1   **Running Head**: CCM regulation in synchronised *Chlamydomonas*

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Title of Article:

Dynamics of carbon concentrating mechanism induction and protein re-localisation during the dark to light transition in synchronised *Chlamydomonas*

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One sentence summary: In synchronised *Chlamydomonas reinhardtii* cells, the carbon concentrating mechanism is induced prior to dawn, which coincides with the re-localisation of key proteins to the chloroplast pyrenoid.

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Abstract

In the model green alga *Chlamydomonas reinhardtii*, a carbon concentrating mechanism (CCM) is induced under low CO_2 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 CO_2. Here, we have investigated the dynamics of CCM activation in synchronised cells grown in dark/light cycles, as compared to induction under low CO_2. The specific focus was to undertake detailed time course experiments comparing physiology and gene expression during the dark to light transition. Firstly, the CCM could be fully induced one hour before dawn, as measured by the $K_{0.5}$ for inorganic carbon (Ci). This occurred in advance of maximum gene transcription and protein accumulation and contrasted with the co-ordinated induction observed under low CO_2. Between two hours and one hour before dawn, the proportion of Rubisco and the thylakoid lumen carbonic anhydrase, CAH3, 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 synchronised cells during the dark to light transition as an alternative means of investigating CCM induction.

INTRODUCTION

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 $CO_{2(aq)}$. 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 CO_2 around Rubisco, thereby favouring the carboxylation reaction and improving growth in CO_2-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 CO_2 and HCO_3^- and operate in parallel within each cellular compartment (Moroney et al., 2011); and third, the localisation of Rubisco to a chloroplast microcompartment called the pyrenoid to minimise CO_2 leakage (Ma et al., 2011, Meyer et al., 2012). The CCM is induced at low (air level) CO_2 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 CO_2-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 CO_2 (for example, Spalding et al., 1983; Colombo et al., 2002; Thyssen et al., 2003). Low CO_2-induced genes investigated in this study include several Ci transporter candidates, some of which are amongst the most highly induced genes under low CO_2 (Brueggeman et al., 2012). *HLA3* encodes a plasma membrane-localised ABC-type transporter (Duanmu et al., 2009), *LCI1* encodes another plasma membrane transporter (Ohnishi et al., 2010), and *LCIA*, *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 *CAH1* gene encodes a periplasmic carbonic anhydrase and is highly CO_2-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 CO_2, although the gene is minimally CO_2-responsive and the protein is expressed equally under high and low CO_2 (Karlsson et al., 1998).

76 Expression of other CCM-related genes was also determined, including *LCR1*, which

77 encodes a MYB-domain transcription factor upstream of *CAH1* and *LCI1* and downstream of

78 CCM master regulator CIA5 (Yoshioka et al., 2004). LCIB and LCIC, which form a peri-

79 pyrenoidal complex associated with carbon recapture (Wang and Spalding, 2013), were also

80 included in this study, as were *RBCS* and *rbcL*, which encode the small and large subunits

81 of Rubisco, the major pyrenoid component (Borkhsenious et al., 1998).

82 In addition to CO_2 responsiveness, CCM activity in Chlamydomonas is associated with light

83 and photosynthesis. In particular, accumulation of Ci in Chlamydomonas cells and

84 chloroplasts is light-dependent (Spalding and Ogren, 1982) and may be driven by ATP from

85 cyclic electron transport (Spalding et al., 1984). HLA3 is a member of the ATP-binding

86 cassette superfamily, which suggests light-dependency, and *HLA3* expression is inhibited by

87 DCMU and in photosystem I and photosystem II mutants (Im and Grossman, 2002).

88 Although originally identified as a high light-induced gene, *HLA3* may in fact be responding

89 to low CO_2 levels brought about by increased photosynthesis at higher light intensities,

90 consistent with the induction of other CCM genes under these conditions (Im and Grossman,

91 2002). Similarly, *CAH1* transcription requires light, low CO_2 and photosynthetic electron flow

92 (Dionisio-Sese et al., 1990). At the protein level, the LCIB-LCIC complex relocalises from the

93 stroma to the area around the pyrenoid both in response to light and to CO_2 levels (Yamano

94 et al., 2010). However, cells acclimating to low CO_2 induce external CA activity and active

95 HCO_3^- transport even in the dark, although induction is delayed compared to cells switched

96 to low CO_2 in the light (Bozzo and Colman, 2000). This suggests that light, while an

97 important regulator of CCM activity, is not an absolute requirement for expression of CCM

98 components. Indeed, in synchronised cells grown at ambient CO_2, genes encoding putative

99 Ci transporters and mitochondrial carbonic anhydrases are transcriptionally upregulated in

100 the light, whereas other key CCM gene transcripts (*CAH3*, stromal carbonic anhydrase

101 *CAH6*, *LCIB*) reach maximum levels during the last half of the dark period (Tirumani et al.,

102 2014).

103 While the majority of experiments investigating CCM induction have used cells in

104 asynchronous cultures grown in continuous light, the aim of the present study was to

105 investigate the dynamics of CCM induction and molecular regulation in synchronised cells

106 grown in dark/light cycles, focussing on a detailed time course during the dark to light period

107 transition. Given the apparent down-regulation of the CCM in the dark (Marcus et al., 1986),

108 we hypothesised that induction of the CCM during the dark to light transition might involve

109 non-transcriptional/translational mechanisms regulating existing CCM components, unlike

110 the CO_2 response, which requires strong transcriptional up-regulation and *de novo* protein

111 synthesis.

112 To investigate possible mechanisms of CCM regulation in synchronised cells, whole cell

113 affinity for Ci ($K_{0.5}$) as well as the relative abundance of known CCM (CO_2-responsive) gene

114 transcripts and proteins were measured across the dark to light transition and

115 unsynchronised cultures adapting to low CO_2 were used as a control. We also set out to

116 determine the extent that the thylakoid lumenal CAH3 and Rubisco relocalised from the

117 stroma to the pyrenoid during the dark to light transition, since this preferential localisation

118 appears to be required for a fully functioning CCM (Blanco-Rivero et al., 2012, Meyer et al.,

119 2012).

120 Unsynchronised cells adapting to low CO_2 showed rapid increases in mRNA levels of CCM

121 genes, followed by increased protein abundance and then CCM activity. In synchronised

122 cells, CCM activity was initially down-regulated in the dark but full CCM activity was inducible

123 before dawn and also prior to maximum mRNA and protein accumulation. Rubisco and

124 CAH3 were both found to have re-localised to the pyrenoid, coincident with increased CCM

125 activity.

126 **RESULTS**

127 **The CCM is partially repressed in the dark but can be induced one hour before dawn**

128 CCM induction during the dark to light transition was measured at the whole cell and

129 molecular level to identify factors affecting CCM regulation in synchronised cultures. Whole

130 cell affinity for Ci ($K_{0.5}$) was derived using an oxygen electrode and HCO_3^--addition

131 responses, following a light pre-treatment used to deplete residual inorganic carbon

132 supplies. In synchronised cells, $K_{0.5}$ for Ci was measured from two hours before dawn to six

133 hours after dawn (Fig. 1A). A relatively high $K_{0.5}$ value (60 µM Ci) was found towards the end

134 of the dark period (-2 h), indicating that the CCM was at least partially repressed in the dark,

135 even after the short light pre-treatment. From this time point, CCM inducibility rapidly

136 increased, so that one hour before dawn, cells were able to express a fully functioning CCM

137 (as indicated by a low $K_{0.5}$ of 11 µM Ci). This high affinity for Ci was maintained through

138 dawn and the first six hours of the light period (mean $K_{0.5}$ of 11 to 19 µM Ci).

139 In order to provide a direct comparison for dark-to-light time courses, CCM induction in

140 asynchronous cultures grown in continuous light and switched to low CO_2 was also

141 investigated. The very high $K_{0.5}$ value (240 µM Ci) of high CO_2-adapted cells indicated that

142 the CCM was fully repressed at the beginning of the time course (Fig. 1B). Affinity for Ci

143 rapidly increased following the transfer to low CO_2, with the measured $K_{0.5}$ reduced to 105

144 µM Ci within one hour of the shift to low CO_2. Cells showed maximum CCM induction

145 (highest affinity for Ci) after 4-6 h at low CO_2, as indicated by low $K_{0.5}$ values (25-40 µM Ci).

146   The maximum $K_{0.5}$ value measured for high CO_2-adapted cells was greater than that

147   observed in dark-adapted (-2 h) cells, as was the overall decrease in $K_{0.5}$ during each time

148   course (ten-fold and six-fold, respectively), which suggests that the CCM was repressed to a

149   greater extent at high CO_2 than two hours before dawn.

150 **Maximum gene expression occurs after CCM induction during the dark to light**

151 **transition**

152   In this comparative study between CCM regulation during the dark to light transition and the

153   conventional CO_2 response, qRT-PCR was used to track expression of CO_2-responsive

154   genes. Gene expression was quantified relative to the control gene *GBLP* and to the first

155   time point. In synchronised cells two hours before dawn, *CCP1*, *CCP2*, *LCI1*, *LCIA*, *LCIB*

156   and *LCIC* were all present at similar levels to high CO_2-adapted cells (Supplemental Table

157   S1). *CAH1*, *LCR1* and *rbcL* were present in greater abundance while *HLA3* and *RBCS* were

158   present in lesser abundance in dark/light-grown (-2D) compared to high CO_2-grown (0 h)

159   cells. Maximum levels of *CAH1*, *CCP1*, *LCI1*, *LCIA*, *LCIB* and *LCIC* mRNA were not

160   significantly different between dark/light and CO_2 time courses (Supplemental Table S2).

161   Some differences were observed in the maximum abundance of *CCP2, HLA3*, *LCR1*, *rbcL*

162   and *RBCS* but these were below one order of magnitude and smaller than the differences

163   observed at the beginning of the time courses. Overall, this indicates that transcriptional

164   regulation during CCM induction in response to light is similar to that of CO_2.

165   Indeed, in synchronised cells, many CO_2-responsive genes (*CCP1*, *CCP2*, *LCI1*, *LCIA*, *LCIB*

166   and *LCIC*) were upregulated during the light period to a similar extent as for the CO_2

167   response (Fig. 2, Supplemental Tables S3 and S4). However, the majority of transcriptional

168   upregulation did not occur until several hours after maximum CCM activity was observed

169   (Fig. 1A , Fig. 2; see also Supplemental Fig. S1 and Table S2), contrasting with the

170   concurrent CCM activation and low CO_2-induced gene induction observed in cells adapting

171   to low CO_2 (Fig. 1B, Fig. 3; see also Supplemental Fig. S1 and Table S2).

172   Although CO_2-responsive genes were consistently upregulated in the light, there were

173   systematic differences in the timing of maximum mRNA expression compared to the CO_2

174   response. For example, inorganic carbon transporter transcripts (*CCP1*, *CCP2*, *HLA3*, *LCI1*,

175   *LCIA*), *LCR1* and *CAH1* reached maximum levels between two and four hours after dawn,

176   whereas *LCIB* and *LCIC* levels were maximal just one hour into the light period (Fig. 2,

177   Supplemental Table S2). In contrast, CCM induction following transfer from high to low CO_2

178   showed a more co-ordinated response. CO_2-responsive transcripts accumulated rapidly,

179   reaching maximum levels after approximately two hours and then showing a steady decline

180   over the next four hours (Fig. 3, Supplemental Table S2).

181   Additionally, because $K_{0.5}$ measurements required an initial period of pre-acclimation in the

182   light, gene expression was also measured in synchronised cells harvested before dawn after

183   a brief period of illumination in the oxygen electrode chamber (Fig. 2: -2L and -1L). This light

184   pre-treatment resulted in the strong upregulation of the Ci transporter genes *CCP1*, *CCP2*

185   *HLA3*, *LCI1* and *LCIA* but did not affect the expression of *CAH1* and *LCR1*. *LCIB* and *LCIC*,

186   although having a lower level of induction to begin with, also responded to the pre-dawn light

187   exposure.

188   *RBCS* (both *RBCS1* and *RBCS2*) mRNA levels remained largely unchanged throughout the

189   dark/light time course, including the pre-dawn light treatment (Fig. 2). *rbcL* transcripts

190   appeared to decrease in abundance by up to 80% both in response to the light treatment

191   and from dawn to six hours into the light, however overall abundance of both *RBCS* and *rbcL*

192   transcripts remained very high (at least one order of magnitude higher than the highly

193   abundant reference gene *GBLP*; Supplemental Tables S1 and S2). Similarly, only minimal

194   changes were detected in the abundance of genes encoding Rubisco (*RBCS* and *rbcL*)

195   throughout the CO_2 time course (Fig. 3).

196   **CCM repression in the dark is independent of key CCM protein abundance**

197   The abundance of several CCM proteins during the dark to light transition was measured

198   using immunoblots to determine whether increased mRNA abundance resulted in increased

199   protein levels (Fig. 4). To complement qRT-PCR data, pre-dawn samples (-2, -1) were

200   harvested either directly from the dark (D) or after light pre-treatment (L). Although CO_2-

201   responsive transcripts were present only at low levels two hours before dawn, their

202   corresponding proteins were easily detectable at this time. LCIB and LCIC were present at

203   high levels two hours before dawn, while CCM activity was still low. These proteins reached

204   maximum levels two to four hours into the light period, several hours after maximum CCM

205   activity occurred. CAH1 was also detectable in the dark and did not show major changes in

206   abundance during the dark to light transition. No major change in either RBCS or rbcL

207   abundance was detected during this time course. Pre-dawn exposure to light (samples -2L

208   and -1L) had minimal effect on the abundance of all proteins probed.

209   In contrast, for cells adapting to low CO_2, immunoblot analyses of LCIB, LCIC and CAH1

210   showed co-ordinated increases in mRNA abundance and protein concentration (Fig. 5; see

211   also Supplemental Fig. S1). Unlike in dark-harvested (partially CCM-repressed)

212   synchronised cells, LCIB, LCIC and CAH1 were almost undetectable in high CO_2-adapted

213   cells (Fig. 5: 0 h). However, all three proteins rapidly accumulated in response to low CO_2,

214   reaching maximum abundance after approximately three (LCIB and LCIC) to five hours

215 (CAH1) and maintaining these high levels for remainder of the time course. No changes in

216 rbcL or RBCS abundance were observed during the acclimation to low CO_2.

217 Re-localisation of Rubisco and CAH3 to the pyrenoid coincides with CCM induction at

218 end of dark period

219 Immunogold labelling was used to probe Rubisco and CAH3 localisation, which are both

220 known to be preferentially localised in the pyrenoid in response to low CO_2 (Fig. 6). Two

221 hours before dawn (-2D), approximately 75% of Rubisco-labelled particles were present in

222 the pyrenoid while, one hour later, almost 90% of labelled Rubisco was found in the pyrenoid

223 (-1D) and this high count was maintained two hours into the light period (2 h; Fig. 6A). This

224 change in Rubisco localisation coincided with the measured change in $K_{0.5}$ but was

225 independent of the dark period light pre-treatment (-2L and -1L).

226 In contrast, relative CAH3 localisation changed both before dawn in the dark and in

227 response to pre-dawn light exposure (Fig. 6B). Two hours before dawn approximately 22%

228 of CAH3 particles were in the pyrenoid (-2D) but this proportion increased to 35% one hour

229 before dawn (-1D) and reached 40% two hours into the light period (2 h). The percentage of

230 CAH3 in the pyrenoid also responded directly to the pre-dawn light treatment (increasing to

231 33% at -2L and 39% at -1L).

232 In general, mean cell area increased in response to light exposure both before and after

233 dawn (Fig. 6C). Pyrenoid area also increased in line with cell area across the time course

234 (Fig. 6D).

235 DISCUSSION

236 Since Marcus et al. (1986) identified oscillations in both CCM activity and photosynthetic

237 characteristics in synchronised cells, the Chlamydomonas CCM has been characterised to a

238 much greater extent in asynchronous cells (see reviews: Moroney and Ynalvez, 2007, Wang

239 et al., 2011). The aim of this study was thus to reconcile changes at the molecular level with

240 regulation of overall CCM activity and cellular dynamics of key CCM constituents in a

241 detailed time course during the dark to light transition. Several key differences in CCM

242 induction were observed in synchronised, as compared to asynchronous cultures, as well as

243 the differential expression of known CCM genes in response to light. This study has

244 highlighted the possible importance of additional regulatory mechanisms since the CCM was

245 inducible before dawn, without concomitant increases in mRNA or protein levels of known

246 CCM components.

Comparison of CCM induction in response to light and CO$_2$

This study systematically characterised an eight hour period covering the dark to light transition as well as the first six hours of acclimation to low CO$_2$ for comparison. Co-ordinated increases in CCM mRNA and protein abundance support the idea that CCM induction in response to low CO$_2$ relies on the *de novo* synthesis of many components. In contrast, synchronised 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 pre-dawn light exposure.

The genes analysed by qRT-PCR in the current study (*CAH1*, *CCP1*, *CCP2*, *HLA3*, *LCI1*, *LCIA*, *LCIB*, *LCIC*, *LCR1*) have all been assigned putative roles in the CCM and identified as transcriptionally upregulated in response to low CO$_2$, in at least three of four microarray or transcriptomic studies published to date (Brueggeman et al., 2012, Supplementary Dataset 1). Maximum abundance of these gene transcripts occurred after two hours at low CO$_2$ and the average fold-change from high CO$_2$ levels was generally within five-fold of the values obtained by Brueggeman et al., (2012) (see also Supplemental Table S4).

In synchronised 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 CO$_2$ (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 CO$_2$ time course (Supplemental Table S2). Strong upregulation of CCM gene transcripts was thus observed in synchronised cells but, crucially, this occurred only during the light period. Maximum mRNA levels were reached approximately two hours into the light period when cells had been expressing maximum CCM activity for the previous three hours.

Tirumani et al., (2014) also showed that Ci transporter transcripts were upregulated in the light in synchronised 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 (see for example, the discussion in Brueggeman et al., 2012 and Fang et al., 2012). Tirumani et al., (2014) also showed that gene expression for carbonic anhydrases (*CAH3*, *CAH6*) and *LCIB* was higher in the dark, although this induction was suppressed by high CO$_2$. As with the different responses of CCM genes to pre-dawn light treatment (discussed below), this suggests that CCM gene transcription responds to multiple signals, and to light and low CO$_2$ in particular. In the current study, expression of *LCIB* was measured from two hours before dawn, which may be why this dark induction of key CCM genes was not observed. Expression of *CAH3* and

282   *CAH6* was not measured in this study because these genes are minimally responsive to

283   CO_2. Finally, Tirumani et al., (2014) found that CAH3 protein only accumulates during the

284   light period but, since they did not measure CCM activity or the abundance of CAH6 and

285   LCIB, it is difficult to determine to what extent gene induction in the dark affects CCM

286   expression.

287   In the CO_2 response, *de novo* CCM protein expression was correlated with a large decrease

288   in $K_{0.5}$. qRT-PCR and immunoblot analyses for LCIB and LCIC showed maximum protein

289   abundance occurring approximately one hour after maximum mRNA abundance in both CO_2

290   and dark/light time courses. However, LCIB and LCIC were only slightly induced in the light

291   period compared to the large induction observed in response to low CO_2. In contrast, CAH1

292   levels showed no increase during the light period despite increased transcription, which

293   again distinguishes this response from the large increases in transcription and translation

294   observed in response to low CO_2. Overall, during the dark to light transition, minimal

295   induction of CAH1, LCIB and LCIC and the relatively high protein content in the dark were

296   consistent with both the lower $K_{0.5}$ and the inducibility of the CCM in the dark (Fig. 1A and 4).

Transcriptional response to light pre-treatment and inducibility of the CCM in the dark

298   Both the timing and speed of CCM induction in synchronised cells suggest that regulation

299   during the dark to light transition may not depend on the *de novo* synthesis of known CCM

300   proteins, in contrast to the low CO_2 response. The differential regulation of mRNA

301   expression and protein levels were further illustrated by the light pre-treatment used for

302   comparability with the protocol for $K_{0.5}$ measurements. Transcripts for inorganic carbon

303   transporters (*CCP1*, *CCP2*, *HLA3*, *LCI1*, *LCIA*) all responded strongly to the light pre-

304   treatment, generally reaching levels at least as high as those achieved one hour into the light

305   period of a conventional dark/light cycle. *LCIB* and *LCIC* transcripts, although induced to a

306   far lesser extent during the light period, showed a similar level of induction under pre-dawn

307   illumination. The speed and magnitude of this transcriptional response to light may be in part

308   due to the greater light levels in the oxygen electrode chamber (photon flux density 200-300

309   µmol photons m^{-2} s^{-1}) compared to growth conditions in the incubator (50 µmol photons m^{-2}

310   s^{-1}). For example, mRNA levels of a CO_2-responsive mitochondrial carbonic anhydrase

311   (CAH4) increase linearly with increasing light intensity up to 500 µmol photons m^{-2} s^{-1}

312   (Eriksson et al., 1998), well above the intensities used in this study.

313   However, not all CCM-related gene transcripts showed the same pattern of induction in

314   response to pre-dawn light exposure. *LCR1*, although slightly induced during the light period,

315   did not respond to light before dawn. More notably, *CAH1* mRNA levels also did not change

316   in response to pre-dawn light exposure, despite being strongly upregulated during the

following light period. This indicates that *LCR1* and *CAH1* transcription may be regulated in a different manner to other CO_2-responsive genes. Rawat and Moroney (1995) also identified strong oscillations of *CAH1* transcripts in synchronised 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 to 0 h (CO_2) 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 CO_2 response compared to 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 CO_2-induced CCM activity. Secondly, the transcriptional responsiveness of inorganic carbon transporter genes to pre-dawn 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 post-translational mechanisms might be important for CCM induction in synchronised cells during the dark to light transition.

Dynamics of protein abundance and re-localisation during CCM induction across the dark to light transition

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

Rubisco accumulated within the pyrenoid during the two hours 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 localisation, which suggests that changes in localisation 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 CO_2 (Borkhsenious et al., 1998).

CAH3 localisation, like Rubisco, may also be controlled by an endogenous signal that co-ordinates CCM induction. Two hours prior to dawn, 20% of CAH3 was localised to the pyrenoid but one hour later the proportion of CAH3 in the pyrenoid had nearly doubled and

this high proportion was maintained two hours into the light period. However, in cells harvested two hours before dawn and exposed briefly to light, the proportion of CAH3 in the pyrenoid was also high. This suggests that CAH3 localisation could also be directly regulated by light. This is consistent with qualitative data from immunofluorescence experiments showing that, in synchronised cells CAH3 is distributed throughout the stromal thylakoids in the middle of the dark period (-6 h) but is pyrenoid-localised in the middle of the light period (6 h; Tirumani et al., 2014). However, the current quantitative study extends the implications of these findings by showing relocalisation of CAH3 just prior to dawn (between -2 and -1 h) in the dark and in response to pre-dawn illumination (at -2 h).

CAH3 re-localisation has also been observed in cells acclimating to low CO_2 and in the current study CAH3 was present in the pyrenoid at similar relative abundance in dark- versus light-adapted cells, compared to those adapted to high versus low CO_2 (19% and 37% respectively; Blanco-Rivero et al., 2012). In the CO_2 response, this shift in localisation 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 synchronised cells. The low CO_2-inducible protein, LCI5, is also rapidly phosphorylated in response to low CO_2 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 CO_2 and light; however, this would not explain the shift in CAH3 localisation in the dark. Whatever the mechanism, the high relative localisation of CAH3 in the pyrenoid after the light pre-treatment two hours 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 CO_2 and during the dark to light transition, this study has identified several key differences that distinguish the response of synchronised 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 localisation of Rubisco and CAH3 to the pyrenoid and highlights the potential importance of post-translational regulation of the CCM. Further work will be needed to determine the mechanism of relocalisation of Rubisco and CAH3 as well as to elucidate the pathways triggered in response to the different signals. In any case, studies focussing on how components are modified, their subcellular localisation and interactions will be necessary to further our understanding of the CCM. Investigating these changes in synchronised cultures of cells during the dark to light

385 transition will provide additional means of probing these important aspects of CCM
386 regulation.

387 **MATERIALS AND METHODS**

388 **Algal strain and culture conditions:** The *Chlamydomonas reinhardtii* wild-type strain 2137
389 mt+ (Spreitzer and Mets, 1981) was maintained in the dark on Tris-acetate medium
390 (Spreitzer and Mets, 1981) agar plates supplemented with Kropat's trace elements (Kropat
391 et al., 2011). Liquid cultures were grown in an Innova 42 incubator (New Brunswick
392 Scientific, Enfield, CT, USA) at 25°C with 50 μmol photons m^{-2} s^{-1} illumination, aeration and
393 shaking (125 rpm). Starter cultures were inoculated into Tris-minimal (Tris-acetate without
394 acetate) medium from freshly replated Tris-acetate agar cells and grown to mid-log phase
395 (1-2 x 10^6 cells mL^{-1}). Experimental cultures were inoculated from starter cultures and
396 harvested at mid-log phase. High CO_2 cultures were bubbled with 5% CO_2 for six days (one
397 starter followed by one experimental culture) before switching to bubbling with air (low CO_2;
398 0.04%) for the CO_2 response time course. Synchronised cultures were continuously bubbled
399 with air, grown in 12 h:12 h dark/light cycles and harvested when at mid-log phase during the
400 third light period after inoculation. Synchronicity of cell division under these conditions was
401 confirmed by cell counts over a 72 h period from inoculation to mid-log phase (harvest).

402 **Oxygen evolution measurements:** Apparent affinity for Ci was determined using the
403 oxygen evolution method described by Badger et al. (1980). Cells grown in Tris-minimal
404 liquid medium were harvested and resuspended in 25 mM HEPES-KOH (pH 7.3) to a
405 density of 1.5 x 10^7 cells mL^{-1}. Aliquots of cells (1 mL) were added to a Clark type oxygen
406 electrode chamber (Rank Brothers, Cambridge, UK) attached to a circulating water bath set
407 to 25 °C. The chamber was closed for a light pre-treatment (200-300 μmol photons m^{-2} s^{-1}
408 illumination for 10-25 min), allowing cells to consume internal Ci stores. When net oxygen
409 evolution ceased, aliquots of $NaHCO_3$ were added to the cells at 30 s intervals and the rate
410 of oxygen evolution was recorded every second using a PicoLog 1216 data logger (Pico
411 Technologies, St Neots, UK). Cumulative concentrations of $NaHCO_3$ after each addition
412 were as follows: 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 and 2000 μM. Some of the RNA and
413 protein extraction and quantitation methods were conducted after 25 min illumination in the
414 electrode chamber to mimic a standard light pre-treatment plus Ci addition protocol.

415 **Chlorophyll extraction:** Chlorophyll was extracted from cells for normalisation of oxygen
416 evolution measurements and loading of proteins for SDS-PAGE and immunoblots.
417 Chlorophyll was extracted in 90% acetone (10 min at 60 °C) and the absorbance of the
418 supernatant was measured at 647 and 664 nm. Chlorophyll concentration was calculated
419 using the equations of Jeffrey and Humphrey (1975).

Analysis of gene expression by qRT-PCR: Quantitative reverse transcriptase PCR (qRT-PCR) was used to determine the relative abundance of CCM gene transcripts. Total RNA was extracted from 1×10^7 cells using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). cDNA was synthesised from 500 ng total RNA using SuperScript II reverse transcriptase (Life Technologies), RNaseOUT (Life Technologies) and oligo(dT)$_{18}$ primers (Thermo Scientific, Waltham, MA, USA). Relative gene expression was determined using a Rotor-Gene Q Real-Time PCR Cycler (Qiagen, Hilden, Germany). Reactions (10 µl) used SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, St Louis, MO, USA) and gene expression was calculated relative to the first time point and the reference gene *GBLP* (*CBLP/RACK1*; Schloss, 1990) according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers were designed using the web-based tools Primer3 (*CCP1, CCP2, GBLP, LCIA, LCIB, LCIC, rbcL, RBCS*; Rozen and Skaletsky, 2000) or QuantPrime (*CAH1, HLA3, LCI1, LCR1*; Arvidsson et al., 2008). Primer sequences are listed in Supplemental Table S5.

Detection of CCM proteins using immunoblots: Cells (2.5×10^7) were harvested by centrifugation, resuspended in 500 µl protein extraction buffer (50 mM Bicine pH 8.0, 10 mM NaHCO$_3$, 10 mM MgCl$_2$, and 1 mM dithiothreitol) and sonicated in 15 mL tubes for 15 min at 4 °C on high power using a Diagenode bioruptor (Diagenode, Liège, Belgium). Optimal lysis conditions were previously determined by visually inspecting samples under a light microscope. Unbroken cells were pelleted by centrifugation at 4,400 x *g* 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% polyacrylamide gel using SDS-PAGE. Sample loading was normalised by chlorophyll amount (extraction described above) for both dark/light and CO$_2$ time courses (1.5 µg per lane). Samples were also normalised by protein (5 µg per lane) and probed using all antibodies, which gave similar results to normalisation by chlorophyll, as summarised in Supplemental Fig. S1.

For immunodetection, proteins were transferred electrophoretically to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were probed with primary antibodies raised against Rubisco (RBCS and rbcL), LCIB, LCIC or CAH1 and donkey secondary antibodies (ECL anti-rabbit IgG, horse radish peroxidase-linked whole antibody, GE Healthcare, Amersham, UK). 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 localisation: Synchronised Chlamydomonas cells were grown to mid-log 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 -

455   1D) or after a light pre-treatment (-2L and -1L) in the oxygen electrode chamber. A final

456   sample was also prepared from cells harvested two hours into the light period.

457   For fixation of cells for electron microscopy, glutaraldehyde (electron microscopy grade) was

458   added to a final concentration of 0.5% (v/v) to cultures prior to harvest by centrifugation.

459   Cells were then resuspended in 0.5% (v/v) glutaraldehyde and 1% (v/v) H_2O_2 in Tris-minimal

460   medium and fixed for 30 min to 2 h at 4°C. All subsequent steps were carried out at room

461   temperature (c. 20°C).

462   Samples were postfixed and osmicated for 1 h in 1% (v/v) OsO_4 , 1.5% (w/v) $K_3[Fe(CN)_6]$

463   and 2 mM $CaCl_2$. After osmication, samples were stained in 2% (w/v) uranyl acetate for 1 h

464   and serially dehydrated for 5 min each in 75, 95, and 100% ethanol followed by two

465   incubations in 100% acetonitrile. Samples were embedded in epoxy resin containing 34%

466   Quetol 651, 44% nonenyl succinic anhydride, 20% methyl-5-norbornene-2,3-dicarboxylic

467   anhydride and 2% catalyst dimethyl-benzylamine (Agar Scientific, Stansted, UK) and the

468   resin was refreshed four times over two days. Samples were degassed and cured at 60°C

469   for at least 3 h. Sections (50 nm) were obtained with a Leica Ultracut UCT Ultramicrotome

470   (Leica Microsystems) and mounted on bare 300 mesh nickel grids.

471   For immunogold labelling of Rubisco and CAH3, fixed and embedded samples were treated

472   to remove superficial osmium and unmask epitopes (Skepper, 2000). Grids were incubated

473   face down on droplets of 4% metaperiodate for 15 min and then washed with dH_2O.

474   Samples were then incubated in 1% periodic acid for 5 min and washed in dH_2O. Non-

475   specific binding sites were blocked by incubating grids in TBS-TT (Tris buffered saline with

476   0.001% Triton X-100 and 0.001% Tween20) containing 0.1% BSA (bovine serum albumin)

477   for 5 min. Primary antibodies were diluted in TBS-TT plus 0.1% BSA and incubated in humid

478   chambers for 16 h. Grids were then washed in TBS-TT and dH_2O. Grids were incubated for

479   1 h with secondary antibodies (Goat anti-rabbit 15 nm gold conjugates, BBI Solutions,

480   Cardiff, UK) diluted 1:200 in TBS-TT plus 0.1% BSA, washed with TBS-TT and dH_2O and

481   dried. The CAH3 antibody was from Agrisera AB (Vännäs, Sweden).

482   Samples were examined with a Technai G^2 transmission electron microscope at 120 kV (FEI

483   Company, Hillsboro, OR, USA) and imaged with AMT Image Capture Engine software

484   (Advanced Microscopy Techniques, Woburn, MA, USA). Image analysis (area

485   measurements and particle counts) was performed using ImageJ (Abramoff et al., 2004).

486   The fraction of gold particles in the pyrenoid was calculated for cells in which the pyrenoid

487   and much of the chloroplast was visible. For each cell, the number of gold particles in the

488   pyrenoid (density multiplied by area) was expressed as a percentage of the total number of

489   particles in the chloroplast (stroma plus pyrenoid). Nonspecific labelling was determined by

490　calculating the density of labelling in the cytosol and this value was deducted from both the

491　pyrenoidal and stromal densities.

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634   **Figure Legends**

635   **Figure 1.** Whole cell affinity for Ci ($K_{0.5}$) measured in wild-type Chlamydomonas during CCM

636   induction in (A) synchronised cells during the dark to light transition and (B) in response to

637   low CO_2 in asynchronous cultures. Synchronised cells were grown in 12 h:12 h dark/light

638   cycles under low CO_2 and harvested during the third dark to light transition after dilution

639   (dawn = 0 h). Asynchronous cells were grown to mid-log phase in high CO_2 and harvested

640   following the switch to low CO_2 (t = 0 h). Values are mean ± 1 s.e. of three to five

641   independent experiments.

642   **Figure 2.** Expression profiles of CCM genes in synchronised cells during the dark to light

643   transition. mRNA abundance was determined using qRT-PCR and normalised to mRNA

644   levels in -2D cells and to *GBLP* (control gene) expression. Growth and harvest conditions

645   were as described in Fig. 1. During the dark period (-2 and -1 h), mRNA was harvested from

646   cells taken either straight from the dark (D) or after a brief illumination in the oxygen

647   electrode chamber (L) to mimic the light pre-treatment necessary for $K_{0.5}$ measurements.

648   Values are mean ± 1 s.e. of three to seven separate flasks harvested across at least three

649   independent experiments.

650   **Figure 3.** Expression profiles of CCM genes in asynchronous cells adapting to low CO_2.

651   mRNA abundance was determined using qRT-PCR and normalised to mRNA levels in high

652   CO_2-adapted cells and to *GBLP* (control gene) expression. Values are mean ± 1 s.e. of three

653   separate flasks harvested during a single experiment.

654   **Figure 4.** Expression profiles of CCM-related proteins in synchronised cells during the dark

655   to light transition. Soluble protein extracts were separated using SDS-PAGE and used for

656   immunoblot analyses with antibodies raised against CAH1, LCIB, LCIC and Rubisco (rbcL

657   and RBCS). Sample loading was normalised by chlorophyll content. During the dark period

658   (-2 and -1 h), protein was harvested from cells taken either straight from the dark (D) or after

659   a brief illumination in the oxygen electrode chamber (L) to mimic the light pre-treatment

660   necessary for $K_{0.5}$ measurements.

661   **Figure 5.** Expression profiles of CCM-related proteins in asynchronous cells adapting to low

662   CO_2. Soluble protein extracts were separated using SDS-PAGE and used for immunoblot

663   analyses with antibodies raised against CAH1, LCIB, LCIC and Rubisco (rbcL and RBCS).

664   Sample loading was normalised by chlorophyll content.

Figure 6. Localisation of mobile CCM components in synchronised cells during the dark to light transition. Immunogold localisation was used to determine the relative abundance in the pyrenoid of (A) primary photosynthetic carboxylase and pyrenoid component, Rubisco, and (B) thylakoid lumen-localised carbonic anhydrase, CAH3 (n=53). Cell area (C) and pyrenoid area (D) were also determined (n=80). Bars represent mean ± 1 s.e.

Figure 1.

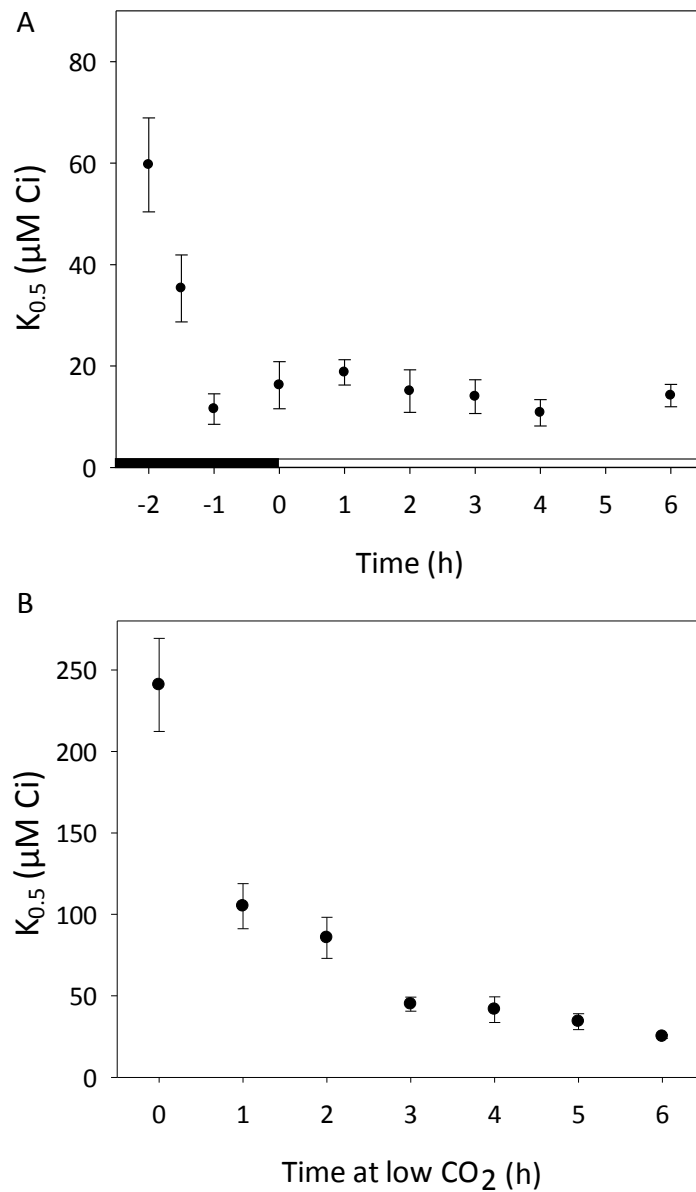**Figure 1.** Whole cell affinity for Ci ($K_{0.5}$) measured in wild-type Chlamydomonas during CCM induction in (A) synchronised cells during the dark to light transition and (B) in response to low CO_2 in asynchronous cultures. Synchronised cells were grown in 12 h:12 h dark/light cycles under low CO_2 and harvested during the third dark to light transition after dilution (dawn = 0 h). Asynchronous cells were grown to mid-log phase in high CO_2 and harvested following the switch to low CO_2 (t = 0 h). Values are mean ± 1 s.e. of three to five independent experiments.
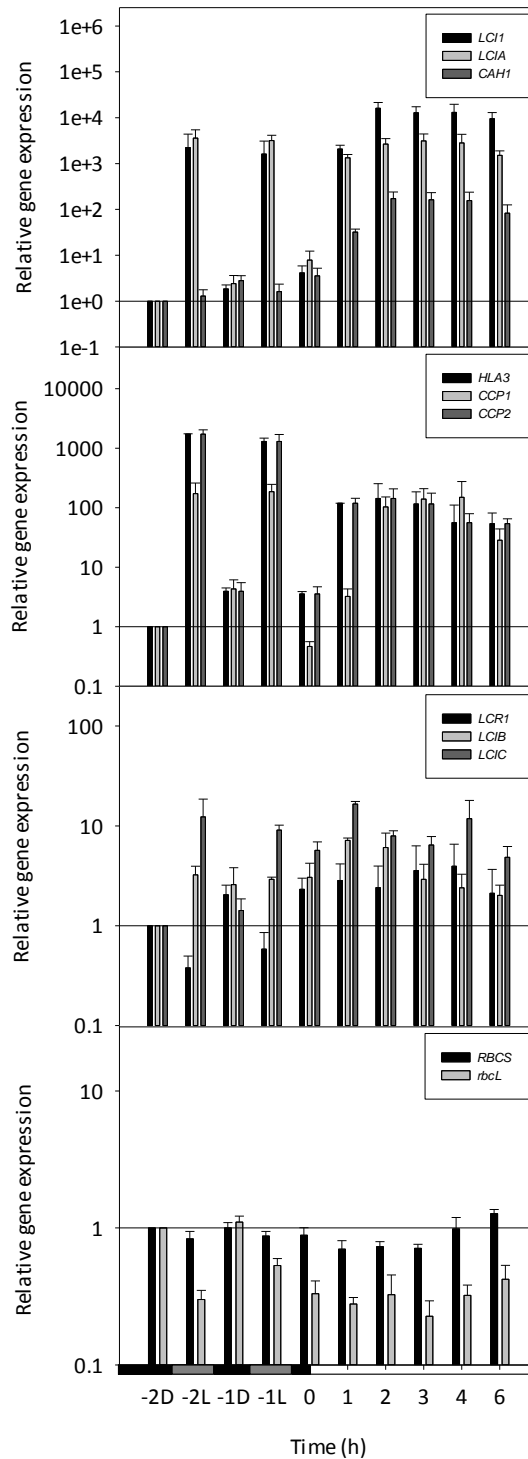
670

Figure 2.

Figure 2. Expression profiles of CCM genes in synchronised cells during the dark to light transition. mRNA abundance was determined using qRT-PCR and normalised 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 25 min illumination in the oxygen electrode chamber (Lto mimic the light pre-treatment necessary for $K_{0.5}$ measurements. Values are mean ± 1 s.e. of three to seven separate flasks harvested across at least three independent experiments.
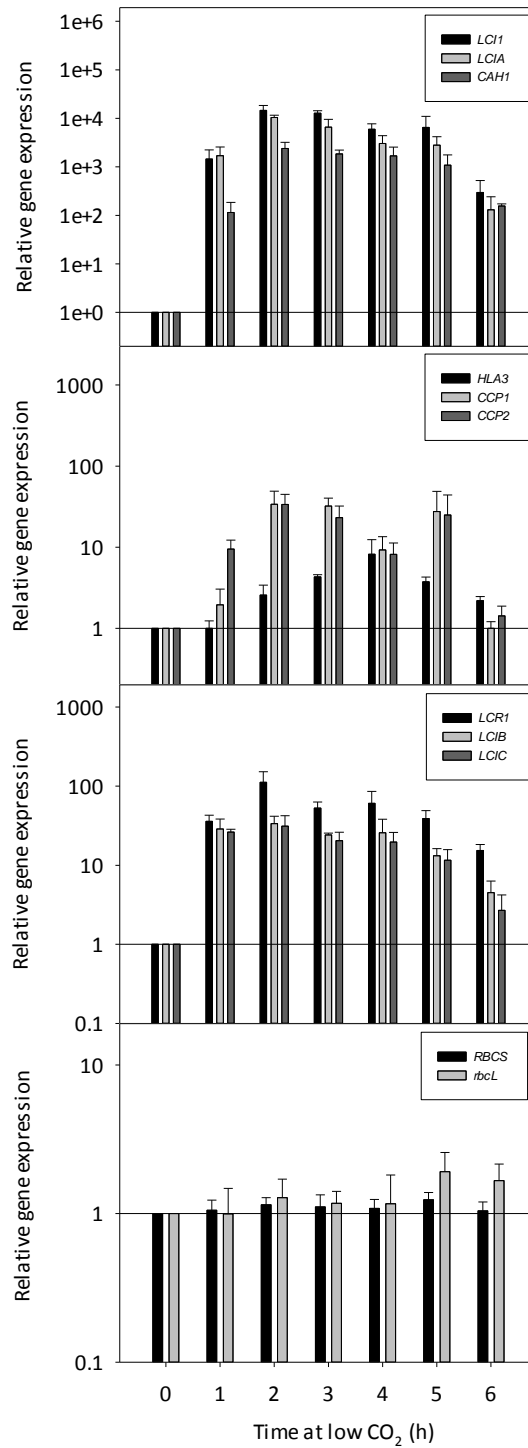
671

Figure 3.

Figure 3. Expression profiles of CCM genes in asynchronous cells adapting to low CO_2. mRNA abundance was determined using qRT-PCR and normalised to mRNA levels in high CO_2-adapted cells and to *GBLP* (control gene) expression. Values are mean ± 1 s.e. of three separate flasks harvested during a single experiment.
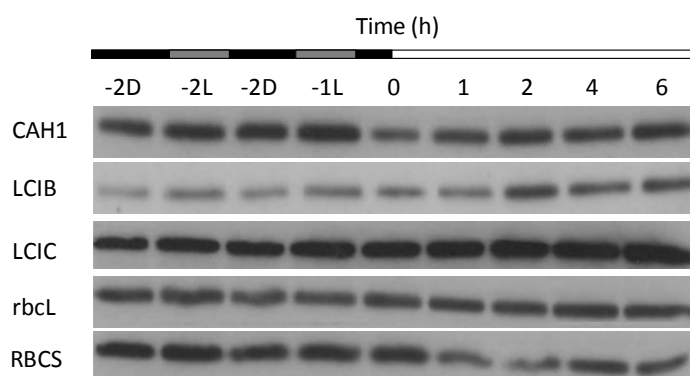
672

Figure 4.

Figure 4. Expression profiles of CCM-related proteins in synchronised 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 normalised 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 25 min illumination in the oxygen electrode chamber (L) to mimic the light pre-treatment necessary for $K_{0.5}$ measurements.

673

Figure 5.
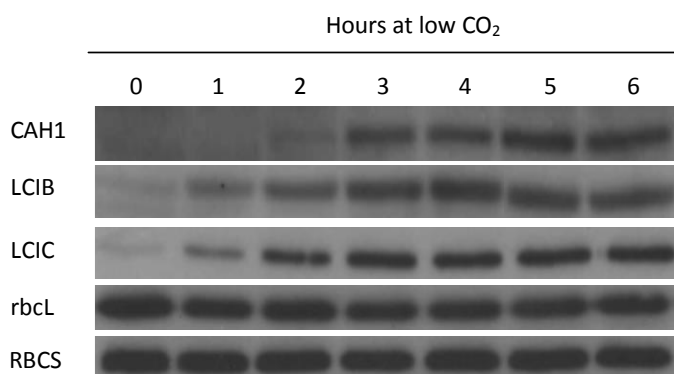
Figure 5. Expression profiles of CCM-related proteins in asynchronous cells adapting to low CO_2. Soluble protein extracts were separated using SDS-PAGE and used for immunoblot analyses with antibodies raised against CAH1, LCIB, LCIC and Rubisco (rbcL and RBCS). Sample loading was normalised by chlorophyll content.

674

Figure 6.

Figure 6. Localisation of mobile CCM components in synchronised cells during the dark to light transition. Immunogold localisation was used to determine the relative abundance in the pyrenoid of (A) primary photosynthetic carboxylase and pyrenoid component, Rubisco, and (B) thylakoid lumen-localised carbonic anhydrase, CAH3 (n=53). Cell area (C) and pyrenoid area (D) were also determined (n=80). Bars represent mean ± 1 s.e.

675

676   **Supplemental Material**

677   **Supplemental Table S1.** Relative mRNA levels of CCM genes at the first time point of
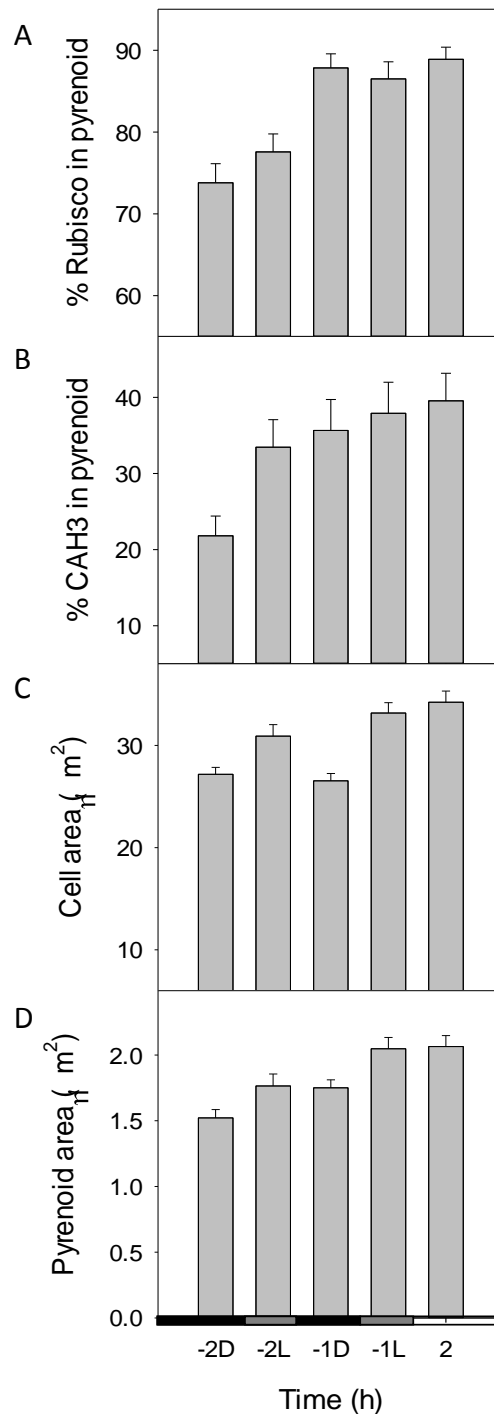
678   dark/light (-2D) and CO_2 response (0 h) time course experiments. Mean ΔCt ($Ct_{(CCM\ gene)}$ −

679    $Ct_{(GBLP)}$) values of three biological replicates are shown with one standard error of the mean

680   (s.e.). The final column compares ΔCt values for the dark/light time course to the CO_2

681   response. Transcripts were determined to be present in greater ('>', $p<0.05$), lesser ('<',

682    $p<0.05$) or equal ('=', $p\geq0.05$) abundance in -2D cells (D/L) compared to 0 h cells (CO_2) as

683   supported by a t-test. Note that a ten-fold increase in mRNA abundance will result in a

684   decrease in Ct of approximately 3.3.

Gene	D/L time course, ΔCt	CO₂ time course, ΔCt	D/L vs CO₂ (-2D vs 0 h)
CAH1	7.90 ± 0.22	12.67 ± 0.66	>
CCP1	13.79 ± 0.80	14.00 ± 0.69	=
CCP2	12.32 ± 0.50	11.25 ± 0.45	=
HLA3	12.56 ± 0.17	8.83 ± 0.96	<
LCI1	10.99 ± 0.11	10.75 ± 0.31	=
LCIA	13.20 ± 0.51	13.66 ± 0.16	=
LCIB	4.24 ± 0.16	5.88 ± 0.61	=
LCIC	8.15 ± 0.22	8.59 ± 0.47	=
LCR1	6.48 ± 0.96	10.15 ± 0.52	>
rbcL	-5.51 ± 0.02	-3.36 ± 0.52	>
RBCS	-5.06 ± 0.05	-5.45 ± 0.16	<

685

686 **Supplemental Table S2.** Relative mRNA levels of CCM genes at maximum expression
687 during dark/light (excluding the pre-dawn light treatment) and CO_2 response time course
688 experiments. Mean ΔCt ($Ct_{(CCM\ gene)} - Ct_{(GBLP)}$) values of three biological replicates are shown
689 with one standard error of the mean (s.e.). The time when maximum mRNA expression
690 occurred is also shown as Max. (h).The final column compares ΔCt values for the dark/light
691 time course to the CO_2 response. Transcripts were determined to be present in greater ('>',
692 $p<0.05$), lesser ('<', $p<0.05$) or equal ('=', $p\geq0.05$) abundance in D/L compared to CO_2 cells
693 as supported by a t-test.

Gene	D/L time course		CO_2 time course		D/L vs CO_2 (max. exp.)
	ΔCt	Max. (h)	ΔCt	Max. (h)	
CAH1	-3.20 ± 0.17	3	-3.12 ± 0.41	2	=
CCP1	0.34 ± 0.29	4	1.04 ±0.44	2	=
CCP2	8.39 ± 0.27	2	6.94 ± 0.89	2	<
HLA3	6.64 ± 0.26	4	5.19 ± 0.36	4	<
LCI1	5.94 ± 0.26	2	4.39 ± 0.24	2	=
LCIA	0.64 ± 0.34	3	0.85 ± 0.36	2	=
LCIB	3.45 ± 0.27	1	3.99 ± 0.32	2	=
LCIC	3.51 ± 0.10	1	4.52 ± 0.27	2	=
LCR1	1.61 ± 0.18	4	0.64 ± 0.58	2	<
rbcL	-5.76 ± 0.04	-1	-5.36 ± 0.17	5	>
RBCS	-3.80 ± 0.09	6	-5.51 ± 0.02	5	>

694

695 **Supplemental Table S3.** Relative expression of CCM genes in synchronised cells during
696 the dark to light transition. mRNA abundance was determined using qRT-PCR and values
697 shown are normalised to mRNA levels from the first time point (-2D) cells and to *GBLP*
698 (control gene) expression. See also Fig. 2 and Fig. S1.

Gene	Time (h)								
	-2L	**-1D**	**-1L**	**0**	**1**	**2**	**3**	**4**	**6**
CAH1	1.3	2.8	1.6	3.3	26	130	160	120	64
CCP1	170	3.9	190	0.7	100	140	180	200	120
CCP2	1700	3.4	1300	2.9	90	97	92	61	48
HLA3	61	2.0	250	1.9	4.1	150	150	200	58
LCI1	2200	1.3	1600	5.9	2300	12,000	10,000	11,000	7000
LCIA	3600	2.1	3200	7.4	970	1800	2400	2000	1100
LCIB	3.2	2.0	2.9	2.4	5.3	4.4	2.4	1.8	1.4
LCIC	12	1.3	9.1	4.3	13	5.7	5.4	7.7	3.5
LCR1	0.38	2.0	0.58	2.3	2.8	2.4	3.6	4.0	2.1
rbcL	0.30	1.1	0.53	0.33	0.28	0.33	0.23	0.32	0.42
RBCS	0.83	1.0	0.87	0.89	0.70	0.73	0.71	0.98	1.3

699

700   **Supplemental Table S4.** Relative expression of CCM genes in asynchronous cells adapting
701   to low CO_2. mRNA abundance was determined using qRT-PCR and normalised to mRNA
702   levels in high CO_2-adapted cells (t = 0 h) and to *GBLP* (control gene) expression. Values
703   listed under Bruegg. refer to the relative induction of CCM genes observed in wild-type
704   Chlamydomonas cells after 180 min adapting to very low CO_2 (Brueggeman et al., 2012,
705   Supplemental Dataset 1). See also Fig. 3 and Fig. S1.

Gene	Time at low CO_2 (h)						Bruegg.
	1	2	3	4	5	6	
CAH1	120	2400	1800	1700	1100	160	670
CCP1	4.7	29	22	8.6	22	0.86	2000
CCP2	17	32	20	8.2	16	1.4	120
HLA3	0.93	3.4	6.0	10	5.2	3.4	40
LCI1	1500	15,000	13,000	5900	6500	300	3000
LCIA	1700	10,000	6600	3000	2800	130	4200
LCIB	29	34	24	26	13	4.5	26
LCIC	26	31	20	20	12	2.7	16
LCR1	36	110	53	61	39	15	53
rbcL	1.0	1.3	1.2	1.2	1.9	1.7	-
RBCS	1.1	1.1	1.1	1.1	1.2	1.0	-

706

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

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
CAH1	TGTGCACCAGGTGACTGAGAAG	TCGCAAAGATGGGCTCAAGCAG
CCP1	TACTCGTCCACGATGGACTG	ATGTGCTCCACGTTCTCCTC
CCP2	AGTACAGCACTACCATTGAC	ATGTGCTCCACGTTCTCCTC
GBLP	AACACCGTGACCGTCTCC	TGCTGGTGATGTTGAACTCG
HLA3	AGAAGCTTAAGGACCAGGATGGC	AGTTGACGTGGGACAGCAGA
LCI1	TCCAGTTCGAGCTGTTTGTGTTCC	AGCAGGAAGAAGATGGCGTTGATG
LCIA	CTCCTCCTCAAGTGTATGAGAACG	CAGAGCAAATAGCAGCTTGG
LCIB	GAGCTGATCAAGCACTTC	CCTCAATCTTGTCCTTCA
LCIC	CGTCCATGGAGTTCATTGC	ATGCGCTTCTCCAGGTAGC
LCR1	GCGCACAGTCCCTTCGTCTAATTC	AGCGATCTCGGTCCAACTGTTAC
rbcL	GGTGACCACCTTCACTCTGG	TCACCGAAGATTTCAACTAAAGC
RBCS	GCGTGTCTTGCCTGTACTACG	CTGCTTCTGGTTGTCGAAGG

Supplemental Figure S1. Summary of relative changes in mRNA (solid line) and protein normalised by chlorophyll (dotted line) accompanying CCM induction in (left) synchronous cultures during the dark to light transition and (right) asynchronous cultures adapting to low CO_2. The black dashed line in the dark/light time course represents relative protein levels determined by protein-normalised SDS-PAGE and immunoblots. The vertical red dashed lines indicate the time at which CCM activity reached maximum levels as determined by oxygen evolution measurements (Fig. 1).