Expression Analysis of Genes Associated with the Induction of the Carbon-Concentrating Mechanism in *Chlamydomonas reinhardtii*[^1][^OA]

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Acclimation to varying CO2 concentrations and light intensities is associated with the monitoring of environmental changes by controlling genetic and physiological responses through CO2 and light signal transduction. While CO2 and light signals are indispensable for photosynthesis, and these environmental factors have been proposed as strongly associated with each other, studies linking these components are largely limited to work on higher plants. In this study, we examined the physiological characteristics of a green alga, *Chlamydomonas reinhardtii*, exposed to various light intensities or CO2 concentrations. Acclimation to CO2-limiting conditions by *Chlamydomonas* requires the induction of a carbon-concentrating mechanism (CCM) to allow the uptake of inorganic carbon (Ci) and increase the affinity for Ci. We revealed that the induction of the CCM is not solely dependent on absolute environmental Ci concentrations but is also affected by light intensity. Using a cDNA array containing 10,368 expressed sequence tags, we also obtained global expression profiles related to the physiological responses. The induction of several CCM-associated genes was strongly affected by high light as well as CO2 concentrations. We identified novel candidates for Ci transporters and CO2-responsive regulatory factors whose expression levels were significantly increased during the induction of the CCM.

By sensing CO2 availability, a number of aquatic photosynthetic organisms, including the unicellular green alga *Chlamydomonas reinhardtii*, induce a carbon-concentrating mechanism (CCM). This mechanism allows cells to concentrate inorganic carbon (Ci) intracellularly, resulting in increased photosynthetic affinity for Ci and the accumulation of Ci in close proximity to Rubisco, despite the low affinity and low selectivity of Rubisco for CO2 (Badger et al., 1980). In *Chlamydomonas*, this CCM induction is controlled by the transcriptional regulators CCM1/CIA5 (Fukuzawa et al., 2001; Xiang et al., 2001; Miura et al., 2002) and LCR1 (Yoshioka et al., 2004). Although the importance of CCM1/CIA5 as a master regulator of CCM has been demonstrated (Miura et al., 2004; Wang et al., 2005), it is unclear what sort of signal is actually perceived by cells and how CCM1/CIA5 is activated during induction of the CCM. When *Chlamydomonas* cells are grown in elevated CO2 (1% [v/v] in air or higher), they exhibit a relatively low affinity for external Ci. Therefore, the induction of the CCM is thought to depend on the environmental CO2 concentration. Recent studies in cyanobacteria have proposed several theories regarding the induction signal of the CCM (Kaplan and Reinhold, 1999; Giordano et al., 2005). These include direct sensing of external or internal Ci levels, the detection of changes in the concentrations of photorespiratory or Calvin cycle intermediates, or changes in the redox potential of the photosynthetic electron transport chain. In support of the latter theory, a link between high-light (HL) stress and Ci-limiting stress was recently suggested using cyanobacteria (Hihara et al., 2001; McGinn et al., 2003).

At low light intensities, an increase in the photon flux density correlates with increased photosynthetic carbon fixation (Elrad et al., 2002). However, as light intensities are elevated, the kinetic imbalance between the rates of photon excitation and thermally activated electron transport causes saturation of the rate of photosynthesis. Photosynthesis is then incapable of using all of the energy absorbed by the light-harvesting complexes. Excess light energy causes the relative depletion of Ci concentration in cells. In contrast, the exposure of cells to limiting Ci conditions results in the absorption of excess light energy by photosynthetic pigments. Therefore, the absorption of light and Ci uptake need to be well balanced for the efficient conversion of CO2 into carbohydrates. Furthermore, considering that blocking photosynthetic electron transport markedly decreases the expression levels of low-CO2 (LC)-inducible genes (Dionisio-Sese et al., 1990; Im and Grossman, 2002) and the ability of cells to accumulate Ci (Badger et al., 1980),

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Both Light Intensity and CO₂ Concentration Affect Photosynthetic Characteristics and CCM Induction in *Chlamydomonas*

It has been reported that HL stress alone did not trigger the induction of the CCM in cyanobacteria (McGinn et al., 2003; Woodger et al., 2003). To understand whether the affinity for Ci and CCM induction is affected by light intensities in *Chlamydomonas*, the photosynthetic rates and the half-saturation constants for Ci ($K_{0.5(Ci)}$) of cells grown under different environmental conditions for 12 h were measured using an O₂ electrode (Fig. 1). When cells were grown under high-CO₂ (HC) conditions, they exhibited low affinity for Ci ($K_{0.5(Ci)}$ was 263–335 μM) over a wide range of light intensities (30–1,000 μmol photons m⁻² s⁻¹). On the other hand, when cells were grown under LC conditions (less than 0.1% CO₂), they showed significantly higher affinity for Ci ($K_{0.5(Ci)}$ was 19–45 μM). A slight increase of $K_{0.5(Ci)}$ was observed under 0.01% and 0.04% CO₂ conditions as the light intensity increased, possibly because the suppression of CO₂ fixation caused by the limitation of Ci supply enhanced the extent of photo-inhibition in *Chlamydomonas* (Murata et al., 2007). Actually, the values of $V_{\text{max}}$ decreased from 164 to 93 μmol O₂ mg⁻¹ chlorophyll h⁻¹ and from 98 to 90 μmol O₂ mg⁻¹ chlorophyll h⁻¹ under 0.01% and 0.04% CO₂ conditions, respectively, as the light intensities increased from 30 to 1,000 μmol photons m⁻² s⁻¹. When cells were grown under medium-CO₂ (MC) conditions at 30 μmol photons m⁻² s⁻¹, they showed low affinity for Ci ($K_{0.5(Ci)}$ was 271 μM). As the light intensity increased, however, the affinity for Ci increased significantly ($K_{0.5(Ci)}$ was 201 μM at 120 μmol photons m⁻² s⁻¹, 149 μM at 600 μmol photons m⁻² s⁻¹, and 69 μM at 1,000 μmol photons m⁻² s⁻¹).

It was possible that the increase in photosynthetic activity due to HL may have caused a drastic decrease in the Ci levels of the culture medium. To examine this possibility, we measured the Ci concentrations in the medium with and without cells in different conditions by gas chromatography (Table I). The Ci concentrations in medium without cells aerated with air containing 5%, 1.2%, and 0.04% CO₂ for 12 h were 5,180, 1,390, and 49.0 μM, respectively. When cells were cultured at 120 μmol photons m⁻² s⁻¹ for 12 h, the Ci concentrations in the medium were 4,840, 1,110, and 8.2 μM, respectively. The Ci concentrations under HL conditions at 1,000 μmol photons m⁻² s⁻¹ were 4,920, 927, and 7.5 μM, respectively, after 12 h of growth. In these experiments, the cell densities of the cultures after 12 h of growth were kept at less than 3 × 10⁷ cells mL⁻¹, which corresponded to an optical density at 730 nm of 0.4, to avoid shading effects of the cells. A slight decrease in the Ci concentrations from 5,180 to 4,840 μM and from 1,390 to 1,110 μM was observed under the 5% CO₂ and 1.2% CO₂ conditions, respectively, at 120 μmol photons m⁻² s⁻¹. In contrast, when the cells were aerated with ordinary air containing 0.04% CO₂, the Ci concentration dropped from 49 to 8.2 μM. These results were consistent with the previous finding that the proportion of CO₂ removed from the supplied gas dramatically increased when the CO₂ concentration was less than 0.5% (Vance and Spalding, 2005). A slight decrease in the Ci concentration from 1,110 to 927 μM was observed under the 1.2% CO₂ conditions when the cells were exposed to varying CO₂ concentrations and light intensities.
condition when the light intensity increased from 120 to 1,000 μmol photons m\(^{-2}\) s\(^{-1}\). However, the Ci concentration of 927 μM was more than 100-fold higher than 8.2 or 7.5 μM under the 0.04% CO\(_2\) condition. These results indicate that the increase in affinity for Ci under the 1.2% CO\(_2\) condition was apparently affected by light intensity.

To evaluate the physiological changes during the CCM induction process, the levels of photosynthetic CO\(_2\)-exchange activity at 50 μL L\(^{-1}\) CO\(_2\) and the Ci concentration in the culture medium were measured simultaneously using an open gas-analysis system and gas chromatography (Fig. 2). First, cells grown under HC at medium-light (ML) conditions were transferred to HC at HL conditions, and samples were taken for measurements at 0.3, 1, 2, and 6 h after transfer. Under HC at ML conditions, cells showed a low level of light-dependent CO\(_2\)-exchange activity of 18.0 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\). The level of the CO\(_2\)-exchange activity doubled to 36.2 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) at 6 h after transfer to HC at HL conditions. Considering that the CCM was not induced under HC at ML conditions, the CO\(_2\)-exchange activity was probably caused by the high photosynthetic rate due to HL. The Ci concentrations under HC at ML and HC at HL conditions at 6 h were 4,840 and 4,820 μM, respectively.

Next, cells grown under HC at ML conditions were transferred to LC at ML conditions. The level of the CO\(_2\)-exchange activity was 85.2 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) at 6 h with a Ci concentration of 8.7 μM. The increase in the CO\(_2\)-exchange activity was 4.7-fold compared with that under HC at ML conditions. This increase was higher than the 2.0-fold change when the light intensity was changed from ML to HL. These data coincide with the previous finding that there is a CO\(_2\)-dependent inducible CO\(_2\) uptake system in *Chlamydomonas*, as determined by measurements of the CO\(_2\)-exchange activity (Spalding and Ogren, 1985).

When cells under HC at ML conditions were transferred to LC at HL conditions, the level of the CO\(_2\)-exchange activity was 91.2 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) at 6 h with a Ci concentration of 8.1 μM. When cells under HC at ML conditions were transferred to MC at HL conditions, the level of the CO\(_2\)-exchange activity was 56.1 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) at 6 h with a Ci concentration of 1,070 μM. By increasing the light intensity from 120 to 1,000 μmol photons m\(^{-2}\) s\(^{-1}\), the CO\(_2\)-exchange activity under MC at HL conditions was 3.9-fold higher than that under MC at ML conditions, although the difference between the Ci concentrations in the respective media was negligible. The CO\(_2\)-exchange activity of 56.1 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) at 6 h under MC at HL conditions was 1.5-fold higher than that of 36.2 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) at 6 h under HC at HL conditions. Considering that the CO\(_2\)-exchange activity of 56.2 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) under MC at HL conditions at 12 h, with a low K\(_{0.5}\)(Ci) value of 69 μM supporting the operation of the CCM (Fig. 1), was almost the same as that of 56.1 μmol CO\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\) under MC at HL conditions at 6 h, the increase of CO\(_2\)-exchange activity under MC at HL conditions at 6 h could be supported not only by an increase in the photosynthetic rate due to HL, as in the case of HC at HL conditions, but also by the CCM. These results indicate that the increase in CO\(_2\)-exchange activity was partly supported by the CCM and that the increase is affected by light intensity as well as Ci concentrations in the culture medium.

### Table I. Measurement of Ci concentrations in medium with or without cells under different CO\(_2\) concentrations and light intensities

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<th>Medium with Cells</th>
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<tr>
<td>0.04</td>
<td>49.0 ± 4.9</td>
<td>8.2 ± 1.0</td>
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Identification of Transcriptome Dynamics in Response to Changes of Light Intensity and CO\(_2\) Concentration

To identify the genes that possibly contribute to the physiological responses to HL and/or LC stresses, we obtained expression profiles under various light intensities and CO\(_2\) concentrations using a cDNA array containing 10,368 EST clones. *Chlamydomonas* cells grown under HC at ML conditions, used as a control, were transferred to HC at HL, LC at ML, or MC at HL conditions, and then the cells were collected at 0.3, 1, 2, and 6 h after transfer to each of the stress conditions. mRNA samples were isolated from these cells and used for array analyses. Fold change values were calculated for duplicate RNA samples from each stress condition and compared with the control value, and expression ratios for each time point were generated. We drew scatterplots of hybridization signals to confirm the quality of our array analyses (data not shown) as described in the previous article dealing with the same macroarray membranes (Miura et al., 2004). We regarded genes with an expression ratio of more than 2.5-fold as up-regulated and those with an expression ratio of less than 0.4-fold as down-regulated, because the same cutoff values were used in our previous papers (Miura et al., 2004; Yoshioka et al., 2004) and the previously identified CCM-related genes could be reliably selected using these criteria.

Based on these criteria, we identified 387 HL- and/or LC-inducible genes (Fig. 3). The transcript levels of 277, 32, and 132 genes were significantly up-regulated under LC at ML, HC at HL, and MC at HL conditions, respectively. Only one gene, *Li818r-1*, was up-regulated...
under all three conditions. Of the 277 genes induced under LC at ML conditions, 40 genes were also induced under MC at HL conditions. Considering that the CCM was induced under both LC at ML and MC at HL conditions (Figs. 1 and 2), it is possible that these 40 genes up-regulated under both LC at ML and MC at HL conditions are strongly related to the induction and/or function of the CCM.

To assign functions of the genes, BLASTN searches against the *Chlamydomonas* genome database (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) were performed and the annotations were confirmed by BLASTP and RPS-BLAST searches against the GenBank database. All up-regulated genes under LC at ML and MC at HL conditions are listed in Table II, and all up-regulated genes under HC at HL conditions are listed in Table III. The refined annotations of all genes spotted on our cDNA array are provided as supplemental information (Supplemental Table S2).

**Screening Genes Induced during Induction of the CCM**

The cDNA array experiments revealed that the levels of 40 different transcripts were up-regulated
under LC at ML and MC at HL conditions but not under HC at HL conditions (Table II). Previously identified LC-inducible genes were included in this group, such as LciA, which is also annotated as Nar1,2 (Miura et al., 2004; Mariscal et al., 2006), LciB, LciC, and LciD (Miura et al., 2004; Wang and Spalding, 2006), Sta2 (Miura et al., 2004), Cah1 (Fukuzawa et al., 1990), Mca (Eriksson et al., 1996), Cah3 (Karlsson et al., 1998), Ccp1 and Ccp2 (Chen et al., 1997; Pollock et al., 2004), Lci1, Lci3, and Lci5 (Burow et al., 1996), Shin1 (Im and Grossman, 2002), and Hla3 (Im and Grossman, 2002).

We previously reported that the expression of 51 genes was induced within 1 h under LC conditions and that almost all of these genes were regulated by CCM1/CIA5 (Miura et al., 2004). Of the 51 genes, the expression of 24 genes was significantly induced under both LC at ML and MC at HL conditions (denoted in Table II). Another 24 genes were induced under LC at ML conditions but not under the MC at HL conditions. The other three genes, 135f03, 138e10, and 011d04, were not assigned as inducible genes in this study because of the low reliabilities of their normalized values.

As described above, the level of CO₂-exchange activity increased significantly supported by the CCM when cells were exposed to LC at ML and MC at HL conditions. The increase of the CO₂-exchange activity after transfer to MC at HL conditions was delayed compared with that of LC at ML conditions (Fig. 2). Similar correspondences were observed in the expression patterns of the 40 inducible genes between LC at ML and MC at HL conditions. Under LC at ML conditions, the induction of mRNA expression of these genes started within 1 h. In contrast, under MC at HL conditions, the induction was delayed in comparison with those of LC at ML conditions and started at 6 h. From these results, it was thought possible that the 40 up-regulated genes are, directly or indirectly, related to the induction and/or function of the CCM.

Of the 40 genes, CO₂-dependent induction of 15 genes has been reported previously. We carried out semi-quantitative reverse transcription (RT)-PCR analyses of the other 25 genes using specifically designed primer sets (Supplemental Table S1) with a number of PCR cycles sufficient to obtain visible product in at least one treatment (Fig. 4A). Of the 25 genes, induction of 23 genes was confirmed, all except for 168f04 and 164h03. Thus, we named these 23 genes Lci7 to Lci29 and provide detailed annotations. The transcripts of Lci9, Lci13, Lci19, Lci20, Lci24, and Lci26 were barely detected in the Ccm1 mutant C16, indicating that these six genes were clearly regulated by the CCM1. On the other hand, the transcripts of several genes, such as Lci7, Lci8, Lci22, Lci25, and Lci29, were increased in the C16 background.

### Ci Transporter Candidates

Although Ci uptake systems have not been elucidated in eukaryotic plants, including *Chlamydomonas*, previous studies have identified several candidate genes responsible for Ci uptake, such as Lci1 (Burow et al., 1996), LciA (Miura et al., 2004; Mariscal et al., 2006), LciB, LciC, and LciD (Miura et al., 2004; Wang and Spalding, 2006), and Hla3 (Im and Grossman, 2002). Considering that cyanobacteria have five types of Ci uptake systems (Omata et al., 1999; Ohkawa et al., 2000a, 2000b; Shibata et al., 2001, 2002a, 2002b; Price et al., 2004) and that *Chlamydomonas* acclimates to variable CO₂-limiting conditions (Wang and Spalding, 2006), it is reasonable to assume the presence of multiple Ci transport systems in *Chlamydomonas*.

We previously reported that LciB and LciC were strongly up-regulated within 1 h by CO₂-limiting stress through CCM1/CIA5 (Miura et al., 2004). The time-course array experiments used here showed that LciB and LciC were rapidly induced within 0.3 h (6.9- and 10.2-fold, respectively) and that mRNA accumulation peaked within 2 h and then decreased under LC at ML conditions. A genetic approach succeeded in the isolation of a *pmp1* allelic mutant, *ad1*, and molecular analysis revealed that the *Ad1/Pmp1* protein was encoded by *LciB* (Wang and Spalding, 2006). Considering that *ad1*, like *pmp1*, could not grow under LC at ML conditions, the LciB protein is essential to acclimate to CO₂-limiting conditions.

The LciB gene is a member of a unique, small gene family that consists of LciB, LciC, LciD, and LciE (Wang and Spalding, 2006; Grossman et al., 2007; Spalding, 2008). The transcript level of LciD also increased under LC at ML (16.5-fold at 1 h) and MC at HL (3.0-fold at 6 h) conditions. Since LciE was not spotted on our cDNA array, we confirmed its CCM1-dependent induction under LC at ML conditions by RT-PCR (Fig. 4B). As described in previous reports, LciD and LciE are linked with another pair of CO₂-responsive genes, Ccp2 and Ccp1, respectively, in an inverted repeat.
Table II. Up-regulated genes under LC at ML and MC at HL conditions revealed by cDNA array analyses

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Notes:
- **EST clone identifiers of the cDNA array membrane.
- *GenBank accession numbers for the EST clones.
- Relative expression levels calculated by dividing the expression levels of cytoplasmic DNA-treated cells by those of wild-type cells under LC at ML conditions for 1 h.
- Transcript identifiers in the joint Genome Institute Chlamydomonas genome database version 3.
- Genes with e-values of less than e-10 are annotated.
- A microarray hybridization experiment was performed.

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(Wang and Spalding, 2006; Grossman et al., 2007; Spalding, 2008). Considering that Ccp1 and Ccp2 were induced under LC at ML and MC at HL conditions (Table II) and that some stress-responsive genes have a tendency to form gene clusters in the Chlamydomonas genome (Galván et al., 2002), this set of genes...
Table III. Up-regulated genes under HC at HL conditions revealed by cDNA array analyses

Time course points when the relative expression levels are greater than 2.5-fold are underlined. Dashes indicate not applicable.

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*EST clone identifiers of the cDNA array membrane. **GenBank accession numbers for the EST clones. †Transcript identifiers in the Joint Genome Institute Chlamydomonas genome database version 3. ‡Genes with e-values of less than e-10 are annotated. §Motifs are annotated by the conserved domain database using RPS-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). ¶Genes whose induction is regulated by CCM1/CIA5 (Miura et al., 2004). ¶¶could function in a coordinated manner when the CCM is in operation. The arrangement of the gene loci of LciD, LciE, Ccp1, and Ccp2 implies that the other family members, LciB and LciC, could also have a “head-to-head gene pair.” The LciB locus is located on scaffold 4:1163348 to 1165522 in the Chlamydomonas genome, and an unidentified gene (transcript identifier, acegs_kg.scaffold_4000041) is linked with LciB in a head-to-head orientation. The predicted amino acid sequence of the gene product has six transmembrane regions and a significant level of similarity with the triose-P transporter family (pfam03151). Although the transcript level of this gene (array identifier, 154d06) was not affected by
changes in CO₂ concentration or light intensity (Supplemental Table S2), it is possible that the gene product functions in association with the LciB protein. The LciC locus is located on scaffold 12:578432 to 581515, but a head-to-head gene pair was not detected for LciC.

Lci10, encoding an Arabidopsis (*Arabidopsis thaliana*) T20K12.220 (Q9M2D2) homolog, was up-regulated under LC at ML (18.1-fold at 2 h) and MC at HL (5.7-fold at 6 h) conditions. The Arabidopsis mutant of DiT2 is one of the classic mutants in the photorespiratory pathway. The mutant is deficient in the transport of dicarboxylates across the chloroplast envelope membrane and requires HC levels for survival (Renné et al., 2003). The predicted amino acid sequence of the Lci19 protein was also shown to have significant similarities with anion transporters in some bacteria and to have eight transmembrane regions.

**Putative Regulatory Factors of the CCM**

Although the signal that induces the CCM has not been elucidated, there is strong evidence that CCM1/ CIA5 is a master regulator of the CCM in *Chlamydomonas*. Figure 4. Expression profiles of genes identified by cDNA array analyses. A, RT-PCR analyses of genes identified by cDNA array analyses in the wild-type strain C9 and the Ccm1 mutant C16. The genes whose inducible expression was confirmed by RT-PCR are designated Lci7 to Lci29. The Cblp gene was used as a loading control. HC, High CO₂ at medium light; LC, low CO₂ for 2 h at medium light. Asterisks indicate that the RNA samples were collected at 0.5 h after transfer to LC conditions. B, RT-PCR analyses of LciB family genes in C9 and C16. C, Northern-blot analyses of Lii81r-1 and Lii81r-3. Total RNA samples isolated from wild-type C9 and C16 were hybridized with 32P-labeled gene-specific probes for Lii81r-1 and Lii81r-3. Images of the ethidium bromide-stained gel are shown as loading controls. ML, High CO₂ at medium light; HL, high CO₂ at high light for 1 h. D, RT-PCR analyses of Lii81r-1 and Lii81r-3 in C9 and C16.
monas (Fukuzawa et al., 2001; Xiang et al., 2001; Miura et al., 2002, 2004; Wang et al., 2005). It is suggested that CCM1/CIA5 is constitutively expressed and may be posttranslationally modified by the addition of some molecules that can dramatically change the molecular size of the protein (Wang et al., 2005). The evidence that the transcriptional level of Ccm1 (array identifier, 019b03) was extremely low and was not affected by changes of light intensity or CO2 concentration (Supplemental Table S2) also suggested that there could be a posttranslational modification of CCM1/CIA5 for its activation.

An earlier study of ours revealed that LCR1 was a CO2-responsive MYB DNA-binding transcriptional regulator in Chlamydomonas and functions as a transmitter of a LC signal from CCM1/CIA5 to at least three CO2-responsive genes, Cah1, Lci1, and Lci6 (Yoshioka et al., 2004). To identify LC-inducible transcriptional factors regulating CO2-responsive genes other than LCR1, we obtained expression profiles of cells treated with cycloheximide at a concentration of 10 µg ml−1 under LC at ML conditions for 1 h (Table II). The induction of several genes, such as LciA, LciB, LciC, LciD, and Sta2, occurred within 0.3 h, and the inhibition of protein synthesis had no effect on the expression of these genes. On the other hand, the induction of Cah1 and Lci1 expression was cycloheximide sensitive, confirming that de novo protein synthesis of LCR1 was required to fully induce these genes, as described previously (Yoshioka et al., 2004). The expression of other CCM-related genes (e.g. Mca, Hla3, Lci3, Lci18, and Lci24) was also cycloheximide sensitive. Although we cannot eliminate the possibility that cycloheximide itself has a direct effect on expression, it is possible that there could be LC-responsive transcriptional factors other than LCR1 regulating these cycloheximide-sensitive genes. From our array data, three unidentified genes encoding putative regulatory factors were significantly induced during induction of the CCM.

Lci14, encoding a mitochondrial matrix protein homolog in Chlamydomonas (Haring and Beck, 1997), was induced under LC at ML (23.3-fold at 2 h) and MC at HL (3.2-fold at 6 h) conditions. The function of this mitochondrial matrix protein homolog is unknown, but the amino acid sequence showed significant similarity with that of PRL1-interacting factor L of Arabidopsis (Németh et al., 1998). Since the mutation of PRL1 results in transcriptional derepression of Glc-responsive genes in Arabidopsis (Németh et al., 1998), PRL1-interacting factor L is also expected to be associated with Glc signaling. A homolog of PRL1 was also found in the Chlamydomonas genome, but the EST clone was not spotted on our array. The C-terminal region of Lci14 protein was shown to have a putative GTPase domain (COG0523) and a WW domain (smart00456). The WW domain binds to proteins with particular Pro motifs and to other domains typical of proteins in signal transduction processes, suggesting that the Lci14 protein could be associated with signal transduction. Lci14 was predicted to localize to the nucleus, not to the mitochondria, and to have no transmembrane domains. In addition, mRNA expression of Lci14 was induced within 0.3 h under LC at ML conditions, and Lci14 was not induced in the Ccm1 mutant C16. This rapid induction and Ccm1 dependence of the expression of Lci14 were similar to those of Lcr1, encoding a MYB transcription factor (Yoshioka et al., 2004), suggesting that the Lci14 protein could function in regulating some parts of the CCM.

Lci16, encoding an HVA22/DP1 gene product-related protein and harboring a TB2/DP1, HVA22 family domain (PF03134), was up-regulated under LC at ML (6.7-fold at 2 h) and MC at HL (2.7-fold at 6 h) conditions. It has been reported that the expression of barley (Hordeum vulgare) HVA22 is induced by several environmental stresses, such as dehydration, salinity, and extreme temperatures, as well as by a plant stress hormone, abscisic acid (Brands and Ho, 2002).

Lci25 was also up-regulated under LC at ML (2.6-fold at 2 h) and MC at HL (2.6-fold at 6 h) conditions. The C-terminal region of the predicted amino acid sequence was shown to have similarity to the U-box domain (PF04564). The U-box motif is a conserved domain found in diverse isoforms of E3 ubiquitin ligase in eukaryotes. While little is known about whether protein degradation is involved in LC stress acclimation, it has been reported that several stress-inducible proteins harboring a U-box domain play important roles in the cellular metabolism of plants under cold temperature, drought, high salinity, and mechanical wounding stress conditions in Arabidopsis (Yan et al., 2003; Cho et al., 2006).

Photosynthesis and CO2 Fixation

Under LC at ML conditions, the transcript levels of photosynthetic genes, such as PsbP (array identifier, 021d10), Gdh1 (024a11), Agat (010d06), Aat1 (145g10), GspP (003b05), Smt1 (159g02), Fhs (169e06), Sgat (021c08), Gs (033a04), Cfg3 (168e10), and Car8 (167b10), showed transient induction (Supplemental Table S2). On the other hand, genes for the Calvin cycle, such as RbcS1 (119e05), Ald3 (004b01), Pgk (011f10), Gap3 (012e07), and Csbp (119b07), were down-regulated under LC at ML conditions (Supplemental Table S2). A decrease in biosynthesis of the Rubisco small subunit during acclimation to CO2-limiting conditions is also controlled at the translational level (Winder et al., 1992). However, these genes were not significantly affected by HL conditions. These observations coincided with the fact that the chloroplast chaperon Cpn60α (165e06) and Cpn60β (124b11), which are molecular chaperons for Rubisco, were only significantly affected under LC at ML conditions.

Starch Synthesis

Lci8 was up-regulated under LC at ML and MC at HL conditions. Under LC at ML conditions, the level of the Lci8 mRNA rapidly increased (8.5-fold at 0.3 h)
and maintained a maximum level until 6 h (41.2-fold). A portion of the predicted amino acid sequence contained a starch-binding domain (PF00686). *Sta2*, encoding granule-bound starch synthase I, was also up-regulated under LC at ML and MC at HL conditions. Considering that *Chlamydomonas* develops pyrenoid structures and that starch sheaths cover the pyrenoid under LC conditions (Ramazanov et al., 1994), *Lci8* and *Sta2* could be necessary for the elongation and formation of starch sheaths and developing pyrenoid structures. A developed pyrenoid structure was also observed in cells grown under MC at HL conditions (data not shown). The transcript of *Sta2* was reported to accumulate under LC at HL conditions (Im et al., 2003). Under conditions of excess excitation, starch synthesis may serve as an electron valve that can productively use excess reductant and ATP generated by photosynthetic electron transport for energy storage. In contrast, the transcript levels of *Pgm* (array identifier, 170a08), whose product catalyzes the conversion of Glc-6-P into ADP-Glc (Ball, 1998), were down-regulated under LC at ML conditions (Supplemental Table S2).

**Up-Regulated Genes under HC at HL Conditions**

As shown in Table III, 32 genes were significantly induced under HC at HL conditions, with seven LC-inducible genes included in this group. Considering that the CCM was not induced under HC at HL conditions (Figs. 1 and 2), these LC-inducible genes may not be directly related to the CCM.

A previous study reported that six LHC-like (Lhl) genes, *Lhl1*, *Lhl2*, *Lhl3*, *Lhl4*, *Li818r-1*, and *Li818r-3*, which are distant relatives of light-harvesting chlorophyll a/b-binding proteins in higher plants, are encoded by the *Chlamydomonas* genome (Elrad and Grossman, 2004). The transcript levels of these genes, except for *Lhl3*, are significantly increased by HL stress (Teramoto et al., 2004). Our study showed that *Li818r-1* demonstrated early induction under all three conditions (Table III). Considering that *Li818r-1* is not induced in C16 (Miura et al., 2004) and that this gene is also up-regulated by sulfur deprivation (Zhang et al., 2004), *Li818r-1* could be regulated by multiple signaling pathways. The EST clone of *Li818r-3* was not spotted on the array, but northern-blot analysis using a specific probe revealed that *Li818r-3* was strongly induced by HL stress (Fig. 4C). On the other hand, the transcript level of *Li818r-3* was down-regulated by CO₂-limiting stress (Fig. 4, C and D). The expression responses of *Li818r-1* and *Li818r-3* were impaired in C16 under LC at ML conditions but not under HC at HL conditions, suggesting that multiple regulatory pathways, including the CCM1 pathway, regulate these genes. Although their physiological functions have not been characterized, the *Li818r-1* protein has been suggested to play a role in protecting chlorophyll proteins from excitation pressure under LC as well as HL conditions (Richard et al., 2000; Iwai et al., 2007).

The predicted amino acid sequence of the EST clone 019e02 showed a high level of sequence similarity to the Arabidopsis PG5 protein, and this gene was designated *CrPgr5*. *CrPgr5* was up-regulated under LC at ML (3.1-fold at 2 h) and HC at HL (2.8-fold at 0.3 h) conditions. It has been reported that the PSI in the pgr5 mutant is damaged by oxidative stress under HL and LC conditions, even under low light intensities, because of a complete lack of transient induction of nonphotochemical quenching (Munekage et al., 2002). The HL and LC stresses also generated oxidative stress in *Chlamydomonas* cells, as shown by the induction of nonphotochemical quenching (Iwai et al., 2007), suggesting that *CrPgr5* functions as a photoprotectant for PSI in *Chlamydomonas* cells.

Both *FtsH1* and *FtsH2* showed rapid up-regulation under HC at HL conditions, and *FtsH1* was also up-regulated under LC at ML conditions. Since the membrane-bound ATPase *FtsH* has been suggested to degrade damaged D1 protein under HL conditions (Spetea et al., 1999), these proteins are possibly involved in the repair of damaged proteins under HL conditions.

Six genes, *Hsp70B*, *Hsp90A*, *Hsp90B*, *Hsp90C*, *Bip1*, and *Crt2*, were up-regulated under HC at HL conditions but down-regulated under LC at ML conditions (Supplemental Table S2). These genes are expected to serve as molecular chaperons in cells. Considering that heat shock protein 70 s contribute to the photoprotection of PSII in *Chlamydomonas* (Schroda et al., 1999; Kropat et al., 2000), these genes may participate in the process of repairing photodamaged PS (e.g. promoting the folding of nascent D1 polypeptides). On the other hand, *Cpn60α* (array identifier, 165e06) and *Cpn60β* (124b11), coding for chaperons in chloroplast, and the gene *HslV* (170a08), coding for proteasome chaperons, were not affected by HL conditions (Supplemental Table S2).

**DISCUSSION**

In this study, we revealed the dynamics and regulation of transcript abundance of known and unknown genes during acclimation to HL and/or LC stresses in the green alga *Chlamydomonas*. These expression profiles could be linked to changes in physiological characteristics, especially for the CCM, under different stress conditions.

Although the signals that induce the CCM have not been precisely determined, several candidates have been proposed (Kaplan and Reinhold, 1999; Giordano et al., 2005). Recent work using *Chlamydomonas* revealed that the CO₂/O₂ ratio did not play any role in the regulation of LC-inducible genes or in CCM induction (Vance and Spalding, 2005). In cyanobacteria, it is reported that Ci concentrations and light intensities do not on their own trigger the expression of CCM-related genes but that a cooperative effect between light and CO₂ is required (McGinn et al., 2003;
Woodger et al., 2003). We have also studied the effect of changing light intensities and CO₂ concentrations on CCM induction in *Chlamydomonas* (Figs. 1 and 2). First, we confirmed that there is a CO₂-dependent inducible CO₂ uptake system in *Chlamydomonas*. Furthermore, under MC conditions, increases of affinity for Ci and CO₂-exchange activity were apparently affected by light intensity. These results suggested that the induction of the CCM in *Chlamydomonas* was dependent not only on a decrease in Ci concentration in the culture medium but also on light intensity. Although increases in the level of CO₂ exchange activity were observed under both LC at ML and MC at HL conditions, the kinetic changes of CO₂-exchange activities were different. The kinetics of the cells exposed to MC at HL conditions for 6 h were similar to those of the cells treated with LC at ML for 1 h, although the actual Ci concentrations were entirely different. This does not mean that the CCM induction under MC at HL conditions was delayed compared with that under LC at ML conditions, because the kinetics at 12 h under MC at HL conditions, when the cells were fully adapted to stress conditions, were the same as those at 6 h under LC at ML conditions (data not shown). Alternatively, these findings suggest that some CCM components are not active under MC at HL conditions. The genes that were induced only under LC at ML conditions seem to contribute to the full induction of the CCM.

Although the CCM induction seems to be enhanced by HL, it remains unclear whether the effect of HL is a direct or indirect effect on the induction process. It is possible that the redox potential of electron carrier(s) in the thylakoid membrane could be involved in the regulation of CCM. In relation to this, it has been shown that the plastocyanine pool is reduced by limiting CO₂ as well as HL (Palmqvist et al., 1990; Iwai et al., 2007). It was also reported that a photosynthetic inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, blocks the expression of LC-inducible genes, such as *Calh* (Dionisio-Sese et al., 1990) and *Lci1* (Im and Grossman, 2002), as well as the active accumulation of Ci (Badger et al., 1980). Recently, proteomic approaches have also been applied to reveal the posttranslationally modified protein LCI5 under LC conditions. The LCI5 protein, which is localized at the stroma surface of the thylakoid membrane, is phosphorylated under LC conditions by redox-dependent protein kinase(s) (Turkina et al., 2006). Although the function of LCI5 is unknown at present, posttranslational modification of proteins during the acclimation process to LC conditions, which cannot be identified by a DNA array analysis, could be associated with the CCM induction. Moreover, because we only know the total Ci concentrations in the medium, there also remains the possibility that the HL effect may be mediated through Ci depletion in the cells but not in the medium.

Further analysis demonstrated that the induction of the CCM in *Synechococcus* PCC7942 was very closely correlated with depletion of the internal Ci pool and that the full induction of the CCM requires oxygen-dependent processes, including photorespiration (Woodger et al., 2005). In addition, there are many lines of evidence that photorespiratory activity is suppressed during the CCM, so a link between photorespiration and induction of the CCM is indicated (Marcus et al., 1983, 1992; Woodger et al., 2003). In our array analyses, 11 photorespiratory genes were significantly induced under LC at ML conditions. Although these genes, except for *Shmt1*, were not regarded as inducible genes under MC at HL conditions on the basis of our criteria, the expression levels of four genes, *Gdh1* (2.3-fold), *Fis* (2.2-fold), *Sgat* (2.4-fold), and *CarB* (2.3-fold), were also slightly affected under MC at HL conditions (Supplemental Table S2). Moreover, considering that mutants with disruptions of *Pgp1* or *Gdh1* showed severe HC-requiring phenotypes (Suzuki et al., 1990; Mamedov et al., 2001; Nakamura et al., 2005), CCM induction seems to operate in coordination with the function of photorespiration in *Chlamydomonas*. Comparison of expression profiles during the CCM derived from the wild type and photorespiratory mutants should provide us with a better understanding of the CCM induction signal.

We showed by our cDNA array experiments that the transcript levels of 40 genes were up-regulated under LC at ML and MC at HL conditions (Table II). Considering that the CCM was induced under MC at HL as well as LC at ML conditions, the genes we identified should be closely related to the functions of the CCM. Previously identified genes that were induced in CO₂-limiting conditions were included in this group, confirming the quality of our array analyses. We also confirmed the induction of 23 genes and named these genes *Lci17* to *Lci29* (Fig. 4A). Interestingly, while several genes showed CCM1-dependent expression, other genes were only slightly induced in the C16 background. These results highlight at least two possibilities. One is that *Chlamydomonas* possesses a CCM1-independent CO₂ signal transduction system, and the other is that CCM1 functions as an amplifier of the CO₂ signal in the CO₂ signaling cascade in *Chlamydomonas*. From both viewpoints, it appears reasonable that genes such as *Lci14*, *Lci16*, and *Lci25*, all encoding putative regulatory factors, were induced during CCM induction.

In this study, a substantial amount of information on the modulation of transcript levels during acclimation to HL and/or LC was obtained using a cDNA macroarray. Although the 10,368 EST clones spotted on our array membranes cover all genes present in our cDNA library (Asamizu et al., 1999, 2000, 2004) and this was a larger analysis compared with all previous studies to identify CCM-related genes, our array membranes does not represent the entire transcriptome of *Chlamydomonas*. For example, 153 of 642 *Chlamydomonas* mRNA sequences deposited in GenBank were not spotted on our array membranes (e.g. *Nar1* encoding nitrite transporter, *Fet2* encoding Fe-assimilating protein 2, and *Sabc* encoding chloroplast ATP-binding protein). Considering that 15,413 protein-coding genes were
predicted from the *Chlamydomonas* genome (Merchant et al., 2007) and that *Chlamydomonas* also possesses a microRNA regulatory system (Molnár et al., 2007), there could be additional factors responsible for the CCM induction and/or function. The results demonstrated the complex nature of plant acclimation to environmental stresses. The central goals of acclimation research include identifying stress-responsive genes, understanding their roles in plants, and ascertaining how environmental signals are transduced and associated. Much work has already been done to identify stress-responsive genes using global array analyses in *Chlamydomonas*. Henceforth, comparative transcriptome analyses comparing expression profiles between different stresses and different species should provide useful information for screening novel stress-responsive genes. Recent studies have revealed that gene-silencing methods can be applied to *Chlamydomonas*, and several stress-responsive genes have been characterized by loss-of-function analyses (Schroda, 2006). As well, through further application of genomic approaches, such as proteome analyses (Förster et al., 2006; Turkina et al., 2006), it should be possible to construct a detailed diagram of regulation in *Chlamydomonas* and to determine the roles of the regulatory networks in acclimation to multiple stresses.

**MATERIALS AND METHODS**

**Cells and Growth Conditions**

The *Chlamydomonas reinhardtii* ‘Dangeard’ wild-type cell C9 (mt−) strain was originally provided from the IAM culture collection at the University of Tokyo. A HC-requiring mutant, C16, has been described previously (Fukuzawa et al., 1998). Cells were cultured in TAP medium (Harris, 1989) for maintenance or a buffered high-salt medium supplemented with 20 mM MOPS (pH 7.2) for photosynthetic growth. For control conditions, *Chlamydomonas* C9 cells were grown under aeration with air enriched with 5% CO₂ (HC) at 120 μmol photons m⁻² s⁻¹ (ML). For HL conditions, cells in HC at ML conditions were shifted to 1,000 μmol photons m⁻² s⁻¹ (HL): for MC, they were enriched with 12% CO₂ and for LC, they were enriched with 0.04% CO₂. Throughout the experiments, the cell densities of the cultures were kept at less than 3 × 10⁷ cells mL⁻¹, corresponding to an optical density at 750 nm of 0.4 to avoid a shading effect of the cells.

**Measurement of Ci Concentrations in Culture Media**

Total Ci concentrations in culture media were measured after methanization in the presence of H₂ gas by use of a gas chromatograph (GC-8A; Shimadzu) with a methanizer (MTN-1; Shimadzu) as described previously (Matsuda and Colman, 1996). The cell suspension was passed through a 0.2-μm Acrodisc syringe filter to remove cells from the culture medium. Subsequently, 10 μL of the cell-free medium was injected directly into the gas-stripping column of the gas chromatograph through a hypodermic needle to minimize contamination with atmospheric CO₂.

**Measurement of the Photosynthetic Rate**

*Chlamydomonas* cells were grown under HC at ML conditions, transferred to each stress condition, and then sampled at 12 h after transfer. Sampled cells were harvested by centrifugation at 600g for 5 min and then resuspended in 50 mM HEPES buffer (pH 7.8) at 20 μg chlorophyll mL⁻¹. Photosynthetic oxygen evolution was measured by a Clark-type oxygen electrode (Hansatech Instruments). Cell suspension (1.5 mL) was put into the measurement chamber of the oxygen electrode and illuminated at 350 μmol photons m⁻² s⁻¹ for 5 to 10 min with bubbling of N₂ gas to deplete dissolved Ci from the suspension. Subsequently, the initial dissolved inorganic carbon concentrations were determined using gas chromatography. The intensity of actinic light was increased to 700 μmol photons m⁻² s⁻¹, and then the required volumes (1-10 μL) of NaHCO₃ stock solutions (15, 150, and 750 μmol) were injected into the cell suspension every 30 s to yield the desired Ci concentration.

**CO₂-Exchange Measurements**

The cells were harvested after stress treatments and resuspended in fresh high-salt medium at 5 μg chlorophyll mL⁻¹. CO₂-exchange activity of the cell suspension was measured at 28°C using an open infrared gas-analysis system that records the rate of CO₂ exchange as a function of time. Standard N₂ gas containing 50 μL L⁻¹ CO₂ and 21% O₂ (Sumitomo Seika Chemicals) was provided at a flow rate of 0.5 L min⁻¹ to the cell chamber in which the cell suspension was placed. The outside and inside diameters of the cell chamber were 5.0 and 3.5 cm, respectively. Actinic light from two light sources (1,000 μmol photons m⁻² s⁻¹ each) was applied to the surface of the cell chamber for 5 min, and the rate of CO₂ exchange was measured during light illumination with a halogen projector lamp to achieve maximal CO₂-exchange activity. The gas leaving the measurement chamber was chilled to 4°C by passing through a cooling tube connected to a cooling unit (NCB-1200; EYELA) and dried by passing it through a glass tube filled with granular CaCl₂. The CO₂ concentration of the gas was analyzed by an infrared CO₂ analyzer (model LJ-7000; LI-COR).

**Preparation of cDNA Microarrays**

A *Chlamydomonas* cDNA membrane array were constructed as described previously (Miura et al., 2004). By assembling the 5-end EST sequences of 50,382 clones (Asamizu et al., 2000, 2004), 10,368 nonredundant EST clones were selected to construct a membrane array. Since the 15,413 genes were predicted on the *Chlamydomonas* genome (Merchant et al., 2007), it is estimated that the cDNA array covers approximately 67% of the protein-coding genes of *Chlamydomonas*. The cDNA inserts from the selected EST clones were amplified by PCR and spotted on six membranes per set. Each membrane contained up to 1,728 cDNAs spotted in duplicate.

**RNA Isolation, Labeling, and Hybridization**

To identify differentially expressed genes under stress conditions, two independent experiments were carried out using different preparations of RNA samples. Since the macroarray carries duplicates of each EST clone, there were four expression ratios for each EST clone. Total RNA was extracted from the samples using an RNeasy Maxi kit (Qiagen) with some modification. *Chlamydomonas* cells were harvested by centrifugation, and the pellet was immediately frozen in liquid nitrogen. Frozen cells were powdered and lysed with RLT buffer (Qiagen) containing 1% (v/v) 2-mercaptoethanol. The lysate was then passed 10 times through a 23-gauge needle fitted to a syringe. The lysate was centrifuged, and the supernatant was used for subsequent steps following the manufacturer’s instructions. Total RNA was used to isolate poly(A)⁺ RNA with the PolyATract kit (Promega). 3²P-labeled target DNA samples were prepared from poly(A)⁺ RNA by incorporation of [α-³²P]dCTP during first-strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen). Labeled targets were used for hybridization as described previously (Miura et al., 2004). After incubation, the membranes were washed four times with 2× SSC and 0.1% SDS at 65°C and once with 0.1× SSC and 0.1% SDS.

**Data Collection and Analysis**

Radioactive images were obtained with a high-resolution scanner, FLA-2000 (Fuji Photo Film), and quantification of the signal intensity was performed using ArrayVision (Amersham Pharmacia Biotech) software. Raw values were measured as the volume of pixels within a circle encompassing the spot. The background for each membrane was calculated from the quantification of 40 sample values, which were located at nonspotted areas on each membrane. Average and s.d values of the background were calculated by using 36 sample values, ignoring the top 5% and bottom 5% of background data values. The average background value was subtracted from the value of each spot on the membrane to give the sample value. To reduce area-specific effects, median normalization was adopted using GeneSpring (Silicon Genetics) software. First,
a trimmed median value was calculated for each membrane using 80% of the data points, ignoring the top 10% and the bottom 10% to prevent the normalization from skewing. Then, the normalized values were calculated by dividing the sample value of each measurement for each probe set by the 50th percentile of the trimmed median values on the membrane. Fold changes were calculated by dividing the average difference for each experimental sample by the average difference for the control samples. Those determined to be up-regulated by the HL and CO₂-limiting treatment were selected as shown, at any time point, by the presence of gene expression in HL or CO₂-limiting treated samples and fold changes of at least 2.5. We also conducted significance analysis of the results using Student’s t-test. Genes with P values of less than 0.05 were considered differentially expressed with respect to the treatment conditions.

Sequence Analyses

Subcellular localization was predicted using the PSORT (Nakai and Horton, 1999) program. The putative cleavage sequence of the transit peptide was predicted using the ChloroP (Emanuelsson et al., 1999) program. Membrane regions were predicted using the SOSUI (Hirokawa et al., 1998) program. The putative cleavage sequence of the transit peptide was predicted using the ChloroP (Emanuelsson et al., 1999) program. The putative cleavage sequence of the transit peptide was predicted using the ChloroP (Emanuelsson et al., 1999) program. The putative cleavage sequence of the transit peptide was predicted using the ChloroP (Emanuelsson et al., 1999) program.

Northern-Blot Analyses

Ten micrograms of total RNA was electrophoresed on a denaturing agarose gel and blotted onto a nylon membrane, Biodyne B (Pall). cDNA probes corresponding to each gene were labeled with [α-32P]dCTP using random primers pd(N)6 (Takara Bio) and Klenow fragment DNA polymerase (Takara Bio). The cDNA clones were isolated from a Chlamydomonas cDNA library (Asamizu et al., 1999, 2000, 2004). Hybridization was performed with ExpressHyb hybridization solution (Clontech) at 68°C for 12 to 16 h.

RT-PCR Analyses

We performed RT reactions using SuperScript III reverse transcriptase (Invitrogen) and the oligo(dT)15 primer (Toyobo). PCR was done with ExTaq (Takara Bio). Primer sequences used in RT-PCR analyses are listed in Supplemental Table S1.

Supplemental Data

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

Supplemental Table S1. Oligonucleotide primers used in semiquantitative RT-PCR analyses.

Supplemental Table S2. Expression profiles of all spotted genes in the cDNA array.

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LITERATURE CITED


Burow MD, Chen ZY, Mouton TM, Moroney JV (1996) Isolation of cDNA clones of genes induced upon transfer of Chlamydomonas reinhardtii cells to low CO₂. Plant Mol Biol 31: 443–448


Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSU: classification and...
secondary structure prediction system for membrane proteins. Bioinformatics 14: 378–379


