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The Novel Myb Transcription Factor LCR1 Regulates the CO_2-Responsive Gene *Cah1*, Encoding a Periplasmic Carbonic Anhydrase in *Chlamydomonas reinhardtii* [W]

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Chlamydomonas reinhardtii **acclimates to CO_2-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 CO_2 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 *Icr1* (for low-CO_2 stress response). The photosynthetic affinity for inorganic carbon of *Icr1* was reduced compared with that of wild-type cells. Expression of three low-CO_2-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 CO_2 levels and controlled by the regulatory factor CCM1. These results suggest that LCR1 transmits the low CO_2 signal to at least three CO_2-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) under CO_2-limiting conditions, allowing effective usage of inorganic carbon (Ci) sources in spite of low CO_2 availability (Kaplan and Reinhold, 1999). This acclimation to CO_2-limiting stress suggests the existence of sensory mechanisms and signal transduction pathways in response to the change of external CO_2 concentration. In *Chlamydomonas reinhardtii*, several genes are regulated by CO_2 availability, including *Aat1* coding for an Ala:α-ketoglutarate aminotransferase (Chen et al., 1996), *Mca* for a mitochondrial carbonic anhydrase (Eriksson et al., 1996), *Ccp* for a chloroplast envelope protein LIP-36 (Chen et al., 1997), and *Pgp1* for a phosphoglycolate phosphatase (Mamedov et al., 2001). Among them, regulation of *Cah1*, encoding a periplasmic carbonic anhydrase, is the most well studied (Dionisio-Sese et al., 1990; Fukuzawa et al., 1990; Fujiwara et al., 1996). The expression of *Cah1* is induced under low-CO_2 conditions (bubbled with ordinary air containing 0.04% [v/v] CO_2) in light, whereas it is repressed under high-CO_2 conditions (air enriched with 5% [v/v] CO_2) or in the dark. Recently, a regulatory gene, *Ccm1* (*Cia5*), encoding a zinc-finger protein, has been identified from high-CO_2-requiring mutants in Chlamydomonas (Fukuzawa et al., 2001; Xiang et al., 2001). *Ccm1* is essential for control of CCM induction and the expression of CO_2-responsive genes, including *Cah1*. An enhancer element consensus (EEC), GANTTNC, which is essential for CO_2-responsive transcriptional activation, was also identified in the *Cah1* upstream region (Kucho et al., 2003). Although physiological responses to changing CO_2 concentration have been examined extensively, the molecular mechanisms involved in CO_2 signaling are still poorly understood because few regulatory mutants have been identified. Therefore, isolation of other regulatory factors is required to understand the molecular mechanisms of the CO_2-signal transduction pathway. Promoter/reporter screening systems are a powerful method for isolating regulatory mutants, suggesting that novel regulatory factors could be identified in Chlamydomonas using the promoter/reporter system.

In this study, we isolated the regulatory mutant *Icr1* (for low-CO_2 stress response), based on loss of *Cah1*-promoter activity. We identified a novel Myb-related gene, *Lcr1*, which functions to regulate CCM activity and low-CO_2-inducible expression of *Cah1*. The role and significance of LCR1 in CO_2-signal transduction pathways in the eukaryotic photosynthetic organism Chlamydomonas are discussed.

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

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arylsulfatase (Ars) enzyme activity (Pro_{Cah1}:*Ars*). The strain CAO3 (Pro_{Cah1}:*Ars*, *Nia1*, *cw15*, and *mt*$^-$), which carries a promoterless *Ars* reporter gene fused to the *Cah1* promoter and exhibits CO_2-responsive Ars expression (Kucho et al., 1999), was crossed with CC2678 (*nia1-305*, *cw15*, *sr-1*, and *mt*$^+$), which has wild-type phenotypes for photosynthetic activity and *Cah1* expression. A single progeny lacking functional *Nia1* gene and exhibiting CO_2-responsive Ars induction was isolated and named Q304P3 (Pro_{Cah1}:*Ars*, *nia1-305*, *cw15*, and *mt*$^-$). The Q304P3 strain was mutagenized by random insertion of the *Nia1* gene, which was used as a selection marker. Twenty-five thousand nia$^+$ colonies were screened, and 15 colonies were found not to exhibit Ars activity under low-CO_2 conditions in light (Figure 1A). Among them, a mutant named *lcr1* was analyzed further. In this mutant, accumulation of both Pro_{Cah1}:*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-CO_2 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-CO_2 conditions, these three strains had equivalent growth rates. Under low-CO_2 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-CO_2-requiring phenotype.

To evaluate the apparent affinity of the *lcr1* mutant for Ci, the photosynthetic $K_{0.5}$(Ci) value was determined using an O_2 electrode (Figure 2B). The host strain Q304P3, grown under low-CO_2 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-CO_2 conditions, it had lower affinity [$K_{0.5}$(Ci) = 207 μM] than Q304P3 grown under the same conditions [$K_{0.5}$(Ci) = 93 μM]. Because under low-CO_2 conditions the *lcr1* mutant showed higher affinity than Q304P3 grown under high-CO_2 conditions [$K_{0.5}$(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-CO_2 conditions (142 ± 15 and 119 ± 8 μmol·mg^{-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 nia$^-$ strain CC2678 (*nia1-305*), which exhibited wild-type phenotypes for photosynthesis and *Cah1* expression. In 27 of 28 nia$^+$ progeny, the deficiency in *Cah1* induction cosegregated with the nia$^+$ phenotype, and only a single insertion of *Nia1* gene was detected by DNA gel blot analysis (data not shown). These

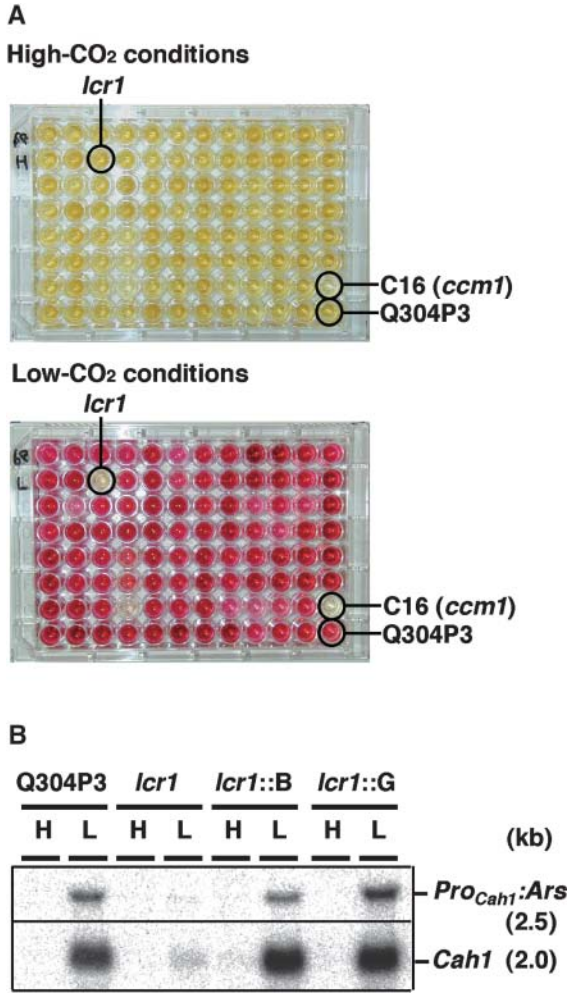

Figure 1. Isolation of Mutants Deficient in *Cah1* Induction under Low-CO_2 Conditions by ARS Screening System.

(A) ARS activity from Pro_{Cah1}:*Ars* in isolated transformants. The *ccm1* mutant C16 without Pro_{Cah1}:*Ars* construct was used as a negative control.

(B) RNA gel blot analyses of Pro_{Cah1}:*Ars* and *Cah1*. RNA gel blots were hybridized with a ^{32}P-labeled pJD54 harboring a promoterless *Ars* gene (Davies et al., 1992) and *Cah1* cDNA clone. *lcr1*::B, *lcr1* transformed with the 5.1-kb genomic fragment Frag-B; *lcr1*::G, *lcr1* transformed with the genomic clone pKK2; H, high-CO_2 conditions; L, transferred from high-CO_2 to low-CO_2 conditions for 2 h.

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

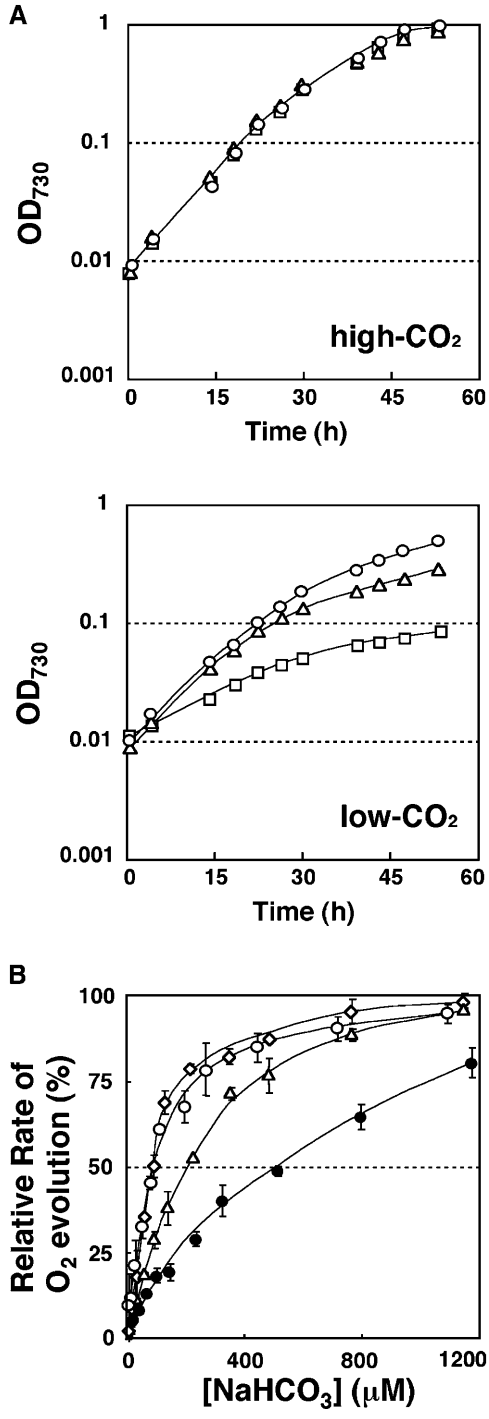

Figure 2. Physiological Characteristics of the *lcr1* Mutant.

(A) Growth curves of the host strain Q304P3 and the *lcr1* mutant under high-CO_2 or low-CO_2 conditions. Circles, triangles, and squares represent Q304P3, the *lcr1* mutant, and the *ccm1* mutant C16, respectively.

(B) Photosynthetic response to Ci concentration of the *lcr1* mutant. High-CO_2 or low-CO_2 cultured Q304P3 (closed or open circles), low-CO_2 cultured *lcr1* (triangles), and complemented *lcr1* (*lcr1*::B; diamonds) were used for measurements of the rate of photosynthetic O_2 evolution. The maximum rates of O_2 evolution were 123, 142, 119, and 120 μmol·mg^{-1} of Chl·h^{-1} for Q304P3-H, Q304P3-L, *lcr1*-L, and *lcr1*::B-L, respectively.

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, *lcr*::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 pI 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, Gln-, 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 CO_2-responsive gene regulation (Kucho et al., 2003), was used as a probe. GST alone did not show DNA

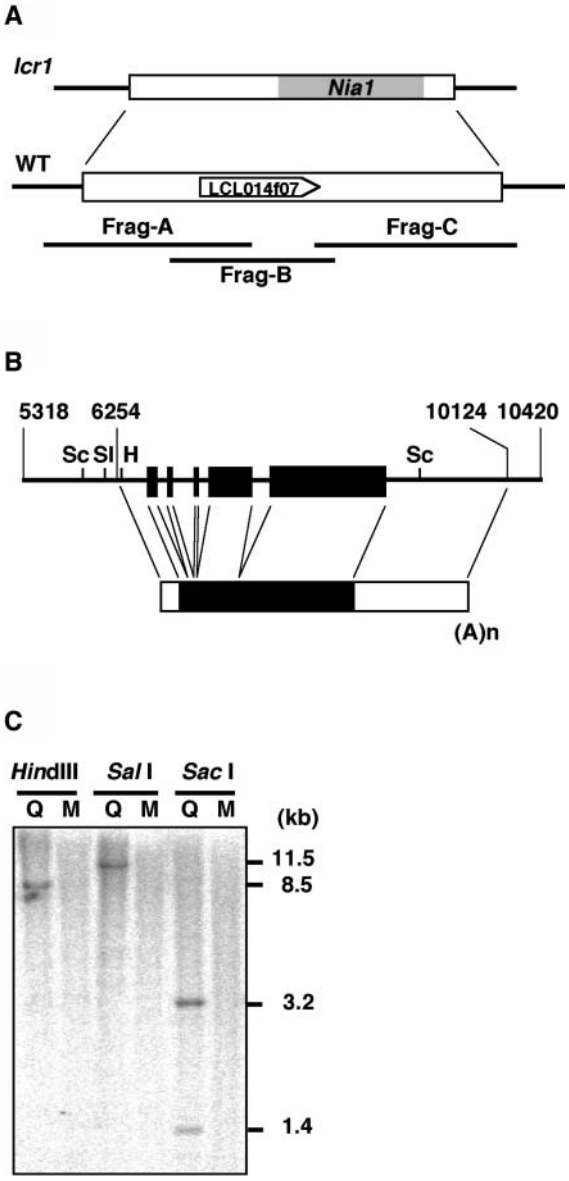

Figure 3. Structure and Copy Number of *Lcr1*.

(A) Insertion site of the *Nia1* tag in the *lcr1* genome and the corresponding wild-type genomic region. Frag-A, Frag-B, and Frag-C represent PCR products designed to span the deleted region. The position and transcriptional direction of the cDNA clone LCL014f07 is displayed.

(B) Structure of the *Lcr1* gene in Frag-B. The closed rectangles indicate the positions of the protein-coding regions. The numbers indicate relative positions of the 16.7-kb genomic sequence containing the deleted region in the *lcr1* mutant. (A)n, poly(A) tail; H, *Hin*dIII; Sl, *Sal*I; Sc, *Sac*I.

(C) Copy number of the *Lcr1* gene. Ten micrograms of both Q304P3 and *lcr1* genomic DNA were digested with the restriction enzymes indicated. The blot was hybridized with a ^{32}P-labeled *Lcr1* cDNA in LCL014f07. Q, Q304P3; M, the *lcr1* mutant.

binding activity (Figure 5B, lane 2). However, GST-Myb showed two shifted bands (Figure 5B, lane 3). These interactions between GST-Myb and the ^{32}P-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 −551 to −501 and −442 to −401 of the *Cah1*-promoter region (Figure 5D).

Previously, we have identified an enhancer, EEC, essential to CO_2-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.

CO_2-Responsive Expression of *Lcr1*

To determine CO_2 responsiveness of *Lcr1*, total RNA was isolated from cells after transfer from high-CO_2 to low-CO_2 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-CO_2 conditions (0 h), a 3.2-kb *Lcr1* mRNA was observed at 1 h after transfer to low-CO_2 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 CO_2-limiting stress.

Next, total RNA was isolated from cells after transfer from low-CO_2 to high-CO_2 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-CO_2 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-CO_2 conditions (Figure 6A, lane 6), it was detected under low-CO_2 conditions in another experiment (Figure 6B, lane 1). This

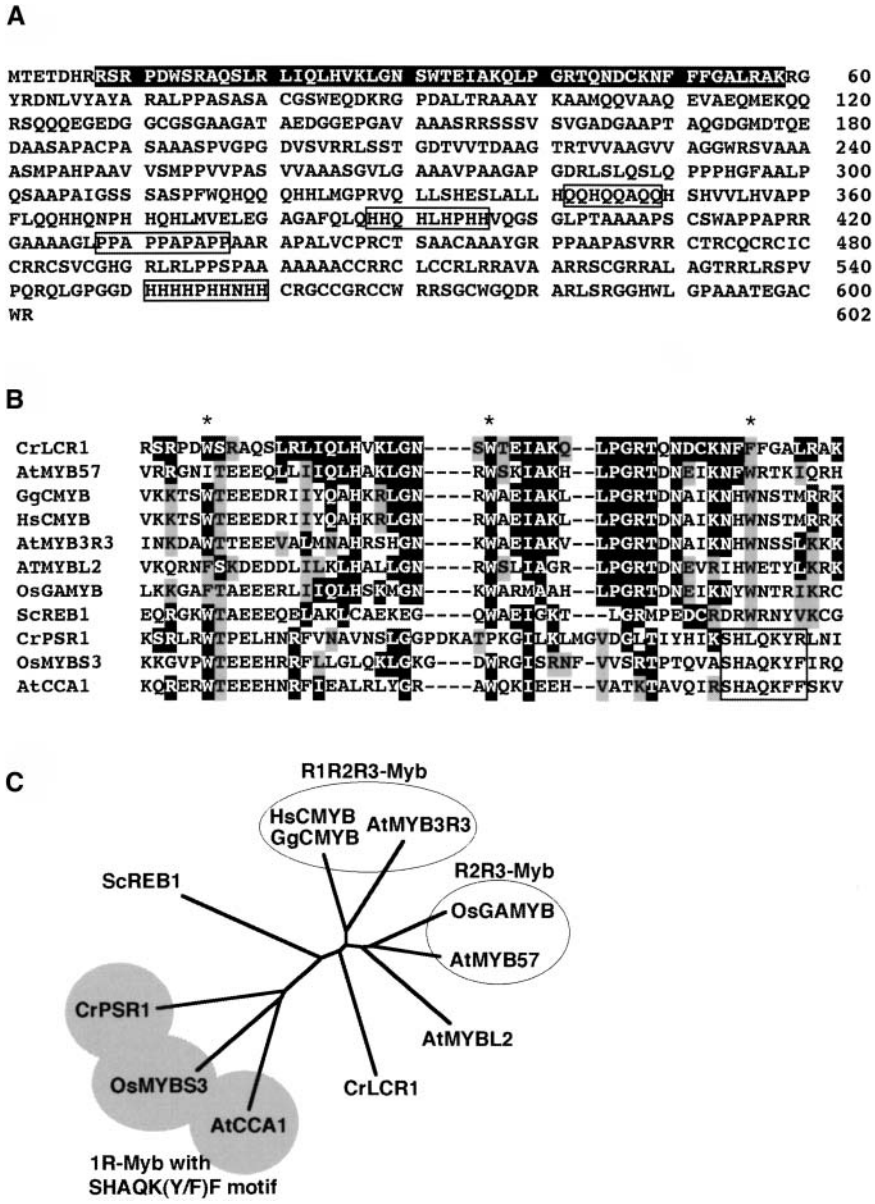

Figure 4. Predicted Amino Acid Sequence of LCR1 and Sequence Comparison with Myb Domains.

(A) Primary structure of LCR1. The highly conserved Myb domain is highlighted, and characteristic sequences in transcription factors (Q, H, or P-rich region) are boxed.

(B) Alignment of Myb domains: c-MYB from chicken; c-MYB from human; AtMyb3R-3, AtMYB57, AtMYBL2, and CCA1 from Arabidopsis; GAMYB and OsMYBS3 from rice; REB1 from yeast; PSR1 from Chlamydomonas. The residues in black and those in gray are identical and similar in LCR1, respectively. Asterisks indicate the three conserved Trp residues present in the Myb domains. The SHAQK(Y/F)F motifs (Lu et al., 2002) are boxed.

(C) Phylogenic relationship of Myb domains. Myb domains listed in **(B)** were aligned by the ClustalW program using the neighbor-joining method.

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-CO_2 signal transduction pathway (Miura et al., 2004). To determine whether CCM1 regulates expression of *Lcr1* under low-CO_2 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-CO_2 conditions (Figure 7A, lane 4). By contrast, in complemented C16, the *Lcr1* mRNA was detected in the case of the wild-type strain C9

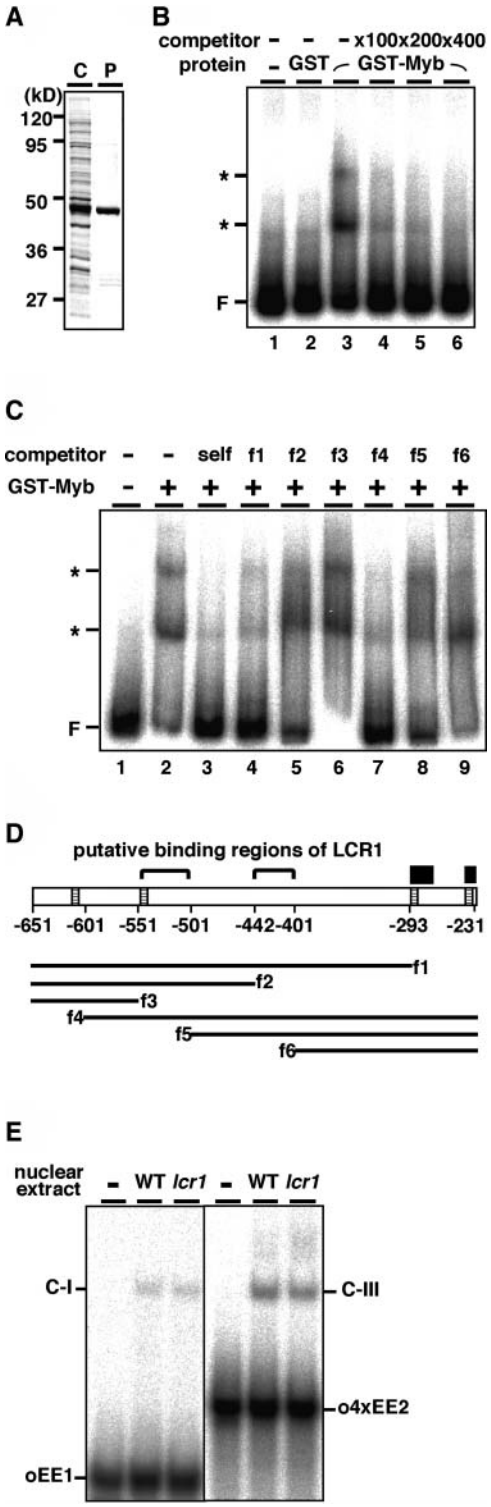

Figure 5. DNA Binding Activity of Myb Domain in LCR1.

(A) Expression of recombinant partial LCR1 fused with GST (GST-Myb). The predicted molecular mass of the GST-Myb, harboring N-terminal 129 amino acids of LCR1, was 43.0 kD. The proteins were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining. C, crude *E. coli* extract; P, purified GST-Myb protein.

(Figure 7A, lane 6). These results revealed that expression of *Lcr1* under low-CO_2 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-CO_2 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-CO_2 conditions or after transfer to low-CO_2 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-CO_2 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-CO_2-inducible genes except *Cah1*, *Lci1*, and *Lci6* were not significantly affected by *Lcr1*, for example, *Mca* (expression ratio of C9 to *lcr1* under low-CO_2 conditions with standard deviation: 2.2 $\pm$ 0.5), *Ccp1* (1.6 $\pm$ 0.1), *Aat1* (1.1 $\pm$ 0.4), and *Pgp1* (1.1 $\pm$ 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

(B) Gel mobility shift assay using the DNA fragment of the *Cah1* promoter. A ^{32}P-labeled 421-bp probe was incubated with GST or GST-Myb. Unlabeled DNA fragments identical to the probe sequence were added as a competitor. F, free probe; asterisks, DNA–protein complexes.

(C) Competition analysis using truncated unlabeled fragments. Two-hundred-fold molar excess of each competitor was added to the reaction mixtures.

(D) Relative positions of the competitors used in **(C)**. Hatched boxes indicate the position of EECs, and closed boxes indicate the position of EE-1 (left) and EE-2 (right) (see **[E]**).

(E) Gel mobility shift assays using the oligonucleotide probes containing EEC sequence. A ^{32}P-labeled 25-bp probe oEE1 and 44-bp probe o4xEE2 (four tandem copies of EE-2) were incubated with the nuclear extracts from the wild type and the *lcr1* mutant. C-I and C-III, DNA–protein complexes (Kucho et al., 2003).

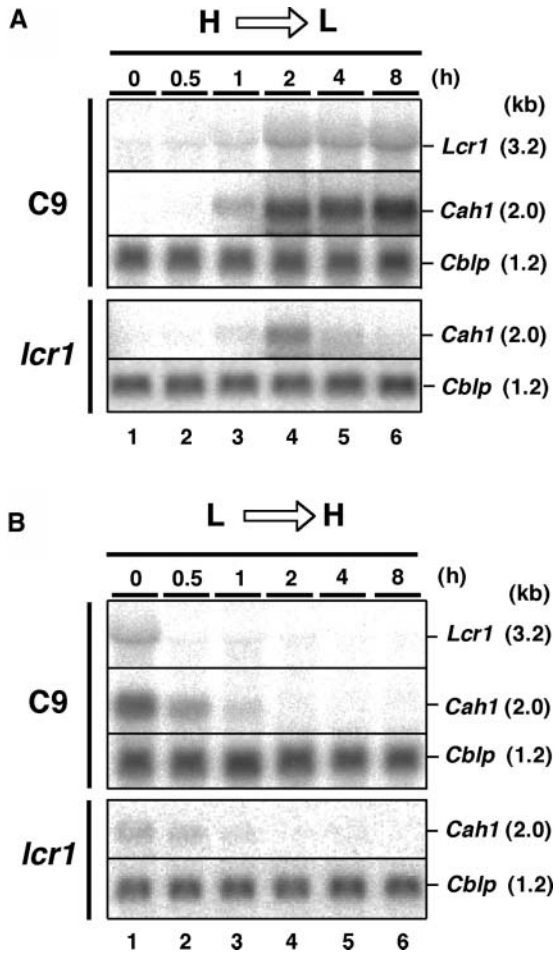

Figure 6. RNA Gel Blot Analyses of *Lcr1* and *Cah1* in Wild-Type Strain C9 and the *lcr1* Mutant.

(A) Accumulation patterns of *Lcr1* and *Cah1* mRNA after transfer from high-CO_2 (H) to low-CO_2 (L) conditions. Total RNA was isolated at 0, 0.5, 1, 2, 4, and 8 h after the change of CO_2 level. RNA gel blots were hybridized with ^{32}P-labeled cDNA probes for *Lcr1* and *Cah1*. A ^{32}P-labeled cDNA probe for *Cblp* encoding the G-protein β subunit, which is expressed constitutively (Schloss, 1990), was used as a loading control. **(B)** Accumulation patterns of *Lcr1* and *Cah1* mRNA after transfer from low-CO_2 (L) to high-CO_2 (H) conditions.

that were deficient in induction of *Cah1* expression under low-CO_2 conditions. Previously, high-CO_2-requiring mutants have been isolated from Chlamydomonas (Moroney et al., 1989; Fukuzawa et al., 1998). One was the regulatory mutant *ccm1* (*cia5*), which cannot induce a set of CCM-related genes. Using the *Pro*$_{Cah1}$*:Ars* screening system, we isolated several regulatory mutants, including a mutant having a mutation in the *Ccm1* gene. This system has advantages compared with methods based on selecting high-CO_2-requiring mutants because we can isolate the regulatory mutants regardless of the high-CO_2-requiring phenotype.

The *lcr1* mutant showed relatively lower growth rate and reduced affinity to Ci under low-CO_2 conditions (Figure 2), indicating that LCR1 is essential for full induction of CCM. LCR1 would regulate genes that play a significant role in the CCM, such as Ci transport. Array analysis and RNA gel blot analyses indicate that three low-CO_2-inducible genes, *Cah1*, *Lci1*, and *Lci6*, are regulated by LCR1. Because inhibition of periplasmic carbonic anhydrases raises the apparent photosynthetic K_m for external 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 *lcr1* mutant exhibited more severe phenotypes than the *Cah1* null mutant (Van and Spalding, 1999). Therefore, the *lcr1* phenotypes cannot be explained only by the defect in *Cah1* induction. Other affected genes, including *Lci1* and *Lci6*, seem to be responsible for the CCM. In particular, *Lci1* is one candidate for the 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, *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. AtMybL2, 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, *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 *cmpABCD* or *ndh3* operons, respectively (Figge et al., 2001; Omata et al., 2001). Although both Chlamydomonas and cyanobacteria possess a CCM, different groups of transcriptional 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

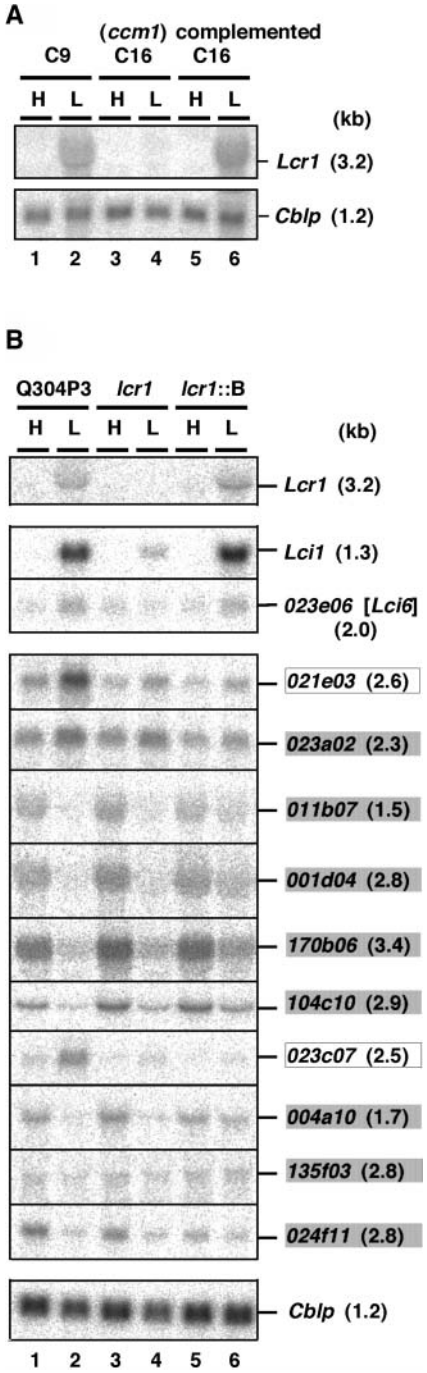

Figure 7. *Lcr1* Expression in the *ccm1* Mutant and Genes Regulated by LCR1.

(A) RNA gel blot analysis of *Lcr1* in the *ccm1* mutant C16 and complemented C16. RNA gel blots were hybridized with a ^{32}P-labeled *Lcr1* cDNA probe. A ^{32}P-labeled cDNA probe for *Cblp* was used as a loading control. H, high-CO_2 conditions; L, transferred from high-CO_2 to low-CO_2 conditions for 2 h.

(B) RNA gel blot analyses of 12 genes that were downregulated in the *lcr1* mutant. RNA gel blots were hybridized with a ^{32}P-labeled cDNA probe for each clone. Shaded boxes indicate cDNA clones whose mRNA levels did not change between Q304P3 and *lcr1*. Open boxes indicate cDNA 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.

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 CO_2-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 CO_2 conditions (Kucho et al., 2003), whereas *Lcr1* mRNA was detected only under low-CO_2 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-CO_2 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 CO_2-limiting stress is described as follows (Figure 8). When cells are exposed to

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

Array ID	Clone ID	C9L/C9H	C9L/*lcr1*L	Gene	Product	Note[a]
021e03	AV622398	1.8 ± 0.4	**8.4 ± 3.0**	–	Unknown	–
023a02	AV624504	1.5 ± 1.3	**6.7 ± 4.2**	–	Unknown	–
002b01(6)	AV388733	**57 ± 27**	**6.1 ± 1.5**	*Cah1**	Periplasmic carbonic anhydrase	Fukuzawa et al. (1990)
023e06(2)	AV625166	1.9 ± 0.2	**4.4 ± 1.2**	*Lci6**	Unknown	This report
011b07	AV634389	1.3 ± 0.6	**3.7 ± 1.1**	–	Unknown	–
001d04(5)	AV393788	1.3 ± 0.4	**3.6 ± 0.9**	*Pf1*	Pyruvate formate-lyase	Chlamydomonas
170b06(2)	BP097279	1.8 ± 0.9	**3.3 ± 0.9**	–	Unknown	–
104c10	AV395790	1.5 ± 0.2	**3.5 ± 1.1**	*Phe1*	Pherophorin I precursor	*Volvox carteri*
023c07	AV624911	1.9 ± 0.2	**3.3 ± 0.8**	*Hspg*	Putative heparan sulfate proteoglycan	*Ovis aries*
004a10	AV397861	1.8 ± 0.9	**3.3 ± 0.3**	–	Expressed protein	Arabidopsis
135f03	AV620059	**7.6 ± 4.8**	**3.2 ± 0.6**	–	Unknown	–
024f11(2)	AV626744	1.2 ± 0.2	**3.0 ± 0.3**	–	Unknown	–
019d11(3)	AV619923	**57 ± 38**	**2.7 ± 0.9**	*Lci1**	Low-CO_2-inducible membrane protein	Burow et al. (1996)

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.

[a] Organisms or references are listed.

CO_2-limiting stress, the CO_2-limiting signal is transmitted to CCM1, which is constitutively expressed regardless of CO_2 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-CO_2 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-CO_2-inducible genes will give us new insights into the mechanisms of CO_2-responsive transcriptional regulation. Characterization of other mutants defective in CO_2 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 CO_2-responsive gene regulation and CO_2-signal transduction.

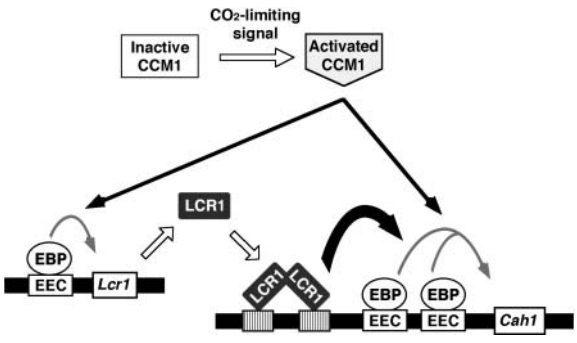

Figure 8. Schematic Drawing of a Possible Mechanism for Transcriptional Activation of *Cah1* in Response to CO_2-Limiting Stress.

Hatched box indicates a putative enhancer that is recognized by LCR1. EBP, EEC binding proteins.

METHODS

Strains, Culture Conditions, and Transformation

For high-CO_2 conditions, cells were cultured in HS medium (Harris, 1989) supplemented with 20 mM Mops [3-(*N*-morpholino)-propanesulfonic acid], pH 7.2, under aeration with air enriched 5% (v/v) CO_2 at 28°C. For low-CO_2 conditions, cultures were bubbled with ordinary air containing 0.04% (v/v) CO_2 in the same medium. Cultures were illuminated by white fluorescent lamps at an intensity of 120 $\mu mol \cdot m^{-2} s^{-1}$. For Ars assays and detection of the Pro_{Cah1}:*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 analysis 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 *Nia1* gene (Fernandez et al., 1989) by the glass beads method as described previously (Kucho et al., 1999). For complementation, cells were cotransformed with the genomic fragment and pSP124S harboring the *ble* gene (Lumbreras et al., 1998). nia^+ or ble^+ colonies were transferred to 96-well microtiter plates and assayed twice for Ars induction under low-CO_2 conditions as described previously (Kucho et al., 2003).

RNA Gel Blot Analyses

Ten micrograms of total RNA were electrophoresed in a denaturing agarose gel and blotted onto a nylon membrane, Biodyne B (Pall, New York, NY). The cDNA probes corresponding to each gene were labeled with [α-^{32}P]dCTP using random primers pd $(N)_9$ (Takara Bio, Otsu, Japan) and Klenow fragment DNA polymerase (Takara). The cDNA clones were isolated from a Chlamydomonas cDNA library (Asamizu et al., 2000). The accession numbers are listed in Table 1. Hybridization was performed with ExpressHyb hybridization solution (Clontech, Palo Alto, CA) at 68°C for 12 to 16 h.

Measurement of Photosynthetic Rate

The rates of photosynthesis were measured in a Clark-type O_2 electrode, Chloroview 1 (Hansatech, King's Lynn, UK), and the CO_2-compensation

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′-GTGGACTGCTACTGCACTCAGG-3′, and C44-3-3r, 5′-CGCCAACATTAGCATACGTCAC-3′; C44-5-2f, 5′-CTGTGGACCGCACAGCAGCACT-3′, and C44-5-2r, 5′-GCTGCAGGTATGCCTGTGTATC-3′. Three primer sets were used to amplify genomic fragments for complementation: Frag-A, C44-3-2, 5′-ATGATGGTCTCAGTGACCGGGTCCGCTGCCTTCAAGGGAC-3′, and C44-cpmA-R, 5′-TGACGACGGTATCACCAGTGGATGAGAG-3′; Frag-B, C44-cpmB-F, 5′-AATGTACAACCAACAGGCGGGAAGGGTC-3′, and C44-cpmB-R, 5′-TGAACAAGCAAACGGGGGTTACGCGCAT-3′; Frag-C, C44-cpmC-F, 5′-GCATGCTTGTGAGAGTTGCTGGAAGACT-3′, and C44-5-2f, 5′-CTGTGGACCGCACAGCAGCACT-3′. PCR was performed with Ex Taq polymerase (Takara) in a reaction mixture (1× Ex Taq buffer is 0.25 mM deoxynucleotide triphosphate, 5% DMSO [v/v], and 1 M betaine) under the following conditions: 40 cycles annealed at 65°C extended for 6 min for Frag-A and Frag-B and 40 cycles annealed at 61°C extended for 6 min for Frag-C. One of the genomic clones containing the flanking region pKK2 was used as a template for the PCR.

Identification and Analysis of Gene Structures

In silico identification of cDNA clones was performed using BLASTN and the Paracel Transcript Assembler programs using Chlamydomonas ESTs. Multiple sequence alignments and the phylogenenic tree were generated using the ClustalW program (http://clustalw.genome.ad.jp/). Molecular weight and pI value were calculated by the Compute pI/Mw tool (http://kr.expasy.org/tools/pi_tool.html). Motifs were annotated by the conserved domain database using Reverse Position Specific BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Localization and classification of proteins were predicted using iPSORT (http://hypothesiscreator.net/iPSORT/) and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html), respectively. The 5′-upstream sequences of *Lci1* and *Lci6* were obtained from the Chlamydomonas genome sequence version 2 in DOE Joint Genome Institute (http://genome.jgi-psf.org/chlre2/chlre2.home.html).

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-aided cloning with the following primer set: LCIR-N, 5′-CCGAATTCATGACGGAGACCGACCACCGCCGAAGC-3′, and LCIR129-SalI, 5′-GGTCGACCCTAGTCCTCTCCCTCTTGCTGCTGGCT-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

The gel mobility shift assays using GST-Myb protein were performed essentially as described previously (Kucho et al., 2003) except as follows. The probe was amplified by PCR with appropriate plasmid DNA using the following primer set: pKpn-3, 5′-ATGGTACCTCAGCTTCTCTCCCGCCAGCAT-3′, and CAup-Kpn6-2, 5′-ATGGTACCTTCGTAAGTCGGACTCGCACCT-3′, and followed by 5′-end labeled with [γ-^{32}P]ATP. Binding reactions were performed by incubating 25 ng of probe (2.0×10^4 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 (Kucho 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-CO_2 conditions or cells transferred from high-CO_2 to low-CO_2 conditions for 1 h using PolyATract System 1000 (Promega, Madison, WI). Target labeling and hybridization were performed as described previously (Miura et al., 2004). Data analyses were performed as follows. Radioactive 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 located at nonspotted areas in each membrane were quantified. 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 (μ_{mem}) 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 $S = (c/\mu_{mem}) \times 18.08$ (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 (S_A/S_B). The expression ratios (C9L/C9H or C9L/*lcr1*L) 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 (S_A) were >25, corresponding to 0.1% of total signal, were selected to assign to be differentially expressed. We confirmed that normalized values of numerators (S_A) 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 AB168090 and AB168091, respectively. The accession number of the genomic sequence containing the deleted region in the *lcr1* mutant is AB168089. The accession numbers for the sequences mentioned in Figure 4 are as follows: P01103 (chicken c-MYB); P10242 (human c-MYB); AAF25950 (Arabidopsis MYB3R-3); NP_186802 (Arabidopsis MYB57); CAA92280 (Arabidopsis AtMYBL2); NP_850460 (Arabidopsis CCA1); CAA67000 (rice GAMYB) and AAN63154 (rice OsMYBS3); P21538 (yeast REB1); AAD55941 (Chlamydomonas PSR1).

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