
The Novel Myb Transcription Factor LCR1 Regulates the CO2-Responsive Gene Cah1, Encoding a Periplasmic Carbonic Anhydrase in Chlamydomonas reinhardtii

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Chlamydomonas reinhardtii acclimates to CO2-limiting stress by inducing a set of genes for a carbon-concentrating mechanism (CCM). This set includes the gene Cah1, which encodes a periplasmic carbonic anhydrase. Although physiological aspects of CO2 response have been extensively studied, regulatory components, such as transcription factors involved in the acclimation, have not been well described in eukaryotic microalgae. Using an arylsulfatase gene driven by the Cah1 promoter, a regulatory mutant of Cah1 was isolated and named lcr1 (for low-CO2 stress response). The photosynthetic affinity for inorganic carbon of lcr1 was reduced compared with that of wild-type cells. Expression of three low-CO2-inducible genes, Cah1, Lci1, and Lci6, were regulated by LCR1 as shown by cDNA array and RNA gel blot analyses. The Lcr1 gene encodes a protein of 602 amino acids containing a single Myb domain, which binds to the Cah1-promoter region. Expression of Lcr1 was induced by lowering CO2 levels and controlled by the regulatory factor CCM1. These results suggest that LCR1 transmits the low CO2 signal to at least three CO2-responsive genes and then fully induces CCM.

INTRODUCTION

Aquatic photosynthetic organisms acclimate to environmental changes, such as light, temperature, and availability of various nutrients, by controlling photosynthetic activity. These photosynthetic organisms induce a set of genes for a carbon-concentrating mechanism (CCM). This set includes the gene Cah1, which encodes a periplasmic carbonic anhydrase. Although physiological aspects of CO2 response have been extensively studied, regulatory components, such as transcription factors involved in the acclimation, have not been well described in eukaryotic microalgae. Using an arylsulfatase gene driven by the Cah1 promoter, a regulatory mutant of Cah1 was isolated and named lcr1 (for low-CO2 stress response). The photosynthetic affinity for inorganic carbon of lcr1 was reduced compared with that of wild-type cells. Expression of three low-CO2-inducible genes, Cah1, Lci1, and Lci6, were regulated by LCR1 as shown by cDNA array and RNA gel blot analyses. The Lcr1 gene encodes a protein of 602 amino acids containing a single Myb domain, which binds to the Cah1-promoter region. Expression of Lcr1 was induced by lowering CO2 levels and controlled by the regulatory factor CCM1. These results suggest that LCR1 transmits the low CO2 signal to at least three CO2-responsive genes and then fully induces CCM.

RESULTS

Isolation of Regulatory Mutants of Cah1

To obtain regulatory mutants of Cah1, we generated a host strain, Q304P3, in which Cah1-promotor activity is monitored by
arylsulfatase (Ars) enzyme activity (ProCah1::Ars). The strain CAO3 (ProCah1::Ars, Nia1, cw15, and mt1), which carries a promoterless Arn gene fused to the Cah1 promoter and exhibits CO2-responsive Ars induction (Kucho et al., 1999), was crossed with CC2678 (nia1-305, cw15, sr-1, and mt1), which has wild-type phenotypes for photosynthetic activity and Cah1 expression. A single progeny lacking functional Nia1 gene and exhibiting CO2-responsive Ars induction was isolated and named Q304P3 (ProCah1::Ars, nia1-305, cw15, and mt1). The Q304P3 strain was mutagenized by random insertion of the Nia1 gene, which was used as a selection marker. Twenty-five thousand nia1 colonies were screened, and 15 colonies were found not to exhibit Ars activity under low-CO2 conditions in light (Figure 1A). Among them, a mutant named lcr1 was analyzed further. In this mutant, accumulation of both ProCah1::Ars and endogenous Cah1 transcripts was significantly lower than in the host strain Q304P3 (Figure 1B), indicating that the lcr1 mutant is impaired in induction of Cah1.

Physiological Characterization of lcr1

Because CCM-deficient mutants show decreased growth rates under low-CO2 conditions, for example, ccm1 mutant C16 (Fukuzawa et al., 2001) and pmp1 (Spalding et al., 1983), the growth rate of the lcr1 mutant was compared with the host strain Q304P3 and the CCM-deficient mutant C16 (Figure 2A). Under high-CO2 conditions, these three strains had equivalent growth rates. Under low-CO2 conditions, however, the growth rate of the lcr1 mutant was 30% less than that of Q304P3 but much higher than that of the ccm1 mutant C16. This indicates that the lcr1 mutant exhibits a moderately high-CO2-requiring phenotype.

To evaluate the apparent affinity of the lcr1 mutant for Ci, the photosynthetic K0.5(Ci) value was determined using an O2 electrode (Figure 2B). The host strain Q304P3, grown under low-CO2 conditions, had a high affinity for Ci, similar to that reported for wild-type cells (Badger et al., 1980). When the lcr1 mutant was grown under low-CO2 conditions, it had lower affinity [K0.5(Ci) = 207 μM] than Q304P3 grown under the same conditions [K0.5(Ci) = 93 μM]. Because under low-CO2 conditions the lcr1 mutant showed higher affinity than Q304P3 grown under high-CO2 conditions [K0.5(Ci) = 511 μM], the lcr1 mutant partially induces the CCM. There was no significant difference in the maximum photosynthetic rate between the lcr1 mutant and Q304P3 under low-CO2 conditions (142 ± 15 and 119 ± 8 μmol·mol−1 of Chl·h−1, respectively). These results indicate that the lcr1 mutant is partially defective in the induction of the CCM.

Complementation the lcr1 Mutation

To determine whether the lcr1 phenotypes were linked to the insertion of the Nia1 tag, the lcr1 mutant (Nia1) was crossed with a nia1-strain CC2678 (nia1-305), which exhibited wild-type phenotypes for photosynthesis and Cah1 expression. In 27 of 28 nia1 progeny, the deficiency in Cah1 induction cosegregated with the nia1 phenotype, and only a single insertion of Nia1 gene was detected by DNA gel blot analysis (data not shown). These results suggest that the lcr1 mutation was caused by a single Nia1 insertion.

To isolate the Lcr1 gene, we determined the nucleotide sequence of the flanking regions of the inserted Nia1 tag. Five genomic clones containing the flanking regions were isolated from the genomic library of C85-20 strain (Zhang et al., 1994). Nucleotide sequencing of one of the genomic clones, pKK2, revealed that a 13.5-kb genomic region was replaced by the inserted pMN24 DNA in the lcr1 genome (Figure 3A). Introduction of pKK2 into the mutant complemented Cah1 induction (Figure 1B, lcr1::G) and restored the photosynthetic affinity for Ci (data not shown).
Furthermore, introduction of the 5.1-kb PCR product, Frag-B, which consisted of a central portion of the deleted genomic region (Figure 3A), also complemented Cah1 induction (Figure 1B, lcr1::B) and affinity for Ci (Figure 2B). Other PCR products, Frag-A or Frag-C, which contain one of the ends of the deleted region, did not complement the lcr1 phenotypes, indicating that the gene corresponding to Lcr1 is located in Frag-B.

Structure and Copy Number of Lcr1

Based on the genomic sequence of the Frag-B, the cDNA clone LCL014f07 was isolated from a Chlamydomonas cDNA library (Asamizu et al., 2000) by in silico search. This cDNA consists of a 1809-bp open reading frame, a 203-bp 5′-untranslated region, and a 1184-bp 3′-untranslated region (Figure 3B). The sequence of the 936-bp 5′-upstream region did not contain any large open reading frames, indicating that the gene encoding this cDNA was responsible for complementation, and the gene was designated Lcr1. DNA gel blot analysis using the cDNA as a probe demonstrated that the Lcr1 gene is a single copy gene in Chlamydomonas and has been deleted in the lcr1 mutant (Figure 3C). The cDNA was predicted to encode a basic soluble protein of 602 amino acid residues with a molecular mass of 62.7 kD and pl value of 9.67. A similarity search of the GenBank database using the predicted amino acid sequence revealed that the N-terminal region of LCR1 has significant similarity to Myb domains that are involved in DNA binding (Jin and Martin, 1999) (Figure 4A). Although the other region of LCR1, except for the Myb domain, shows no sequence similarity to any other proteins, four characteristic sequence stretches common to transcription factors, Gin-, two His-, and Pro-rich regions, were found (Liu et al., 1999). The Myb domain of LCR1 exhibited higher levels of similarity to the R3 domain of multiple-type Myb proteins, such as Arabidopsis thaliana AtMyb57 and chicken c-Myb (45 and 41% identity, respectively) (Figures 4B and 4C). The Myb domain of Arabidopsis AtMybL2, which is one of the single-type Myb proteins, was 33% identical to that of LCR1. By contrast, other single-type plant Myb proteins (e.g., Chlamydomonas Psr1 and Arabidopsis CCA1) exhibited lower similarity (20 and 16% identity, respectively).

A Recombinant Polypeptide Containing the Myb Domain of LCR1 Binds to the Cah1-Promoter Region

To elucidate the DNA binding activity, the N-terminal region of LCR1 containing the Myb domain was fused to glutathione S-transferase (GST) and expressed in Escherichia coli (Figure 5A). The purified fusion protein, GST-Myb, was tested for its ability to bind to the Cah1-promoter region by gel mobility shift assays (Figure 5B). The Cah1 upstream region from −651 to −231, relative to the transcription initiation site, which is sufficient for CO2-responsive gene regulation (Kucho et al., 2003), was used as a probe. GST alone did not show DNA maximum rates of O2 evolution were 123, 142, 119, and 120 μmol·mg Chl−1·h−1 for Q304P3-H, Q304P3-L, lcr1-L, and lcr1::B-L, respectively.
binding activity (Figure 5B, lane 2). However, GST-Myb showed two shifted bands (Figure 5B, lane 3). These interactions between GST-Myb and the 32P-labeled probe were competed away by addition of excess unlabeled probe (Figure 5B, lanes 4 to 6), indicating that the Myb domain of LCR1 has DNA binding activity specific to the 421-bp probe containing the Cah1-promoter region. To locate the LCR1 binding region, competition analyses using truncated unlabeled fragments were performed (Figure 5C). DNA-LCR1 interaction was successfully competed out using f1 (Figure 5C, lane 4); however, f2 and f6 (lanes 5 and 9) did not interfere with the binding. In addition, the DNA-LCR1 interaction was also competed out by f4 (Figure 5C, lane 7) but not by f3 and f5 (lanes 6 and 8). These results suggest that the Myb domain of LCR1 binds to two regions around /C255 551 to /C255 501 and /C255 442 to /C255 401 of the Cah1-promoter region (Figure 5D).

Previously, we have identified an enhancer, EEC, essential to CO2-responsive expression of Cah1 and demonstrated the presence of binding proteins to EEC (Kucho et al., 2003). To investigate whether the EEC binding proteins are identical to LCR1, another gel mobility shift assay using probes containing the EEC sequence were performed (Figure 5E). The complexes between EEC and the binding proteins (C-I and C-III) were detected with nuclear extracts from both wild-type cells and the lcr1 mutant. These results revealed that EEC binding proteins previously reported were different from LCR1 because EEC binding proteins were expressed in the lcr1 mutant, in which the Lcr1 gene was completely deleted, as in the case of wild-type cells.

**CO2-Responsive Expression of Lcr1**

To determine CO2 responsiveness of Lcr1, total RNA was isolated from cells after transfer from high-CO2 to low-CO2 conditions in light, and RNA gel blot analyses were performed (Figure 6A). In the wild-type strain C9, although there was no detectable band under high-CO2 conditions (0 h), a 3.2-kb Lcr1 mRNA was observed at 1 h after transfer to low-CO2 conditions, and the signal increased to a maximum at 2 h. The amount of Lcr1 mRNA remained steady until 8 h after transfer (Figure 6A, lanes 4 to 6). This accumulation pattern of Lcr1 mRNA was the same as that of Cah1 mRNA in C9. On the other hand, in the lcr1 deletion mutant, the expression of Cah1 was transiently induced, and the level of accumulation was significantly lower than in C9 (Figure 6A, lane 4). These results indicate that LCR1 does not function in initial induction but functions in amplification and maintenance of Cah1 mRNA levels in response to CO2-limiting stress.

Next, total RNA was isolated from cells after transfer from low-CO2 to high-CO2 conditions in light, and RNA gel blot analyses were performed (Figure 6B). In C9, the amount of Lcr1 mRNA decreased to an undetectable level within half an hour after transfer (Figure 6B, lanes 1 and 2). Cah1 mRNA was not detectable 2 h after transfer in both C9 and lcr1 (Figure 6B, lane 4). These results indicate that expression of Lcr1 is repressed under high-CO2 conditions in light and suggest that LCR1 does not influence degradation of Cah1 mRNA. Although Cah1 mRNA was not detected in the lcr1 mutant 8 h after transfer to low-CO2 conditions (Figure 6A, lane 6), it was detected under low-CO2 conditions in another experiment (Figure 6B, lane 1). This
discrepancy may be a result of the oscillation in accumulation of Cah1 mRNA (Fujiwara et al., 1996).

The regulatory gene Ccm1 has been identified in Chlamydomonas (Fukuzawa et al., 2001), and CCM1 is indicated as a master regulator in the low-CO2 signal transduction pathway (Miura et al., 2004). To determine whether CCM1 regulates expression of Lcr1 under low-CO2 conditions, we performed RNA gel blot analysis with the ccm1 mutant C16 and complemented C16 (Figure 7A). In the ccm1 mutant C16, the 3.2-kb Lcr1 mRNA was not detected under low-CO2 conditions (Figure 7A, lane 4). By contrast, in complemented C16, the Lcr1 mRNA was detected in the case of the wild-type strain C9.
These results revealed that expression of Lcr1 under low-CO₂ conditions is regulated by CCM1.

**Global Analysis of Target Genes of LCR1**

To identify genes transcriptionally regulated by LCR1, expression profiles of the lcr1 mutant and wild-type strain C9 were compared under low-CO₂ conditions in light using a cDNA macroarray containing 10,368 EST clones of Chlamydomonas (Miura et al., 2004). Array analysis showed that mRNA levels of 13 cDNAs in the lcr1 mutant were lowered <40% compared with those in C9 (Table 1). To examine whether these expression deficiencies were caused by the loss of LCR1 or by other mutations, RNA gel blot analyses were performed (Figure 7B). Total RNA was isolated from the host strain Q304P3, lcr1, and complemented lcr1 (lcr1::B) grown under high-CO₂ conditions or after transfer to low-CO₂ conditions for 2 h. RNA gel blot analyses with these 13 cDNA probes revealed that only three genes, Cah1, Lci1, and 023e06, were target genes of Lcr1. The 023e06 gene is novel, encoding a putative polypeptide of 445 amino acids whose pI value is 8.84, and was named Lci6 (for low-CO₂ inducible). The Lci6 gene has no significant similarities to any genes in the GenBank database. Downregulation of two genes, 021e03 and 023c07, was caused by a mutation other than Lcr1 because their low expression was not complemented by introduction of Lcr1 (Figure 7B, lanes 3 to 6, boxed genes). Downregulation of the other eight genes was caused by differences in genetic background between C9 and Q304P3 because equivalent expression levels were observed in Q304P3 and lcr1 (Figure 7B, lanes 1 to 4, shaded genes). Array analysis also suggested that all other low-CO₂-inducible genes except Cah1, Lci1, and Lci6 were not significantly affected by Lcr1, for example, Mca (expression ratio of C9 to lcr1 under low-CO₂ conditions with standard deviation: 2.2 ± 0.5), Ccp1 (1.6 ± 0.1), Aat1 (1.1 ± 0.4), and Pgp1 (1.1 ± 0.4).

**DISCUSSION**

The Ars gene has been used as a reporter to examine promoter activity in Chlamydomonas because it is easily visualized (Davies et al., 1992; Villand et al., 1997; Kucho et al., 1999). The Ars gene driven by Cah1 promoter was used to isolate regulatory mutants.
would regulate genes that play a significant role in the CCM, such as $\text{Ci}$ transport. Array analysis and RNA gel blot analyses indicate that three low-CO$_2$-inducible genes, $\text{Cah1}$, $\text{Lci1}$, and $\text{Lci6}$, are regulated by LCR1. Because inhibition of periplasmic carbonic anhydrases raises the apparent photosynthetic $K_m$ for external $\text{Ci}$ at alkaline pH, the periplasmic carbonic anhydrases are thought to contribute to the CCM only at alkaline pH (Kaplan and Reinhold, 1999). However, the effect of the mutation in LCR1 on photosynthetic affinity is significant at neutral pH of 7.0. This suggests that CCM components other than periplasmic carbonic anhydrases contribute to the maintenance of higher photosynthetic affinity in wild-type cells under low-CO$_2$ conditions. In addition, the $\text{lcr1}$ mutant exhibited more severe phenotypes than the $\text{Cah1}$ null mutant (Van and Spalding, 1999). Therefore, the $\text{lcr1}$ phenotypes cannot be explained only by the defect in $\text{Cah1}$ induction. Other affected genes, including $\text{Lci1}$ and $\text{Lci6}$, seem to be responsible for the CCM. In particular, $\text{Lci1}$ is one candidate for the $\text{Ci}$ transporter because it encodes a putative membrane protein containing four transmembrane regions (Burow et al., 1996) and a signal peptide (predicted by iPSORT). Another gene, $\text{Lci6}$, encodes a basic soluble protein, whose function still needs to be identified.

A large number of Myb proteins have been found in various species among animals, plants, and yeast, and they comprise a gene family (Jin and Martin, 1999). Myb proteins are classified into three subfamilies depending on the number of adjacent repeats of the Myb domain: R1R2R3-Myb (three domains), R2R3-Myb (two domains), and 1R-Myb (one domain). LCR1 belongs to the 1R-Myb protein subfamily. This family contains transcription factors, such as CCA1, which functions in circadian control, from Arabidopsis (Wang et al., 1997). Some 1R-Myb proteins, including CCA1, OsMYBS3, and PSR1, are 30 to 40% identical in the Myb domain with each other and possess a SHAQK(Y/F)F motif (Lu et al., 2002) (Figures 4B, boxed, and 4C). However, the Myb domain of LCR1 shows less similarity to these 1R-Myb proteins (~20% identical). These sequence characteristics suggest that LCR1 may have evolved by a different process than the single-type Myb genes harboring a SHAQK(Y/F)F motif. AthMybL2, exhibiting higher similarity to LCR1 in the Myb domain (33% identical), interacts with the transcription factor GL3 and regulates GL2 expression, which controls trichome development (Sawa, 2002). LCR1 may interact with other transcription factors and together regulate expression of the low-CO$_2$-inducible genes. Single-type Myb genes functioning in phosphate, sugar, and light response have been identified in plants (Wykoff et al., 1999; Lu et al., 2002; Kuno et al., 2003). Our finding of a novel single-type Myb gene, $\text{Lcr1}$, functioning in CO$_2$ response, supports the notion that single-type Myb genes are involved in various stress responses in plants. In cyanobacteria, CO$_2$-responsive transcription factors CmpR and NdhR, classified as part of the LysR family, have been isolated and regulate the $\text{cmpABC}$ or $\text{ndh3}$ operons, respectively (Figge et al., 2001; Omata et al., 2001). Although both Chlamydomonas and cyanobacteria possess a CCM, different groups of transcription factors are operating in eukaryotes and prokaryotes in response to CO$_2$-limiting stress.

A single-type Myb protein, PSR1, in Chlamydomonas has demonstrated nuclear localization, although no known nuclear
Localization signal has been found in the predicted PSR1 sequence (Wykoff et al., 1999). LCR1 should be transported to the nucleus because of its DNA binding activity (Figures 5B and 5D), although there is no nuclear localization signal. LCR1 and PSR1 may be carried into the nucleus via associations with other proteins (Schwechheimer and Bevan, 1998). Further analyses are required to determine the nuclear localization of LCR1 in vivo.

In gel mobility shift assays, the fact that two distinct complexes between GST-Myb and Cah1 promoter were detected (Figure 5B) implies the possibility that the complexes corresponding to the lower and upper shifted bands include one and two GST-Myb proteins, respectively. In the competition assay, these interactions required both binding regions, consisting of a portion from –551 to –501 and another one from –442 to –401 because these interactions were not competed out by the DNA fragments f2 and f5, in which one of the binding regions was deleted (Figure 5C, lanes 5 and 8). These results suggest the possibility that LCR1 proteins might dimerize and recognize binding regions as predicted previously about the single-type Myb proteins (Jin and Martin, 1999). It would be interesting to identify the nucleotide sequence recognized by LCR1 and determine whether dimerization occurs.

Previously, we identified an EEC (GANTTNC) that is essential for CO2-responsive induction of Cah1 and demonstrated the presence of EEC binding proteins (Kucho et al., 2003). Because the equivalent complexes were detected using nuclear extracts from both wild-type and the lcr1 null mutant, the EEC binding proteins are different from LCR1 (Figure 5E). This result is consistent with the facts that EEC binding proteins are present in the nuclear extract regardless of CO2 conditions (Kucho et al., 2003), whereas Lcr1 mRNA was detected only under low-CO2 conditions. A better understanding of the relationship between LCR1 and EEC binding proteins will require cloning and characterization of the EEC binding proteins.

In the lcr1 null mutant, the accumulation of Cah1 mRNA under low-CO2 conditions was decreased significantly compared with that seen in wild-type cells; however, Cah1 induction was not abolished (Figure 6A). This result is in agreement with the previous deletion analysis of Cah1 upstream region (Kucho et al., 1999). Deletion of the region from –651 to –294 relative to transcription initiation site, including putative LCR1 binding sites, lowered promoter activity dramatically but did not abolish it. Lcr1, Cah1, and Lci1 genes were regulated by CCM1 (Figure 7A; Fukuzawa et al., 2001), and EECs are found in all 5′-upstream regions of them. By contrast, the expression of Lci6 is not regulated by CCM1 directly (Miura et al., 2004), and the EEC is not found in the 1157-bp 5′-upstream region of Lci6. These findings suggest that the EEC is necessary for gene regulation mediated by CCM1.

Together with this data, a possible mechanism by which expression of Cah1 is induced in response to CO2-limiting stress is described as follows (Figure 8). When cells are exposed to clones whose mRNA levels did not change between lcr1 and complemented lcr1. lcr1::B, lcr1 transformed with the 5.1-kb genomic fragment Frag-B.
CO₂-limiting stress, the CO₂-limiting signal is transmitted to CCM1, which is constitutively expressed regardless of CO₂ conditions (Fukuzawa et al., 2001). Then, CCM1 is posttranscriptionally modified and induces initial expression of both Lcr1 and Cah1 via interactions between activated CCM1 and EEC binding proteins. Newly synthesized LCR1 is transported to the nucleus. Imported LCR1 interacts with unidentified enhancers in the Cah1-promoter region, then amplifies the magnitude of Cah1 induction and maintains the mRNA levels under continuous low-CO₂ conditions. To verify this model, it is necessary to clarify the interaction among the CCM1, LCR1, and EEC binding proteins. Information about the 5′-upstream region of other low-CO₂-inducible genes will give us new insights into the mechanisms of CO₂-responsive transcriptional regulation. Characterization of other mutants defective in CO₂ response and identification of the targets of LCR1 using one-hybrid or two-hybrid screening methods will enable us to further understand the molecular mechanisms of CO₂-responsive gene regulation and CO₂-signal transduction.

**METHODS**

**Strains, Culture Conditions, and Transformation**

For high-CO₂ conditions, cells were cultured in HS medium (Harris, 1989) supplemented with 20 mM Mops [3-[N-(tris(hydroxymethyl)methyl)]-morpholino]-propanesulfonic acid], pH 7.2, under aeration with air enriched 5% (v/v) CO₂ at 28°C. For low-CO₂ conditions, cultures were bubbled with ordinary air containing 0.04% (v/v) CO₂ in the same medium. Cultures were illuminated by white fluorescent lamps at an intensity of 120 μmol·m⁻²·s⁻¹. For Ars assays and detection of the ProCah1::Ars transcript, cells were cultured in HS medium supplemented with 0.4 mM magnesium sulfate and 20 mM Mops, pH 7.2. (HSM + S; Kucho et al., 1999). Cell densities were estimated by absorbance at 730 nm (Harris, 1989). C9 is a wild-type Chlamydomonas reinhardtii strain used as a control strain in macroarray analysis and isolate of LCR1 expression. The ccm1 mutant C16 and complemented C16 strain were described previously (Fukuzawa et al., 2001; C16::pKI4XA). For isolation of regulatory mutants of Cah1, cells were transformed with pMN24 harboring the Ccm1::ble gene (Fernandez et al., 1989) and Nia1::ble gene (Fernandez et al., 1990). For complementation, cells were cotransformed with the genomic fragment and pSP124S harboring the ble gene. For complementation, cells were cotransformed with the genomic fragment and pSP124S harboring the ble gene (Fernandez et al., 1998). For measurement of photosynthetic rate, the rates of photosynthesis were measured in a Clark-type O₂ electrode, Chloroview 1 (Hansatech, King’s Lynn, UK), and the CO₂-compensation

**Table 1. Downregulated Genes in the lcr1 Mutant Revealed by cDNA Array**

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<th>Array ID</th>
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<th>C9/L.C9H</th>
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<th>Product</th>
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<td>021e03</td>
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<td>8.4 ± 3.0</td>
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<td>6.7 ± 4.2</td>
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<td>3.6 ± 0.9</td>
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<td>Pyruvate formate-lyase Chlamydomonas</td>
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<td>57 ± 38</td>
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<td>Lci1</td>
<td>Low-CO₂-inducible membrane protein Burow et al. (1996)</td>
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The expression ratios in excess of 2.5-fold are in bold. Asterisks indicate LCR1-regulated genes (see Figure 7A). Dashes represent gene products and references that are not assigned. Organisms or references are listed.

**Figure 8. Schematic Drawing of a Possible Mechanism for Transcriptional Activation of Cah1 in Response to CO₂-Limiting Stress.**

Hatched box indicates a putative enhancer that is recognized by LCR1. EBP, EEC binding proteins.
concentration was determined using gas chromatography as previously described (Fukuzawa et al., 2001).

Isolation of Genomic Clones and PCR Fragments for Complementation

Genomic clones containing the flanking regions of *Nia1* insertion were isolated by PCR selection with pooled genomic clones using the following primer sets: C44-3-3f, 5'-GTGGACGTTGACTGCTAGCAG-3', and C44-3-3r, 5'-CTGGACCCGACACGAGTAC-3'. Three primer sets were used to amplify genomic fragments for complementation: Frag-A, C44-3-2, 5'-GTGGACCGCACAGCAGCACT-3'; Frag-B, C44-cpmB-F, 5'-TGACGACCGTGATCCAGTAC-3', and C44-cpmB-R, 5'-CTGGACCGCACAGCAGCACT-3'; and Frag-C, C44-cpmC-F, 5'-CGTGGTGTAATCGAGCTGCTGAAACCT-3', and C44-cpmC-R, 5'-CTGGACGGCACATACCAGTGGATGAGAG-3'.

Isolation of Genomic Clones and PCR Fragments

Three primer sets were used to amplify genomic fragments for complementation: Frag-A, C44-3-2, 5'-GTGGACCGCACAGCAGCACT-3'; Frag-B, C44-cpmB-F, 5'-TGACGACCGTGATCCAGTAC-3', and C44-cpmB-R, 5'-CTGGACCGCACAGCAGCACT-3'; and Frag-C, C44-cpmC-F, 5'-CGTGGTGTAATCGAGCTGCTGAAACCT-3', and C44-cpmC-R, 5'-CTGGACGGCACATACCAGTGGATGAGAG-3'.

Identification and Analysis of Gene Structures

Identification and analysis of gene structures were performed essentially as described previously (Kuco et al., 2003).

Protein Expression in *Escherichia coli* and Purification

To obtain the polypeptide containing the Myb domain, the cDNA of *Lcr1* from nucleotide 1 to 387, coding for the 129 amino acid polypeptide, was cloned into pGEX-6p-1 (Amersham, Buckinghamshire, UK) using PCR-cloned polypeptide containing the following primer set: *Lcr1-R*, 5'-CCGAATTTATGAGAGTACAGCAGCAGGACACGAGGAC-3', and *Lcr1-F*, 5'-GCCATCTACGAGCTGCTGACGACTC-3'. The construct was transformed into *E. coli* BL21. The purification of GST-Myb was performed using a GSTrap FF column (Amersham). Protein concentrations were determined using a protein assay reagent kit (Bio-Rad, Hercules, CA).

Gel Mobility Shift Assays

As described previously (Kuco et al., 2003) except as follows. The probe was amplified by PCR with appropriate plasmid DNA using the following primer set: pKpn-3, 5'-ATGGTACCTGCTCTCCCGCACAATC-3', and CUp-Kpn6-2, 5'-ATGGTACCTCGTAAACGTCCACGGCATGACTC-3', and followed by 5'-end labeled with [γ-32P]ATP. Binding reactions were performed by incubating 25 ng of probe (2.0 × 10^6 cpm/μL) with 2.5 μg of recombinant GST or 1.0 μg of GST-Myb. The reaction mixtures were electrophoresed on a 3.5% nondenaturing polyacrylamide gel. The preparation of nuclear extracts and the gel mobility shift assay were performed as described previously (Kuco et al., 2003).

cDNA Macroarray Analysis

ChlamyArray version 3.3 (Japanese consortium of Chlamydomonas macroarray) was used for array analysis. Poly(A)^+ RNA was isolated from cells grown under high-CO2 conditions or cells transferred from high-CO2 to low-CO2 conditions for 1 h using PolyATtract System 1000 (Promega, Madison, WI). Target labeling and hybridization were performed as described previously (Miura et al., 2004). Data analyses were performed as follows. Radiative images were obtained at 50-μm resolution with a high-resolution scanner, FLA-2000 (Fuji Photo Film, Tokyo, Japan), and quantification of the signal intensity was performed using the program ArrayVision (Amersham). Raw value was measured as the volume of pixels within a circle encompassing the spot. The background for each membrane was calculated as follows: 40 sample values, which were selected at nonspun areas in each membrane were quantitated. Average and standard deviation of the background were calculated using 36 sample values, ignoring the top 5% and bottom 5% of the background data. The average of the background was subtracted from the value of each spot on the membrane. This subtracted value was called as a sample value (c). To reduce area-specific effects, mean normalization was adapted. A trimmed mean (μtrimmed) was calculated for each membrane using 80% of data points, ignoring the top 10% and the bottom 10% of the data points to prevent the normalization from skewing. Then the sample value was normalized. After calculating normalized values S = (c/μtrimmed) × 18.08 (a correction factor), the relative signal intensity was calculated as the ratio of two normalized values. This relative signal intensity estimated is called the expression ratio (Sd/Sb). The expression ratios (C9L/C9H or C9L/lcr1L) of the duplicated spots were averaged. Data were obtained from two independent cultures and hybridizations for each condition. If the correlation coefficient between these two experimental data was >0.90, these were used for further analyses. Only ESTs whose averaged expression ratios were >2.5 and normalized values of numerators (Sd) were >25, corresponding to 0.1% of total signal, were selected to assign to be differentially expressed. We confirmed that normalized values of numerators (Sd) were at least twofold higher than the average background (plus 2 × SD of background). Using four expression ratio data per each EST clone, the means and their standard deviations were calculated. Because each EST clone has four expression ratios, if three of four expression ratios were more than 2.5-fold, this EST clone was selected as significant differential expressed genes for further analysis.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers. Accession numbers of *Lcr1* and *Lci6* are AB168890 and AB168891, respectively. The accession number of the genomic sequence containing the deleted region in the *lcr1* mutant is AB168809. The accession numbers for the sequences mentioned in Figure 4 are as follows: P01103 (chicken c-MYB); P10242 (human c-MYB); AA25950 (Arabidopsis MYB3R-3); NP_186802 (Arabidopsis MYB7); CA92280 (Arabidopsis AtMYBL2); NP_850460 (Arabidopsis CCA1); CA94259 (rice GAMYB) and AAN63154 (rice OsMYBS3); P21538 (yeast REB1); AAD55941 (Chlamydomonas PSR1).

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The Novel Myb Transcription Factor LCR1 Regulates the CO₂-Responsive Gene Cah1, Encoding a Periplasmic Carbonic Anhydrase in Chlamydomonas reinhardtii

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