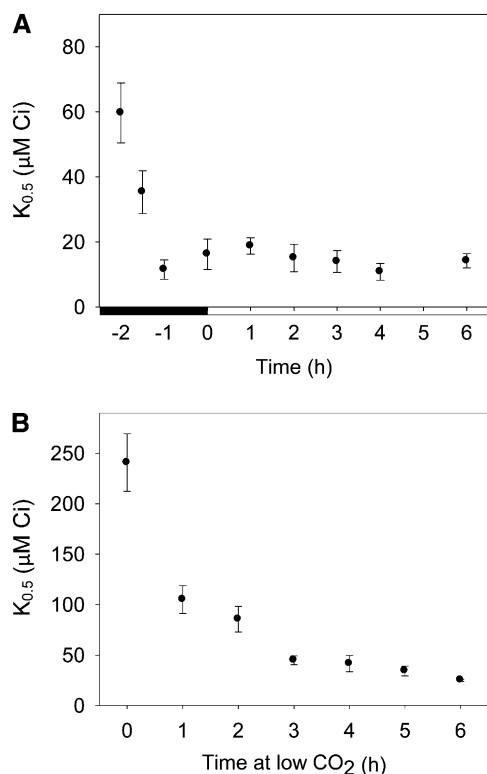


Figure 1. Whole-cell affinity for Ci ($K_{0.5}$) measured in wild-type *C. reinhardtii* during CCM induction in synchronized cells during the dark-to-light transition (A) and in response to low CO₂ in asynchronous cultures (B). Synchronized cells were grown in 12-h/12-h dark/light cycles under low CO₂ and harvested during the third dark-to-light transition after dilution (dawn = 0 h). Asynchronous cells were grown to midlog phase in high CO₂ and harvested following the switch to low CO₂ (time = 0 h). Values are mean \pm 1 SE of three to five independent experiments.

values (25–40 $\mu\text{M Ci}$). The maximum $K_{0.5}$ value measured for high CO₂-adapted cells was greater than that observed in dark-adapted (-2 h) cells, as was the overall decrease in $K_{0.5}$ during each time course (10- and 6-fold, respectively), which suggests that the CCM was repressed to a greater extent at high CO₂ than 2 h before dawn.

Maximum Gene Expression Occurs after CCM Induction during the Dark-to-Light Transition

In this comparative study between CCM regulation during the dark-to-light transition and the conventional CO₂ response, quantitative reverse transcriptase (qRT)-PCR was used to track expression of CO₂-responsive genes. Gene expression was quantified relative to the control gene *Guanidine Nucleotide-Binding Subunit Beta-Like Protein (GBLP)* and to the first time point. In synchronized cells 2 h before dawn, *CCP1*, *CCP2*, *LCI1*, *LCIA*, *LCIB*, and *LCIC* were all present at similar levels to high CO₂-adapted cells (Supplemental Table S1). *CAH1*, *LCR1*, and *rbcl* were present in greater abundance, while *HLA3* and

RBCS were present in lesser abundance in dark-/light-grown (harvested in the dark 2 h before dawn [-2D]) compared with high CO₂-grown (0 h) cells. Maximum levels of *CAH1*, *CCP1*, *LCI1*, *LCIA*, *LCIB*, and *LCIC* mRNA were not significantly different between dark/light and CO₂ time courses (Supplemental Table S2). Some differences were observed in the maximum abundance of *CCP2*, *HLA3*, *LCR1*, *rbcl*, and *RBCS*, but these were below one order of magnitude and smaller than the differences observed at the beginning of the time courses. Overall, this indicates that transcriptional regulation during CCM induction in response to light is similar to that of CO₂.

In synchronized cells, many CO₂-responsive genes (*CCP1*, *CCP2*, *LCI1*, *LCIA*, *LCIB*, and *LCIC*) were up-regulated during the light period to a similar extent as for the CO₂ response (Fig. 2; Supplemental Tables S3 and S4). However, the majority of transcriptional up-regulation did not occur until several hours after maximum CCM activity was observed (Figs. 1A and 2; Supplemental Fig. S1; Supplemental Table S2), contrasting with the concurrent CCM activation and low CO₂-induced gene induction observed in cells adapting to low CO₂ (Figs. 1B and 3; Supplemental Fig. S1; Supplemental Table S2).

Although CO₂-responsive genes were consistently up-regulated in the light, there were systematic differences in the timing of maximum mRNA expression compared with the CO₂ response. For example, Ci transporter transcripts (*CCP1*, *CCP2*, *HLA3*, *LCI1*, and *LCIA*), *LCR1*, and *CAH1* reached maximum levels between 2 and 4 h after dawn, whereas *LCIB* and *LCIC* levels were maximal just 1 h into the light period (Fig. 2; Supplemental Table S2). By contrast, CCM induction following transfer from high to low CO₂ showed a more coordinated response. CO₂-responsive transcripts accumulated rapidly, reaching maximum levels after approximately 2 h and then showing a steady decline over the next 4 h (Fig. 3; Supplemental Table S2).

Additionally, because $K_{0.5}$ measurements required an initial period of preacclimation in the light, gene expression was also measured in synchronized cells harvested before dawn after a brief period of illumination in the oxygen electrode chamber (Fig. 2, harvested 2 h before dawn with light pretreatment [-2L] and -1L). This light pretreatment resulted in the strong up-regulation of the Ci transporter genes *CCP1*, *CCP2*, *HLA3*, *LCI1*, and *LCIA* but did not affect the expression of *CAH1* and *LCR1*. *LCIB* and *LCIC*, although having a lower level of induction to begin with, also responded to the predawn light exposure.

RBCS (both *RBCS1* and *RBCS2*) mRNA levels remained largely unchanged throughout the dark/light time course, including the predawn light treatment (Fig. 2). *rbcl* transcripts appeared to decrease in abundance by up to 80% both in response to the light treatment and from dawn to 6 h into the light; however, overall abundance of both *RBCS* and *rbcl* transcripts remained very high (at least one order of magnitude

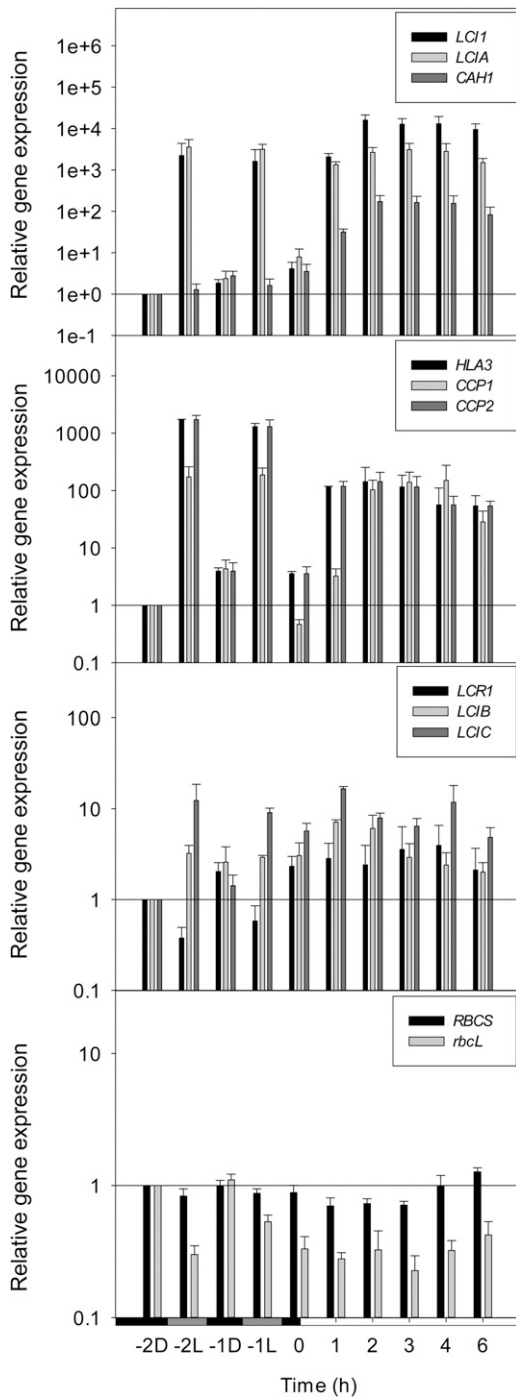


Figure 2. Expression profiles of CCM genes in synchronized cells during the dark-to-light transition. mRNA abundance was determined using qRT-PCR and normalized to mRNA levels in -2D cells and to *GBLP* (control gene) expression. Growth and harvest conditions were as described in Figure 1. During the dark period (-2 and -1 h), mRNA was harvested from cells taken either straight from the dark (D) or after a brief illumination in the oxygen electrode chamber (L) to mimic the light pretreatment necessary for $K_{0.5}$ measurements. Values are mean \pm 1 SE of three to seven separate flasks harvested across at least three independent experiments.

higher than the highly abundant reference gene *GBLP*; Supplemental Tables S1 and S2). Similarly, only minimal changes were detected in the abundance of genes

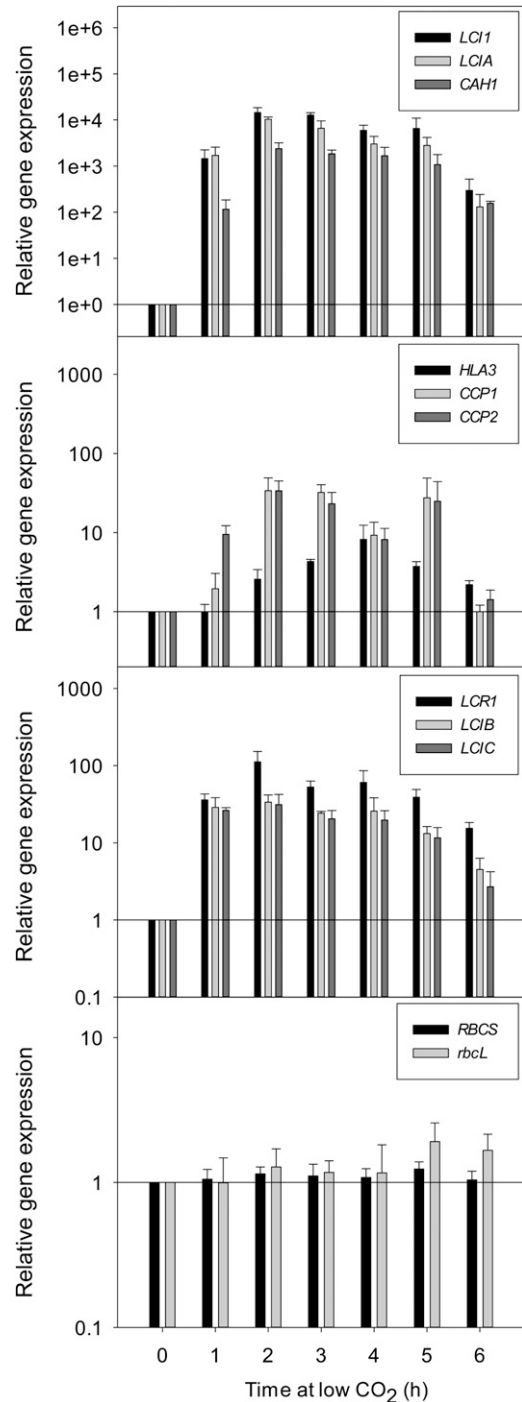


Figure 3. Expression profiles of CCM genes in asynchronous cells adapting to low CO₂. mRNA abundance was determined using qRT-PCR and normalized to mRNA levels in high CO₂-adapted cells and to *GBLP* (control gene) expression. Values are mean \pm 1 SE of three separate flasks harvested during a single experiment.

encoding Rubisco (*RBCS* and *rbcl*) throughout the CO₂ time course (Fig. 3).

CCM Repression in the Dark Is Independent of Key CCM Protein Abundance

The abundance of several CCM proteins during the dark-to-light transition was measured using immunoblots to determine whether increased mRNA abundance resulted in increased protein levels (Fig. 4). To complement qRT-PCR data, predawn samples (–2, –1) were harvested either directly from the dark or after light pretreatment. Although CO₂-responsive transcripts were present only at low levels 2 h before dawn, their corresponding proteins were easily detectable at this time. LCIB and LCIC were present at high levels 2 h before dawn, while CCM activity was still low. These proteins reached maximum levels 2 to 4 h into the light period, several hours after maximum CCM activity occurred. CAH1 was also detectable in the dark and did not show major changes in abundance during the dark-to-light transition. No major change in either RBCS or *rbcl* abundance was detected during this time course. Predawn exposure to light (samples –2L and –1L) had minimal effect on the abundance of all proteins probed.

By contrast, for cells adapting to low CO₂, immunoblot analyses of LCIB, LCIC, and CAH1 showed coordinated increases in mRNA abundance and protein concentration (Fig. 5; Supplemental Fig. S1). Unlike in dark-harvested (partially CCM-repressed) synchronized cells, LCIB, LCIC, and CAH1 were almost undetectable in high CO₂-adapted cells (Fig. 5, 0 h). However, all three proteins rapidly accumulated in response to low CO₂, reaching maximum abundance after approximately 3 (LCIB and LCIC) to 5 h (CAH1) and maintaining these high levels for remainder of the time course. No changes in *rbcl* or RBCS abundance were observed during the acclimation to low CO₂.

Relocalization of Rubisco and CAH3 to the Pyrenoid Coincides with CCM Induction at End of Dark Period

Immunogold labeling was used to probe Rubisco and CAH3 localization, which are both known to be preferentially localized in the pyrenoid in response to low CO₂ (Fig. 6). Two hours before dawn (–2D), approximately 75% of Rubisco-labeled particles were present in the pyrenoid while, 1 h later, almost 90% of labeled Rubisco was found in the pyrenoid (–1D), and this high count was maintained 2 h into the light period (2 h; Fig. 6A). This change in Rubisco localization coincided with the measured change in *K*_{0.5} but was independent of the dark-period light pretreatment (–2L and –1L).

By contrast, relative CAH3 localization changed both before dawn in the dark and in response to predawn light exposure (Fig. 6B). Two hours before dawn, approximately 22% of CAH3 particles were in the pyrenoid (–2D), but this proportion increased to 35% 1 h before

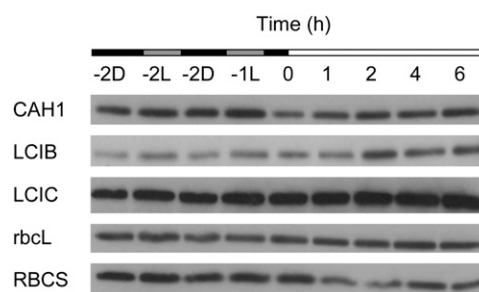


Figure 4. Expression profiles of CCM-related proteins in synchronized cells during the dark-to-light transition. Soluble protein extracts were separated using SDS-PAGE and used for immunoblot analyses with antibodies raised against CAH1, LCIB, LCIC, and Rubisco (*rbcl* and RBCS). Sample loading was normalized by chlorophyll content. During the dark period (–2 and –1 h), protein was harvested from cells taken either straight from the dark (D) or after a brief illumination in the oxygen electrode chamber (L) to mimic the light pretreatment necessary for *K*_{0.5} measurements.

dawn (–1D) and reached 40% 2 h into the light period (2 h). The percentage of CAH3 in the pyrenoid also responded directly to the predawn light treatment (increasing to 33% at –2L and 39% at –1L).

In general, mean cell cross-sectional area increased in response to light exposure both before and after dawn (Fig. 6C). Pyrenoid area also increased in line with cell area across the time course (Fig. 6D).

DISCUSSION

Since Marcus et al. (1986) identified oscillations in both CCM activity and photosynthetic characteristics in synchronized cells, the *C. reinhardtii* CCM has been characterized to a much greater extent in asynchronous cells (for review, see Moroney and Ynalvez, 2007; Wang et al., 2011). The aim of this study was thus to reconcile changes at the molecular level with regulation of overall CCM activity and cellular dynamics of key CCM constituents in a detailed time course during the dark-to-light transition. Several key differences in CCM induction were observed in synchronized, as compared with asynchronous, cultures, as well as the differential expression of known CCM genes in response to light. This study has highlighted the possible importance of additional regulatory mechanisms because the CCM was inducible before dawn, without concomitant increases in mRNA or protein levels of known CCM components.

Comparison of CCM Induction in Response to Light and CO₂

This study systematically characterized an 8-h period covering the dark-to-light transition as well as the first 6 h of acclimation to low CO₂ for comparison. Coordinated increases in CCM mRNA and protein abundance support the idea that CCM induction in response to low

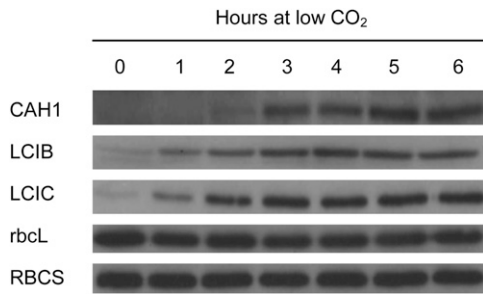


Figure 5. Expression profiles of CCM-related proteins in asynchronous cells adapting to low CO₂. Soluble protein extracts were separated using SDS-PAGE and used for immunoblot analyses with antibodies raised against CAH1, LCIB, LCIC, and Rubisco (rbcl and RBCS). Sample loading was normalized by chlorophyll content.

CO₂ relies on the de novo synthesis of many components. By contrast, synchronized cells, which divide at the start of the dark period, can induce CCM activity in advance of the light period and prior to increased expression of CCM gene transcripts and proteins. Some CCM gene transcripts, mostly encoding Ci transporters, were also found to respond differentially to predawn light exposure.

The genes analyzed by qRT-PCR in this study (*CAH1*, *CCP1*, *CCP2*, *HLA3*, *LCI1*, *LCIA*, *LCIB*, *LCIC*, and *LCR1*) have all been assigned putative roles in the CCM and identified as transcriptionally up-regulated in response to low CO₂, in at least three of four microarray or transcriptomic studies published to date (supplemental data set 1 of Brueggeman et al., 2012). Maximum abundance of these gene transcripts occurred after 2 h at low CO₂, and the average fold change from high CO₂ levels was generally within 5-fold of the values obtained by Brueggeman et al. (2012; Supplemental Table S4).

In synchronized cells, the level of gene induction (fold increase) during the light period was generally within one order of magnitude of that observed in cells adapting to low CO₂ (Supplemental Tables S3 and S4). Perhaps more importantly, the maximum levels of mRNA abundance (relative to *GBLP*) were also within 4-fold of values obtained for the CO₂ time course (Supplemental Table S2). Strong up-regulation of CCM gene transcripts was thus observed in synchronized cells, but, crucially, this occurred only during the light period. Maximum mRNA levels were reached approximately 2 h into the light period, when cells had been expressing maximum CCM activity for the previous 3 h.

Tirumani et al. (2014) also showed that Ci transporter transcripts were up-regulated in the light in synchronized cells of a different wild-type strain (137C/CC-125), which allows greater confidence in the data given that differences between strains and growth conditions are often a major source of variation between experiments (Brueggeman et al., 2012; Fang et al., 2012). Tirumani et al. (2014) also showed that gene expression for carbonic anhydrases (*CAH3* and *CAH6*) and *LCIB* was higher in the dark, although this induction was

suppressed by high CO₂. As with the different responses of CCM genes to predawn light treatment (discussed below), this suggests that CCM gene transcription responds to multiple signals, and to light and low CO₂ in particular. In this study, expression of *LCIB* was measured from 2 h before dawn, which may be why this dark induction of key CCM genes was not observed. Expression of *CAH3* and *CAH6* was not measured in this study because these genes are minimally responsive to CO₂. Finally, Tirumani et al. (2014) found that *CAH3* protein only accumulates during the light period, but

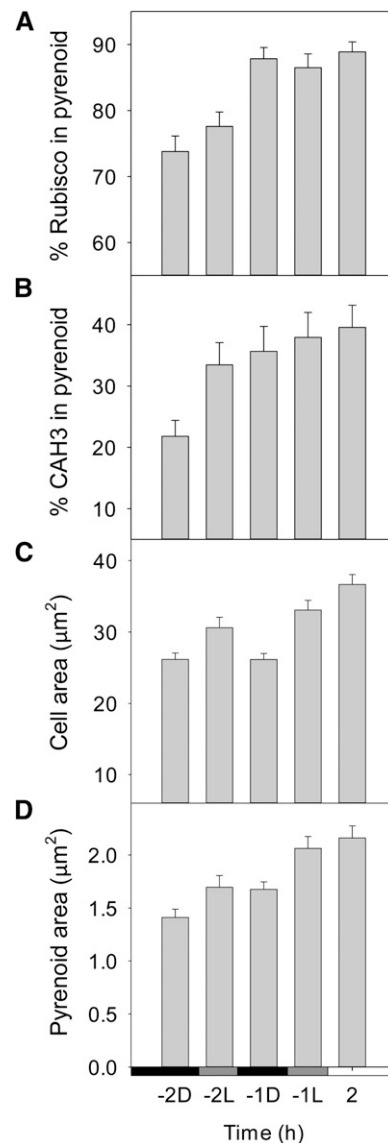


Figure 6. Localization of mobile CCM components in synchronized cells during the dark-to-light transition. Immunogold localization was used to determine the relative abundance in the pyrenoid of primary photosynthetic carboxylase and pyrenoid component Rubisco (A) and thylakoid lumen-localized carbonic anhydrase CAH3 (B; *n* = 53). Cell area (C) and pyrenoid area (D) were also determined (*n* = 80). Bars represent mean ± 1 SE.

because they did not measure CCM activity or the abundance of CAH6 and LCIB, it is difficult to determine to what extent gene induction in the dark affects CCM expression.

In the CO₂ response, de novo CCM protein expression was correlated with a large decrease in $K_{0.5}$. qRT-PCR and immunoblot analyses for LCIB and LCIC showed maximum protein abundance occurring approximately 1 h after maximum mRNA abundance in both CO₂ and dark/light time courses. However, LCIB and LCIC were only slightly induced in the light period compared with the large induction observed in response to low CO₂. By contrast, CAH1 levels showed no increase during the light period despite increased transcription, which again distinguishes this response from the large increases in transcription and translation observed in response to low CO₂. Overall, during the dark-to-light transition, minimal induction of CAH1, LCIB, and LCIC and the relatively high protein content in the dark were consistent with both the lower $K_{0.5}$ and the inducibility of the CCM in the dark (Figs. 1A and 4).

Transcriptional Response to Light Pretreatment and Inducibility of the CCM in the Dark

Both the timing and speed of CCM induction in synchronized cells suggest that regulation during the dark-to-light transition may not depend on the de novo synthesis of known CCM proteins, in contrast to the low CO₂ response. The differential regulation of mRNA expression and protein levels was further illustrated by the light pretreatment used for comparability with the protocol for $K_{0.5}$ measurements. Transcripts for Ci transporters (*CCP1*, *CCP2*, *HLA3*, *LCI1*, and *LCIA*) all responded strongly to the light pretreatment, generally reaching levels at least as high as those achieved 1 h into the light period of a conventional dark/light cycle. *LCIB* and *LCIC* transcripts, although induced to a far lesser extent during the light period, showed a similar level of induction under predawn illumination. The speed and magnitude of this transcriptional response to light may be in part due to the greater light levels in the oxygen electrode chamber (photon flux density, 200–300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) compared with growth conditions in the incubator (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). For example, mRNA levels of a CO₂-responsive mitochondrial carbonic anhydrase (*CAH4*) increase linearly with increasing light intensity up to 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Eriksson et al., 1998), well above the intensities used in this study.

However, not all CCM-related gene transcripts showed the same pattern of induction in response to predawn light exposure. *LCR1*, although slightly induced during the light period, did not respond to light before dawn. More notably, *CAH1* mRNA levels also did not change in response to predawn light exposure, despite being strongly up-regulated during the following light period. This indicates that *LCR1* and *CAH1* transcription may be regulated in a different manner to other CO₂-responsive genes. Rawat and Moroney (1995) also identified strong

oscillations of *CAH1* transcripts in synchronized cells, and *CAH1* was later shown to be under circadian control (Fujiwara et al., 1996). The higher abundance of *CAH1* mRNA in -2D (dark/light) cells compared with 0-h (CO₂) cells is consistent with the previous observation of the presence of *CAH1* mRNA in cells at the end of the dark period (Supplemental Table S1; Rawat and Moroney, 1995). This also explains the greater induction seen in the CO₂ response compared with dark-/light-grown cells, even though the maximum *CAH1* mRNA abundance was similar in both time courses (Supplemental Table S2).

The evidence presented thus far suggests that the increased affinity for Ci ($K_{0.5}$) and inducibility of the CCM in advance of the light period is uncoupled from the transcriptional up-regulation of key CCM elements, which occurred in the light, as distinct from low CO₂-induced CCM activity. Secondly, the transcriptional responsiveness of Ci transporter genes to predawn light treatment, relative to other components, shows there to be differentially regulated elements of the CCM. Together with the relatively high concentrations of key CCM proteins in cells growing through dark and into light periods, this leads us to suggest that posttranslational mechanisms might be important for CCM induction in synchronized cells during the dark-to-light transition.

Dynamics of Protein Abundance and Relocalization during CCM Induction across the Dark-to-Light Transition

The relative localization of mobile CCM components in synchronized cells was investigated as a possible means of modulating CCM activity independently of protein abundance. Importantly, Rubisco and CAH3 were found to be differentially localized during the dark-to-light transition, although with certain key differences in this response.

Rubisco accumulated within the pyrenoid during the 2 h before dawn, coinciding with measured changes in $K_{0.5}$ values and suggesting that the aggregation of Rubisco in the pyrenoid is associated directly with increased CCM activity. Brief exposure to light prior to dawn did not affect Rubisco localization, which suggests that changes in localization during the dark-to-light transition may rely on an endogenous (light-independent) signal. Nevertheless, Rubisco is likely to respond to multiple signals because aggregation is also known to occur in response to low CO₂ (Borkhsenius et al., 1998).

CAH3 localization, like Rubisco, may also be controlled by an endogenous signal that coordinates CCM induction. Two hours prior to dawn, 20% of CAH3 was localized to the pyrenoid, but 1 h later, the proportion of CAH3 in the pyrenoid had nearly doubled, and this high proportion was maintained 2 h into the light period. However, in cells harvested 2 h before dawn and exposed briefly to light, the proportion of CAH3 in the pyrenoid was also high. This suggests that CAH3 localization could also be directly regulated by light. This is consistent with qualitative data from immunofluorescence experiments showing that, in synchronized cells,

CAH3 is distributed throughout the stromal thylakoids in the middle of the dark period (–6 h) but is pyrenoid localized in the middle of the light period (6 h; Tirumani et al., 2014). However, this quantitative study extends the implications of these findings by showing relocation of CAH3 just prior to dawn (between –2 and –1 h) in the dark and in response to predawn illumination (at –2 h).

CAH3 relocation has also been observed in cells acclimating to low CO₂, and in this study, CAH3 was present in the pyrenoid at similar relative abundance in dark- versus light-adapted cells, compared with those adapted to high versus low CO₂ (19% and 37%, respectively; Blanco-Rivero et al., 2012). In the CO₂ response, this shift in localization was linked to the phosphorylation of CAH3 (Blanco-Rivero et al., 2012), but it has not been determined whether a similar mechanism is operating in air-grown synchronized cells. The low CO₂-inducible protein LCI5 is also rapidly phosphorylated in response to low CO₂, and this has been shown to occur in a redox-dependent manner (Turkina et al., 2006). A similar redox-dependent kinase may be responsible for phosphorylation of CAH3 in response to low CO₂ and light; however, this would not explain the shift in CAH3 localization in the dark. Whatever the mechanism, the high relative localization of CAH3 in the pyrenoid after the light pretreatment 2 h before dawn may be partly responsible for the relatively low K_{0.5} value (incomplete repression of the CCM) at this point.

CONCLUSION

Although there are similarities in the transcriptional response of genes during CCM induction in response to low CO₂ and during the dark-to-light transition, this study has identified several key differences that distinguish the response of synchronized cells from that of asynchronous cultures. The inducibility of the CCM before dawn and in advance of mRNA and protein accumulation was directly linked to increased localization of Rubisco and CAH3 to the pyrenoid and highlights the potential importance of posttranslational regulation of the CCM. Further work will be needed to determine the mechanism of relocation of Rubisco and CAH3 as well as to elucidate the pathways triggered in response to the different signals. In any case, studies focusing on how components are modified, including their subcellular localization and interactions, will be necessary to further our understanding of the CCM. Investigating these changes in synchronized cultures of cells during the dark-to-light transition will provide additional means of probing these important aspects of CCM regulation.

MATERIALS AND METHODS

Algal Strain and Culture Conditions

The *Chlamydomonas reinhardtii* wild-type strain 2137 mt+ (Spreitzer and Mets, 1981) was maintained in the dark on Tris-acetate medium (Spreitzer and

Mets, 1981) agar plates supplemented with Kropat's trace elements (Kropat et al., 2011). Liquid cultures were grown in an Innova 42 incubator (New Brunswick Scientific) at 25°C with 50 μmol photons m⁻² s⁻¹ illumination, aeration, and shaking (125 rpm). Starter cultures were inoculated into Tris-minimal (Tris-acetate without acetate) medium from freshly replated Tris-acetate agar cells and grown to midlog phase (1–2 × 10⁶ cells mL⁻¹). Experimental cultures were inoculated from starter cultures and harvested at midlog phase. High CO₂ cultures were bubbled with 5% (v/v) CO₂ for 6 d (one starter followed by one experimental culture) before switching to bubbling with air (low CO₂; 0.04% [v/v]) for the CO₂ response time course. Synchronized cultures were continuously bubbled with air, grown in 12-h/12-h dark/light cycles, and harvested when at midlog phase during the third light period after inoculation. Synchronicity of cell division under these conditions was confirmed by cell counts over a 72-h period from inoculation to midlog phase (harvest).

Oxygen Evolution Measurements

Apparent affinity for Ci was determined using the oxygen evolution method described by Badger et al. (1980). Cells grown in Tris-minimal liquid medium were harvested and resuspended in 25 mM HEPES-KOH (pH 7.3) to a density of 1.5 × 10⁷ cells mL⁻¹. Aliquots of cells (1 mL) were added to a Clark-type oxygen electrode chamber (Rank Brothers) attached to a circulating water bath set to 25°C. The chamber was closed for a light pretreatment (200–300 μmol photons m⁻² s⁻¹ illumination for 10–25 min), allowing cells to consume internal Ci stores. When net oxygen evolution ceased, aliquots of NaHCO₃ were added to the cells at 30-s intervals, and the rate of oxygen evolution was recorded every second using a PicoLog 1216 data logger (Pico Technologies). Cumulative concentrations of NaHCO₃ after each addition were as follows: 2.5, 5, 10, 25, 50, 100, 250, 500, 1,000, and 2,000 μM. Some of the RNA and protein extraction and quantitation methods were conducted after 25-min illumination in the electrode chamber to mimic a standard light pretreatment plus Ci addition protocol.

Chlorophyll Extraction

Chlorophyll was extracted from cells for normalization of oxygen evolution measurements and loading of proteins for SDS-PAGE and immunoblots. Chlorophyll was extracted in 90% (v/v) acetone (10 min at 60°C), and the absorbance of the supernatant was measured at 647 and 664 nm. Chlorophyll concentration was calculated using the equations of Jeffrey and Humphrey (1975).

Analysis of Gene Expression by qRT-PCR

qRT-PCR was used to determine the relative abundance of CCM gene transcripts. Total RNA was extracted from 1 × 10⁷ cells using TRIzol Reagent (Life Technologies). Complementary DNA was synthesized from 500 ng of total RNA using SuperScript II reverse transcriptase (Life Technologies), RNaseOUT (Life Technologies), and oligo(dT)₁₈ primers (Thermo Scientific). Relative gene expression was determined using a Rotor-Gene Q Real-Time PCR Cycler (Qiagen). Reactions (10 μL) used SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), and gene expression was calculated relative to the first time point and the reference gene *GBLP* (*Chlamydomonas Beta Subunit-Like Polypeptide/Receptor of Activated Protein Kinase C1*; Schloss, 1990) according to the method of Livak and Schmittgen (2001). Primers were designed using the Web-based tools Primer3 (*CCP1*, *CCP2*, *GBLP*, *LCIA*, *LCIB*, *LCIC*, *rbcL*, and *RBCS*; Rozen and Skaletsky, 2000) or QuantPrime (*CAH1*, *HLA3*, *LCI1*, and *LCR1*; Arvidsson et al., 2008). Primer sequences are listed in Supplemental Table S5.

Detection of CCM Proteins Using Immunoblots

Cells (2.5 × 10⁷) were harvested by centrifugation, resuspended in 500 μL of protein extraction buffer (50 mM Bicine, pH 8.0, 10 mM NaHCO₃, 10 mM MgCl₂, and 1 mM dithiothreitol) and sonicated in 15-mL tubes for 15 min at 4°C on high power using a Diagenode bioruptor. Optimal lysis conditions were previously determined by visually inspecting samples under a light microscope. Unbroken cells were pelleted by centrifugation at 4,400g for 10 min at 4°C, and protein levels in the supernatant (soluble protein fraction) were quantified using Bradford reagent (Sigma-Aldrich). Proteins were separated on a 12% (w/v) polyacrylamide gel using SDS-PAGE. Sample loading was normalized by chlorophyll amount (extraction described above) for both dark/light and CO₂

time courses (1.5 μg per lane). Samples were also normalized by protein (5 μg per lane) and probed using all antibodies, which gave similar results to normalization by chlorophyll, as summarized in Supplemental Figure S1.

For immunodetection, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were probed with primary antibodies raised against Rubisco (RBCS and *rbcl*), LCIB, LCIC, or CAH1 and donkey secondary antibodies (enhanced chemiluminescence anti-rabbit IgG, horse radish peroxidase-linked whole antibody; GE Healthcare). Membranes were stripped using Restore Plus Western-Blot Stripping Buffer (Thermo Scientific) and reprobed to allow the detection of multiple proteins on a single membrane.

Transmission Electron Microscopy and Immunogold Localization

Synchronized *C. reinhardtii* cells were grown to midlog phase in 12-h/12-h dark/light cycles as described above. Prior to dawn, cells were fixed and harvested either in the dark (-2D and -1D) or after a light pretreatment (-2L and -1L) in the oxygen electrode chamber. A final sample was also prepared from cells harvested 2 h into the light period.

For fixation of cells for electron microscopy, glutaraldehyde (electron microscopy grade) was added to a final concentration of 0.5% (v/v) to cultures prior to harvest by centrifugation. Cells were then resuspended in 0.5% (v/v) glutaraldehyde and 1% (v/v) hydrogen peroxide in Tris-minimal medium and fixed for 30 min to 2 h at 4°C. All subsequent steps were carried out at room temperature (approximately 20°C).

Samples were postfixed and osmicated for 1 h in 1% (v/v) OsO_4 , 1.5% (w/v) $\text{K}_3[\text{Fe}(\text{CN})_6]$, and 2 mM CaCl_2 . After osmication, samples were stained in 2% (w/v) uranyl acetate for 1 h and serially dehydrated for 5 min each in 75% (v/v), 95% (v/v), and 100% ethanol, followed by two incubations in 100% acetone. Samples were embedded in epoxy resin containing 34% (v/v) Quetol 651, 44% (v/v) nonenyl succinic anhydride, 20% (v/v) methyl-5-norbornene-2,3-dicarboxylic anhydride, and 2% (v/v) catalyst dimethyl-benzylamine (Agar Scientific), and the resin was refreshed four times over 2 d. Samples were degassed and cured at 60°C for at least 3 h. Sections (50 nm) were obtained with a Leica Ultracut UCT Ultramicrotome and mounted on bare 300-mesh nickel grids.

For immunogold labeling of Rubisco and CAH3, fixed and embedded samples were treated to remove superficial osmium and unmask epitopes (Skepper, 2000). Grids were incubated face down on droplets of 4% (w/v) metaperiodate for 15 min and then washed with distilled water. Samples were then incubated in 1% (w/v) periodic acid for 5 min and washed in distilled water. Nonspecific binding sites were blocked by incubating grids in Tris-buffered saline with 0.001% (v/v) Triton X-100 and 0.001% (v/v) Tween 20 (TBS-TT) containing 0.1% (w/v) bovine serum albumin (BSA) for 5 min. Primary antibodies were diluted in TBS-TT plus 0.1% (w/v) BSA and incubated in humid chambers for 16 h. Grids were then washed in TBS-TT and distilled water. Grids were incubated for 1 h with secondary antibodies (goat anti-rabbit 15-nm gold conjugates; BBI Solutions) diluted 1:200 in TBS-TT plus 0.1% (w/v) BSA, washed with TBS-TT and distilled water, and dried. The CAH3 antibody was from Agriseria AB.

Samples were examined with a Technai G² transmission electron microscope at 120 kV (FEI Company) and imaged with Advanced Microscopy Techniques Image Capture Engine software. Image analysis (area measurements and particle counts) was performed using ImageJ (Abramoff et al., 2004). The fraction of gold particles in the pyrenoid was calculated for cells in which the pyrenoid and much of the chloroplast was visible. For each cell, the number of gold particles in the pyrenoid (density multiplied by area) was expressed as a percentage of the total number of particles in the chloroplast (stroma plus pyrenoid). Nonspecific labeling was determined by calculating the density of labeling in the cytosol, and this value was deducted from both the pyrenoidal and stromal densities.

Supplemental Data

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

Supplemental Figure S1. Summary of relative changes in mRNA and protein abundance during CCM induction in synchronous and asynchronous cultures.

Supplemental Table S1. Relative mRNA levels of CCM genes at the first time point of dark/light and CO_2 response time course experiments.

Supplemental Table S2. Relative mRNA levels of CCM genes at maximum expression during dark/light and CO_2 response time course experiments.

Supplemental Table S3. Relative expression of CCM genes in synchronized cells during the dark to light transition.

Supplemental Table S4. Relative expression of CCM genes in asynchronous cells adapting to low CO_2 .

Supplemental Table S5. List of primers used in this study.

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LITERATURE CITED

- Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophot Int* 11: 36–42
- Arvidsson S, Kwasniewski M, Riaño-Pachón DM, Mueller-Roeber B (2008) QuantPrime: a flexible tool for reliable high-throughput primer design for quantitative PCR. *BMC Bioinformatics* 9: 465
- Badger MR, Kaplan A, Berry JA (1980) Internal inorganic carbon pool of *Chlamydomonas reinhardtii*: evidence for a carbon dioxide-concentrating mechanism. *Plant Physiol* 66: 407–413
- Blanco-Rivero A, Shutova T, Román MJ, Villarejo A, Martinez F (2012) Phosphorylation controls the localization and activation of the lumenal carbonic anhydrase in *Chlamydomonas reinhardtii*. *PLoS ONE* 7: e49063
- Borkhsenius ON, Mason CB, Moroney JV (1998) The intracellular localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. *Plant Physiol* 116: 1585–1591
- Bozzo GG, Colman B (2000) The induction of inorganic carbon transport and external carbonic anhydrase in *Chlamydomonas reinhardtii* is regulated by external CO_2 concentration. *Plant Cell Environ* 23: 1137–1144
- Brueggeman AJ, Gangadharaiyah DS, Cserhati MF, Casero D, Weeks DP, Ladunga I (2012) Activation of the carbon concentrating mechanism by CO_2 deprivation coincides with massive transcriptional restructuring in *Chlamydomonas reinhardtii*. *Plant Cell* 24: 1860–1875
- Colombo SL, Pollock SV, Eger KA, Godfrey AC, Adams JE, Mason CB, Moroney JV (2002) Use of the bleomycin resistance gene to generate tagged insertional mutants of *Chlamydomonas reinhardtii* that require elevated CO_2 for optimal growth. *Funct Plant Biol* 29: 231–241
- Dionisio-Sese ML, Fukuzawa H, Miyachi S (1990) Light-induced carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiol* 94: 1103–1110
- Duanmu D, Miller AR, Horken KM, Weeks DP, Spalding MH (2009) Knockdown of limiting- CO_2 -induced gene *HLA3* decreases HCO_3^- transport and photosynthetic C_i affinity in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 106: 5990–5995
- Eriksson M, Villand P, Gardeström P, Samuelsson G (1998) Induction and regulation of expression of a low- CO_2 -induced mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiol* 116: 637–641
- Fang W, Si Y, Douglass S, Casero D, Merchant SS, Pellegrini M, Ladunga I, Liu P, Spalding MH (2012) Transcriptome-wide changes in *Chlamydomonas reinhardtii* gene expression regulated by carbon dioxide and the CO_2 -concentrating mechanism regulator *CIA5/CCMI*. *Plant Cell* 24: 1876–1893
- Fujiwara S, Ishida N, Tsuzuki M (1996) Circadian expression of the carbonic anhydrase gene, *Cah1*, in *Chlamydomonas reinhardtii*. *Plant Mol Biol* 32: 745–749
- Giordano M, Beardall J, Raven JA (2005) CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annu Rev Plant Biol* 56: 99–131
- Im CS, Grossman AR (2002) Identification and regulation of high light-induced genes in *Chlamydomonas reinhardtii*. *Plant J* 30: 301–313

- Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, *c*₁, and *c*₂ in higher plants, algae and natural phytoplankton. *Biochem Physiol Pflanz* **165**: 191–194
- Karlsson J, Clarke AK, Chen ZY, Huggins SY, Park YI, Husic HD, Moroney JV, Samuelsson G (1998) A novel α -type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂. *EMBO J* **17**: 1208–1216
- Kropat J, Hong-Hermesdorf A, Casero D, Ent P, Castruita M, Pellegrini M, Merchant SS, Malasarn D (2011) A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. *Plant J* **66**: 770–780
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{- $\Delta\Delta$ CT} method. *Methods* **25**: 402–408
- Ma Y, Pollock SV, Xiao Y, Cunnusamy K, Moroney JV (2011) Identification of a novel gene, *CIA6*, required for normal pyrenoid formation in *Chlamydomonas reinhardtii*. *Plant Physiol* **156**: 884–896
- Manuel LJ, Moroney JV (1988) Inorganic carbon accumulation by *Chlamydomonas reinhardtii*: new proteins are made during adaptation to low CO₂. *Plant Physiol* **88**: 491–496
- Marcus Y, Schuster G, Michaels A, Kaplan A (1986) Adaptation to CO₂ levels and changes in the phosphorylation of thylakoid proteins during the cell cycle of *Chlamydomonas reinhardtii*. *Plant Physiol* **80**: 604–607
- Meyer MT, Genkov T, Skepper JN, Jouhet J, Mitchell MC, Spreitzer RJ, Griffiths H (2012) Rubisco small-subunit α -helices control pyrenoid formation in *Chlamydomonas*. *Proc Natl Acad Sci USA* **109**: 19474–19479
- Miura K, Yamano T, Yoshioka S, Kohinata T, Inoue Y, Taniguchi F, Asamizu E, Nakamura Y, Tabata S, Yamato KT, et al (2004) Expression profiling-based identification of CO₂-responsive genes regulated by CCM1 controlling a carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiol* **135**: 1595–1607
- Moroney JV, Ma Y, Frey WD, Fusilier KA, Pham TT, Simms TA, DiMario RJ, Yang J, Mukherjee B (2011) The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles. *Photosynth Res* **109**: 133–149
- Moroney JV, Ynalvez RA (2007) Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*. *Eukaryot Cell* **6**: 1251–1259
- Ohnishi N, Mukherjee B, Tsujikawa T, Yanase M, Nakano H, Moroney JV, Fukuzawa H (2010) Expression of a low CO₂-inducible protein, LC11, increases inorganic carbon uptake in the green alga *Chlamydomonas reinhardtii*. *Plant Cell* **22**: 3105–3117
- Price GD, Badger MR, Woodger FJ, Long BM (2008) Advances in understanding the cyanobacterial CO₂-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. *J Exp Bot* **59**: 1441–1461
- Rawat M, Moroney JV (1995) The regulation of carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase oxygenase activase by light and CO₂ in *Chlamydomonas reinhardtii*. *Plant Physiol* **109**: 937–944
- Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**: 365–386
- Schloss JA (1990) A *Chlamydomonas* gene encodes a G protein β subunit-like polypeptide. *Mol Gen Genet* **221**: 443–452
- Skepper JN (2000) Immunocytochemical strategies for electron microscopy: choice or compromise. *J Microsc* **199**: 1–36
- Spalding MH (2008) Microalgal carbon dioxide-concentrating mechanisms: *Chlamydomonas* inorganic carbon transporters. *J Exp Bot* **59**: 1463–1473
- Spalding MH, Critchley C, Govindjee , Orgren WL (1984) Influence of carbon dioxide concentration during growth on fluorescence induction characteristics of the green alga *Chlamydomonas reinhardtii*. *Photosynth Res* **5**: 169–176
- Spalding MH, Orgren WL (1982) Photosynthesis is required for induction of the CO₂-concentrating system in *Chlamydomonas reinhardtii*. *FEBS Lett* **145**: 41–44
- Spalding MH, Spreitzer RJ, Orgren WL (1983) Reduced inorganic carbon transport in a CO₂-requiring mutant of *Chlamydomonas reinhardtii*. *Plant Physiol* **73**: 273–276
- Spreitzer RJ, Mets L (1981) Photosynthesis-deficient mutants of *Chlamydomonas reinhardtii* with associated light-sensitive phenotypes. *Plant Physiol* **67**: 565–569
- Spreitzer RJ, Salvucci ME (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. *Annu Rev Plant Biol* **53**: 449–475
- Thyssen C, Hermes M, Sültemeyer D (2003) Isolation and characterisation of *Chlamydomonas reinhardtii* mutants with an impaired CO₂-concentrating mechanism. *Planta* **217**: 102–112
- Tirumani S, Kokkanti M, Chaudhari V, Shukla M, Rao BJ (2014) Regulation of CCM genes in *Chlamydomonas reinhardtii* during conditions of light-dark cycles in synchronous cultures. *Plant Mol Biol* **85**: 277–286
- Turkina MV, Blanco-Rivero A, Vainonen JP, Vener AV, Villarejo A (2006) CO₂ limitation induces specific redox-dependent protein phosphorylation in *Chlamydomonas reinhardtii*. *Proteomics* **6**: 2693–2704
- Van K, Spalding MH (1999) Periplasmic carbonic anhydrase structural gene (*Cah1*) mutant in *Chlamydomonas reinhardtii*. *Plant Physiol* **120**: 757–764
- Wang Y, Duanmu D, Spalding MH (2011) Carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*: inorganic carbon transport and CO₂ recapture. *Photosynth Res* **109**: 115–122
- Wang Y, Spalding MH (2014) LCIB in the *Chlamydomonas* CO₂-concentrating mechanism. *Photosynth Res* **121**: 185–192
- Yamano T, Miura K, Fukuzawa H (2008) Expression analysis of genes associated with the induction of the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiol* **147**: 340–354
- Yamano T, Tsujikawa T, Hatano K, Ozawa S, Takahashi Y, Fukuzawa H (2010) Light and low-CO₂-dependent LCIB-LCIC complex localization in the chloroplast supports the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Cell Physiol* **51**: 1453–1468
- Yoshioka S, Taniguchi F, Miura K, Inoue T, Yamano T, Fukuzawa H (2004) The novel Myb transcription factor LCR1 regulates the CO₂-responsive gene *Cah1*, encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Cell* **16**: 1466–1477