Expression Profiling-Based Identification of CO_2-Responsive Genes Regulated by CCM1 Controlling a Carbon-Concentrating Mechanism in *Chlamydomonas reinhardtii*[1]

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Photosynthetic acclimation to CO_2-limiting stress is associated with control of genetic and physiological responses through a signal transduction pathway, followed by integrated monitoring of the environmental changes. Although several CO_2-responsive genes have been previously isolated, genome-wide analysis has not been applied to the isolation of CO_2-responsive genes that may function as part of a carbon-concentrating mechanism (CCM) in photosynthetic eukaryotes. By comparing expression profiles of cells grown under CO_2-rich conditions with those of cells grown under CO_2-limiting conditions using a cDNA membrane array containing 10,368 expressed sequence tags, 51 low-CO_2 inducible genes and 32 genes repressed by low CO_2 whose mRNA levels were changed more than 2.5-fold in *Chlamydomonas reinhardtii* Dangeard were detected. The fact that the induction of almost all low-CO_2 inducible genes was impaired in the *ccm1* mutant suggests that CCM1 is a master regulator of CCM through putative low-CO_2 signal transduction pathways. Among low-CO_2 inducible genes, two novel genes, *LciA* and *LciB,* were identified, which may be involved in inorganic carbon transport. Possible functions of low-CO_2 inducible and/or CCM1-regulated genes are discussed in relation to the CCM.

Since acclimation to changing environmental conditions is crucial for cell growth and survival, organisms have developed sensing and signaling mechanisms for acclimation to stress conditions. By sensing the CO_2 availability, a number of aquatic photosynthetic organisms induce a carbon-concentrating mechanism (CCM) to concentrate inorganic carbon (Ci) intracellularly, resulting in increased photosynthetic affinity for Ci and accumulation of Ci in close proximity to Rubisco despite the low affinity and low selectivity of Rubisco for CO_2 (Kaplan and Reinhold, 1999; Badger and Spalding, 2000). This induction is controlled by the transcriptional regulator CCM1 (CIA5) in the eukaryotic alga *Chlamydomonas reinhardtii* through a CO_2-signal transduction pathway (Fukuzawa et al., 2001; Xiang et al., 2001). This CO_2-signaling is a highly regulated process not only in microorganisms but also in higher plants whose stomatal numbers are tightly controlled by atmospheric CO_2 partial pressure (Stretton and Goodman, 1998; Lake et al., 2002). During this process of acclimation to CO_2-limiting conditions, several genes are induced in Chlamydomonas cells, such as *Cah1* encoding a periplasmic carbonic anhydrase (CA; Fukuzawa et al., 1990), *Mca* coding for a mitochondrial CA (Eriksson et al., 1996), *Ccp* for a chloroplast envelope protein LIP-36 (Chen et al., 1997), *Aat1* for Ala-α-ketoglutarate aminotransferase (Chen et al., 1996), and *Pgp1* for phosphoglycolate phosphatase (Mamedov et al., 2001). *Cah3,* encoding a chloroplast CA, is slightly induced under CO_2-limiting conditions and is essential for the CCM because *cia3,* which is impaired in the expression of chloroplastic CA, shows a severe high-CO_2 requiring phenotype (Karlsson et al., 1998). Although these low CO_2-inducible genes have been characterized, other components of the CCM are still poorly understood.

Photorespiration has been shown to be necessary for acclimation to CO_2-limiting stress using the high-CO_2 requiring mutant, *pgp1-1* (Suzuki et al., 1990). The necessity of the Ci-uptake system has also been clearly shown by isolation and characterization of a high-CO_2 requiring mutant, *pmp1-1*, defective in bicarbonate transport and Ci accumulation (Spalding et al., 1983). In the CCM-containing cyanobacteria, four Ci-uptake

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systems are reported: two bicarbonate transport systems encoded by *CmpABCD* (Omata et al., 1999) and *SbtA* (Shibata et al., 2002b), and two CO_2 uptake systems consisting of the NdhD3 (Ohkawa et al., 2000a) and NdhD4 types (Shibata et al., 2001).

It is not yet clear how many genes are induced or repressed during acclimation to low-CO_2 conditions. The roles of proteins encoded by low-CO_2 inducible genes in the acclimation to CO_2-limiting conditions are not fully understood in photosynthetic eukaryotes. Quantitative and global analysis of expression profiles by means of cDNA array analyses have been used to improve our overall understanding of the molecular basis of various mechanisms (Lockhart and Winzeler, 2000). In this study, we have identified CO_2-responsive genes by analyzing the transcript profiles of wild-type cells and CCM1-regulated genes by comparing expression of genes in a mutant with that in wild-type cells. Since the *ccm1* mutant C16 exhibits a high-CO_2 requiring phenotype (Fukuzawa et al., 1998), it is possible to identify genes essential to the CCM by analyzing the expression profile of the C16 mutant. Based on transcriptome analyses, the role of the *Ccm1* gene in the induction of the CCM and possible functions of CO_2-inducible and CCM1-regulated genes are discussed in relation to the CCM.

RESULTS

Identification of Differentially Expressed Genes during Changes in CO_2 Levels

We generated a cDNA macroarray using expressed sequence tag (EST) clones collected from wild-type Chlamydomonas cells to allow global analyses of gene expression. By assembling the 5′-end EST sequences of 50,832 clones (Asamizu et al., 1999, 2000; E. Asamizu, Y. Nakamura, K. Miura, H. Fukuzawa, S. Fujiwara, M. Hirono, K. Iwamoto, Y. Matsuda, J. Minagawa, K. Shimogawara, Y. Takahashi, and S. Tabata, unpublished data), 10,368 nonredundant EST clones were selected to construct a membrane array for simultaneous analysis of gene expression. The cDNA inserts from the selected EST clones were amplified by PCR and spotted on membranes in duplicate. The resulting array comprised 6 membranes containing 20,736 spots consist of 10,368 PCR fragments derived from EST clones.

To identify differentially expressed genes during the low-CO_2 acclimation process, two independent hybridization experiments were carried out using different preparations of RNA samples. The array membranes were hybridized with ^{32}P-labeled target cDNA samples produced from poly(A)$^+$ RNA isolated from wild-type cells. To maintain high-CO_2 conditions, air enriched with 5% CO_2 was bubbled through the medium. For low-CO_2 conditions, cells grown in high-CO_2 conditions were transferred to conditions where ordinary air containing 0.04% CO_2 was bubbled through the medium for 1 h. The induction period of 1 h was used because previously identified low-CO_2 inducible genes such as *Cah1* and *Mca* are induced within 1 h of exposure to low CO_2 (Fukuzawa et al., 1990; Eriksson et al., 1996). After hybridization, the image data of the hybridization patterns were converted into digital data and the signal intensities of the spots were adjusted with mean-normalization. Each

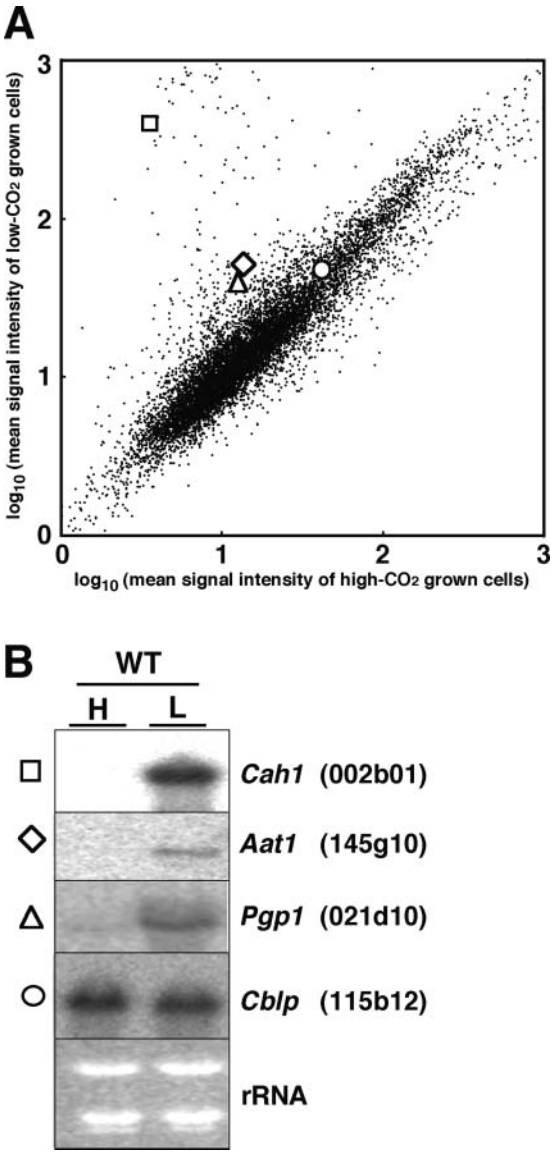

Figure 1. Scatter plot of hybridization signals in the cDNA array (A) and northern-blot analyses of representative low-CO_2 inducible genes (B). Mean signal intensities in hybridization with probes from high-CO_2 grown wild-type cells and low-CO_2 grown wild-type cells were plotted along the *x* and *y* axes, respectively. The signal intensities of *Cah1* (clone ID 002b01, square), *Aat1* (clone ID 145g10, diamond), *Pgp1* (clone ID 021d10, triangle), and *Cblp* (clone ID 115b12, circle) are highlighted. B, Ten micrograms of total RNA from either high-CO_2 grown (H) or 1-h low-CO_2 adapted wild-type cells (L) were hybridized with ^{32}P-labeled *Cah1*, *Aat1*, *Pgp1*, and *Cblp* gene-specific probes.

expression ratio of the signal intensity of the clone in low-CO_2 cells to that in high-CO_2 cells was calculated as described in ''Materials and Methods'' (Fig. 1A; Tables I and II). Because the macroarray carries duplicates of each EST clone, there were 4 expression ratios for each EST clone. If 3 expression ratios out of 4 exceeded 2.5-fold and the average of the expression ratios was more than 2.5, then the EST was selected as a differentially expressed clone. In this initial step, 122 induced clones and 55 repressed clones were identified under CO_2-limiting conditions. Based on the 3′-end sequences of these EST clones, chimerical and redundant clones were removed. In total, 51 low-CO_2 inducible and 32 low-CO_2 repressed transcripts were identified after analysis of the macroarray results.

To annotate the putative biological functions of these 83 differentially expressed genes, consensus sequences of the EST contigs were obtained by using the GenBank EST-sequence databases (Asamizu et al., 1999, 2000; Shrager et al., 2003). Then these sequences were annotated by using the programs BLASTX and RPS-BLAST (Tables I and II). As expected, 7 previously known low-CO_2 inducible genes, *Mca*, *Cah1*, *Lci1*, *Ccp1*, *Pgp1*, *Aat1*, and *Lci5,* were found to be part of the group of 51 genes detected as low-CO_2 inducible in our array analysis. To compare the ratios of the expression levels in high-CO_2 conditions to that in low-CO_2 conditions, mRNA levels corresponding to 3 previously known low-CO_2 inducible genes, *Cah1*, *Aat1*, *and Pgp1,* were measured by northern-blot analyses using the same RNA samples used in the array analysis (Fig. 1). Indeed, the induction ratio of *Cah1* was 57 (low-CO_2/high-CO_2) in the array analysis, which is comparable to the ratio of 57 measured in the northern blot analysis. Similarly, the ratio of *Aat1* was 3.4 in the array and 3.4 in the northern blot, while the ratio of *Pgp1* was 3.6 in the array and 3.3 in the northern (Fig. 1). The similarity in ratios between the array and northern-blot analyses indicates that the cDNA membrane array is useful for identification of differentially expressed genes among the 10,378 EST clones.

Up-Regulation of Genes during Acclimation to CO_2-Limiting Stresses

Seven of the 51 low-CO_2 inducible genes have significant sequence similarities with photorespiration-related genes such as *Aat1*, *Pgp1*, *Sgat*-coding for Ser-glyoxylate aminotransferase, *Fhs*-coding for formyltetrahydrofolate synthetase, *Gdh1*-coding for glycolate dehydrogenase, *Shmt*-coding for Ser hydroxymethyltransferase, and *GscP*-coding for the Gly cleavage system P-protein. Three genes, *Aat1*, *Pgp1*, and *Gdh1,* are low-CO_2 inducible as shown previously (Fig. 1; Miura et al., 2002). Given that the CarB protein, a carbamoyl phosphate synthetase, plays a role in assimilation of ammonium, it seems likely that the *CarB* homolog in Chlamydomonas functions to assimilate ammonium resulting from Gly cleavage in the photorespiratory cycle.

Two genes involved in starch synthesis, *Sta2* and *Sta3*, were also induced under low-CO_2 conditions. The *Sta2* gene, encoding a granule-bound starch synthase I, and the *Sta3* gene, which codes for a soluble starch synthase (ADP-Glc: α-1, 4-D-glucan-4-α-D-glucosyltransferase), play roles in elongation of starch (Ball, 1998). Since another low-CO_2 inducible gene, 024b04, contains the starch-binding domain of CBM_20 (pfam00686), this gene may be involved in starch metabolism. Because Chlamydomonas cells develop pyrenoids under low-CO_2 conditions, which are surrounded by a typical starch sheath (Ramazanov et al., 1995), these starch-related genes may function in pyrenoid development during acclimation to low-CO_2 conditions.

Although four genes encoding chlorophyll *a/b*-binding proteins were repressed under low-CO_2 conditions (Table II), *LI818* encoding an unknown protein similar to chlorophyll *a/b*-binding proteins was highly induced under low-CO_2 conditions (Richard et al., 2000). The gene 142b11, with low level but significant sequence similarity to Arabidopsis *Sag29,* which encodes a senescence-associated membrane protein, is also induced. Since thioredoxin plays a role in redox signaling in many photosynthetic organisms (Huppe et al., 1990), it is noteworthy that *Trxf1,* which codes for thioredoxin f1, was also induced under CO_2-limiting conditions. These genes may have some regulatory roles in controlling enzyme activities or expression of other genes. In the low-CO_2 induced genes, a gene with sequence similarity to *Arabidopsis Ndh* encoding a type-2 NADH dehydrogenase, a gene similar to *Tagetes Ggps* coding for geranylgeranyl pyrophosphate synthase, and *Chlamydomonas Mmp* encoding a mitochondrial matrix protein, were included. Additionally, three of the low-CO_2 inducible genes encode proteins containing several domains related with gene regulation; e.g. 149b12 contains the interferon-induced GTPase domain (pfam05049), 022g04 harbors the glyoxalase domain (pfam00903), and 022b10 has a TB2/DP1, HVA22 domain (pfam03134).

Down-Regulated Genes during Acclimation to CO_2-Limiting Conditions

During acclimation to low-CO_2 stress, 32 genes were repressed within 1 h (Table II). Several photosynthetic genes were among these 32 genes such as genes encoding light harvesting chlorophyll-*a/b* binding proteins (*Lhcb2*, *Lhcb4*, *Lhcbm9*, and *LhcII-3*), a light-harvesting-like protein (019c02, similar to Arabidopsis *Lil3*), proteins for chlorophyll synthesis and chlorophyll assembly (*Gsa* coding for Glu-1-semialdehyde aminomutase, *156h09* similar to pea *HemC* coding for porphobilinogen deaminase, *Cpx1* coding for coproporphyrinogen III oxidase, *ChlH* coding for magnesium chelatase H subunit, *Ggh* coding for geranylgeranyl hydrogenase, *Por1* coding for NADPH: protochlorophyllide oxidoreductase, and *Alad* coding for δ-aminolevulinic acid dehydratase), and compo-

Table I. *Genes induced under low-CO_2 conditions and/or CCM1-dependent genes revealed by cDNA array analyses*

Array ID[a]	Clone ID[b]	WT-LC/ WT-HC	WT-LC/ C16-LC	Gene[c]	Putative Function[c]	Organism[c]	GenBank Hit[d]	Motif(pfam)[e]
135h02 (28)	AV620271	110 (±55)	150 (±99)	*Mca*	Mitochondrial carbonic anhydrase	*C. reinhardtii*	U41190	–
002b01 (6)	AV388733	57 (±27)	77 (±49)	*Cah1*	Periplasmic carbonic anhydrase	*C. reinhardtii*	P20507	–
019d11 (3)	AV619824	57 (±38)	79 (±57)	*Lci1*	Low-CO_2 inducible membrane protein	*C. reinhardtii*	U31976	–
018h08 (4)	AV619223	56 (±18)	52 (±15)	*LciA*	Nitrite transporter homolog (this study)	*C. reinhardtii*	AF149737	–
022b07	AV623489	39 (±14)	50 (±25)	*Ccp1*	Chloroplast envelope protein LIP-36	*C. reinhardtii*	U75345	–
012g07	AV637344	25 (±7.1)	17 (±6.8)	*LciB*	Putative chloroplast protein (this study)	–	–	Dynamin family (pfam00350)
150h12	AV629279	22 (±10)	12 (±5.7)	–	–	–	–	No hit
013d05	AV638622	20 (±0.6)	11 (±3.3)	–	Unknown (AT3g61320)	Arabidopsis	NP_191691	No hit
028e01 (4)	AV630016	18 (±5.5)	18 (±3.4)	*LciC*	Putative chloroplast protein (this study)	–	–	Dynamin family (pfam00350)
019b05 (7)	AV619622	13 (±7.1)	15 (±9.5)	*Ll818*	Chlorophyll a/b-binding protein-like	*C. reinhardtii*	X95326	–
146f04	AV627166	12 (±4.5)	16 (±3.7)	–	–	–	–	No hit
019g12	AV620297	12 (±5.6)	14 (±2.2)	–	–	–	–	No hit
142b11	AV624994	11 (±4.8)	3.6 (±1.7)	*Sag29*	Senescence-associated membrane protein	Arabidopsis	NP_196821	–
024b04 (2)	AV626358	11 (±2.8)	13 (±3.8)	–	–	–	–	CBM_20, Starch binding domain (pfam00686)
149b12	AV628400	9.7 (±3.0)	7.2 (±4.1)	–	–	–	–	Interferon induced GTPase (pfam05049)
154c05	AV630876	9.0 (±1.2)	21 (±14)	–	–	–	–	No hit
028f03 (2)	AV630146	8.9 (±4.2)	14 (±7.0)	–	Unknown (At2g45870)	Arabidopsis	NP_182111	No hit
164b10	BP087144	7.7 (±1.2)	6.5 (±2.8)	–	–	–	–	No hit
135f03	AV620059	7.6 (±4.8)	7.8 (±4.4)	–	–	–	–	No hit
025f01 (3)	AV627578	7.5 (±3.3)	8.4 (±3.9)	*Mmp*	Putative mitochondrial matrix protein	*C. reinhardtii*	Y11586	–
021c08	AV622166	6.3 (±1.2)	3.3 (±0.4)	*Sgat*	Serine-glyoxylate aminotransferase	*Methylobacterium extorquens*	P55819	–
137c09	AV386723	5.7 (±1.4)	14 (±0.8)	*Ggps*	Geranylgeranyl pyrophosphate synthase	*Tagetes erecta*	AAG10424	–
134g10 (3)	AV619535	5.2 (±1.5)	8.3 (±2.0)	–	–	–	–	No hit
102f05	AV627999	4.9 (±1.4)	3.4 (±1.3)	–	–	–	–	No hit
169e06	BP097010	4.8 (±1.4)	6.3 (±1.9)	*Fhs*	10-Formyltetrahydrofolate synthetase	Arabidopsis	NP_564571	–
022g04 (2)	AV624187	4.8 (±1.6)	3.2 (±0.6)	–	Unknown (At5g57040)	Arabidopsis	NC_003076	Glyoxalase (pfam00903)
151c11	AV629419	4.4 (±1.3)	5.9 (±1.8)	*Mcp*	Mitochondrial carrier protein	*Schizosaccharomyces pombe*	NP_593701	–
024a11 (3)	AV626334	4.4 (±0.6)	3.7 (±0.5)	*Gdh1*	Putative glycolate dehydrogenase	*Vibrio cholerae*	NP_233369	–
020c02	AV620842	4.2 (±1.1)	4.6 (±0.7)	–	–	–	–	No hit
138e10	AV622332	3.8 (±0.6)	4.4 (±0.7)	–	–	–	–	No hit
138c12	AV622214	3.8 (±0.4)	4.4 (±1.0)	–	–	–	–	No hit
021d10 (2)	AV635342	3.6 (±1.1)	3.9 (±1.4)	*Pgp1*	Phosphoglycolate phosphatase	*C. reinhardtii*	AB052169	–
145g10	AV626859	3.4 (±0.6)	6.0 (±1.7)	*Aat1*	Alanine aminotransferase	*C. reinhardtii*	U31975	–
011e02 (2)	AV634930	3.4 (±1.0)	4.0 (±1.5)	*Lci5*	Low-CO_2 inducible protein	*C. reinhardtii*	AAK77552	No hit
022f03 (2)	AV624023	3.4 (±0.7)	2.8 (±0.1)	*Shmt*	Serine hydroxymethyltransferase	*C. reinhardtii*	AF442558	–
029g12 (3)	AV631115	3.3 (±1.1)	3.7 (±0.7)	*Sta2*	Granule-bound starch synthase I	*C. reinhardtii*	AF026420	–
123h02	BP098683	3.3 (±0.8)	3.6 (±0.9)	*Sta3*	Soluble starch synthase	*C. reinhardtii*	AF026422	–
030h09	BP086273	3.3 (±1.0)	2.8 (±0.6)	–	–	–	–	No hit

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Array ID[a]	Clone ID[b]	WT-LC/ WT-HC	WT-LC/ C16-LC	Gene[c]	Putative Function[c]	Organism[c]	GenBank Hit[d]	Motif(pfam)[e]
005c07	AV387887	3.2 (±0.8)	3.5 (±0.9)	–	–	–	–	No hit
022b10	AV623502	3.0 (±0.5)	4.7 (±0.6)	–	Unknown (At4g36720)	Arabidopsis	NP_195390	TB2/DP1, HVA22 family (pfam03134)
023d09	AV624492	2.9 (±0.3)	3.0 (±0.4)	–	–	–	–	No hit
148a10	AV627826	2.9 (±0.5)	2.8 (±0.8)	–	–	–	–	Chromate transporter (pfam02417)
022h10 (2)	AV624435	2.8 (±0.4)	2.7 (±0.3)	–	–	–	–	No hit
170b12	BP097305	2.8 (±0.2)	2.6 (±0.3)	*Ndh*	Mitochondrial type 2 NADH dehydrogenase	Arabidopsis	NP_193880	–
167b10	BP095941	2.7 (±0.5)	2.9 (±0.3)	*CarB*	Carbamoyl phosphate synthetase	Arabidopsis	BAB90007	–
011d04	AV634814	2.6 (±0.7)	2.6 (±0.2)	*Trxf1*	Thioredoxin f1	*C. reinhardtii*	AY184800	–
003b05 (2)	AV396054	2.5 (±0.3)	4.1 (±0.9)	*GcsP*	Glycine cleavage system P-protein	Arabidopsis	O49850	–
171b12	BP097792	4.7 (±2.7)	2.1 (±0.9)	–	–	–	–	No hit
021d12	AV622387	2.9 (±0.3)	2.4 (±0.2)	–	–	–	–	No hit
010b02	AV632213	2.8 (±0.5)	2.3 (±0.3)	–	Unknown (At1g65230)	Arabidopsis	NP_176702	No hit
160c11	BP088539	2.6 (±0.2)	2.0 (±0.1)	–	–	–	–	No hit
021e03	AV622398	1.8 (±0.4)	7.3 (±2.4)	–	–	–	–	No hit
023c07	AV624911	1.9 (±0.2)	4.6 (±0.9)	*Hspg*	Putative heparan sulfate proteoglycan	*Ovis aries*	AAD01973	–
139e12	AV623221	2.4 (±0.6)	4.0 (±0.5)	–	–	–	–	Reverse transcriptase (pfam00078)
019h09 (2)	AV620492	1.9 (±0.5)	3.4 (±0.4)	*Ptp*	Putative tyrosine phosphatase	*O. sativa*	AAF81798	–
022a05	AV392559	2.1 (±0.6)	3.2 (±0.6)	*Vdac*	Voltage-dependent anion channel	*O. sativa*	CAB82853	–
142e05	AU301246	2.2 (±0.4)	3.1 (±0.3)	–	–	–	–	No hit
024c01	AV626401	2.1 (±0.4)	3.1 (±0.8)	*Cah3*	Chloroplast carbonic anhydrase	*C. reinhardtii*	U40871	–
022c08	AV623614	2.4 (±0.4)	2.9 (±0.2)	–	Unknown (PA0315)	*Pseudomonas aeruginosa*	NP_249006	No hit
148c05	AV627926	2.3 (±0.1)	2.8 (±0.4)	*Mdh1*	NADP-malate dehydrogenase	*C. reinhardtii*	AJ277281	–
138h02	AV622607	1.8 (±0.1)	2.7 (±0.2)	*Cry*	Crystallin J1C	*T. cystophora*	C46745	–
150d03	AV628957	2.2 (±0.2)	2.5 (±0.4)	–	Unknown (At2g42750)	Arabidopsis	AAM19907	DnaJ domain (pfam00226)

Genes whose expression ratios were in excess of 2.5-fold are underlined. Dashes indicate not applicable. [a]Representative array IDs are shown in the first column. Numbers in parentheses indicate numbers of array IDs grouped into the same contigs, after sequencing the 3′ end of the cDNA. [b]GenBank accession numbers for the EST clones. [c]Genes with e-values $<e^{-10}$ were annotated. [d]Best or most informative GenBank sequence alignments. [e]Motifs were annotated by the conserved domain database using RPS-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

nents for the oxygen-evolving complex (*Psb2* and *Psb3*). LIL3 is a counterpart of the cyanobacterial high light inducible proteins (Jannson, 1999). These chlorophyll-related genes are presumably repressed for photoprotection during CO_2-limiting stress conditions, because light energy is in excess under low-CO_2 conditions. The *Crd1* gene encoding a putative di-iron enzyme was also repressed in low-CO_2 conditions. CRD1 is required for accumulation of photosystem I and light harvesting chlorophyll complex-I under copper-deficient conditions, and the *crd1* mutant exhibits copper-deficiency-conditional chlorosis (Moseley et al., 2000).

A high-CO_2 induced gene, *H43*, coding for a periplasmic protein (Shiraiwa and Kobayashi, 1999), was repressed in low-CO_2 conditions, although this gene is induced under cadmium- and iron-stress conditions (Rubinelli et al., 2002). Several nutrient-related metabolic genes such as *Pgk* coding for phosphoglycerate kinase, 033e05 similar to *Ipmdh* encoding 3-isopropylmalate dehydrogenase, *Uo2* coding for urate oxidase, and *MetC* coding for the Met synthase were also repressed in low-CO_2 conditions. Three genes, *Hsp70*, *Hsp81-2*, and *Cpn60a* encoding molecular chaperons, and 016e07 similar to pea *Sar,* which codes for a DNA-binding protein, were repressed under low-CO_2 conditions. In contrast, three other chaperon-related genes, *Chlamydomonas Hsp22,* 115d09 similar to *Scherffelia Bip*, and 114b09 similar to rice *DnaK*, were not repressed in the *ccm1* mutant C16 but repressed in wild-type irrespective to CO_2 levels.

Table II. *Genes repressed under low-CO_2 conditions and/or CCM1-dependent genes revealed by cDNA array analyses*

ID[a]	Clone ID[b]	WT-HC/ WT-LC	C16-LC/ WT-LC	Gene[c]	Putative Function[c]	Organism[c]	GenBank Hit[d]	Motif(pfam)[e]
033b11	BP094164	9.9 (±5.9)	4.0 (±2.3)	*Gsa*	Glutamate-1-semialdehyde aminomutase	*C. reinhardtii*	U03632	–
156h09 (2)	BP086423	6.2 (±2.4)	4.3 (±1.7)	*HemC*	Porphobilinogen deaminase	*Pisum sativum*	Q43082	–
019c02 (2)	AV619723	5.4 (±2.8)	7.2 (±3.7)	*Lil3*	Light-harvesting-like protein 3	Arabidopsis	T52310	–
007h05 (2)	AV390641	5.1 (±2.7)	3.4 (±1.0)	*Cpx1*	Coproporphyrinogen III oxidase	*C. reinhardtii*	AF133672	–
028b04	AV629643	3.8 (±1.5)	3.4 (±0.5)	*Crt*	Calreticulin, Calcium-binding protein	*C. reinhardtii*	AJ000765	–
169b10	BP096864	3.8 (±1.0)	2.7 (±0.5)	–	–	–	–	No hit
165d02	AV644167	3.7 (±1.4)	4.1 (±1.7)	–	–	–	–	No hit
122h07 (2)	AV391593	3.6 (±1.7)	2.8 (±0.8)	*Psb3*	Oxgen evolving enhancer protein 3	*C. reinhardtii*	X13832	–
031b05	BP086554	3.5 (±2.2)	5.7 (±2.3)	*H43*	High-CO_2 inducible periplasmic protein	*C. reinhardtii*	AB042098	–
033e05	BP094463	3.4 (±1.2)	4.3 (±2.6)	*Ipmdh*	3-Isopropylmalate dehydratase, small subunit	Arabidopsis	NP_181837	–
126f08 (2)	BP098729	3.2 (±0.8)	3.2 (±1.2)	*Lhcbm9*	Light-harvesting chlorophyll a/b-binding protein	*C. reinhardtii*	AF479778	–
002g07 (5)	AV397762	3.0 (±1.2)	3.0 (±1.3)	*LhcII-3*	Light-harvesting chlorophyll a/b-binding protein	*C. reinhardtii*	AB051209	–
107c05	AV386724	3.0 (±0.1)	2.9 (±0.7)	*Ubc1*	Ubiquitin-conjugating enzyme UBC1	Arabidopsis	NP_563951	–
157h02	BP087178	2.9 (±0.3)	2.7 (±0.7)	–	–	–	–	No hit
011f10	AV635306	2.8 (±0.7)	2.7 (±0.3)	*Pgk*	Phosphoglycerate kinase	*C. reinhardtii*	U14912	–
160e07	BP088719	5.0 (±0.9)	1.5 (±0.1)	*Crd1*	Copper response defect 1 protein	*C. reinhardtii*	AF237671	–
022c06 (2)	AV623576	4.4 (±2.0)	2.3 (±1.2)	*Ggh*	Geranylgeranyl hydrogenase	*Mesembryanthemum crystallinum*	AAD28640	–
029e10	AV630927	4.2 (±1.4)	2.3 (±1.4)	*Por1*	NADPH:protochlorophyllide oxidoreductase	*C. reinhardtii*	Q39617	–
170d07	BP097411	3.9 (±0.7)	2.1 (±1.0)	*ChlH*	Magnesium chelatase H-subunit	*C. reinhardtii*	AJ307054	–
016e07 (2)	AV642279	3.8 (±1.8)	1.6 (±0.5)	*Sar*	SAR DNA binding protein	*P. sativum*	T06377	–
152c03	AV642602	3.6 (±1.8)	2.1 (±0.7)	*Hsp70*	70 kD heat shock protein	*C. reinhardtii*	P25840	–
149a01	AV628277	3.5 (±1.0)	2.3 (±0.3)	*Hsp81-2*	Heat shock cognate protein 80	*Lycopersicon esculentum*	P36181	–
008g12	AV391709	3.5 (±1.3)	2.0 (±0.5)	*Lhcb4*	Light-harvesting chlorophyll a/b-binding protein	*C. reinhardtii*	AB051211	–
159g09	BP088102	3.4 (±0.5)	2.4 (±1.0)	*Uo2*	Urate oxidase II	*C. reinhardtii*	AF195795	–
032a05	AV636296	3.3 (±1.4)	2.5 (±2.0)[f]	*Lhcb2*	Light-harvesting chlorophyll a/b-binding protein	*C. reinhardtii*	AF104630	–
031c08	BP086706	3.3 (±0.7)	1.8 (±1.0)	–	–	–	–	No hit
010c10	AV632566	3.1 (±0.7)	1.8 (±0.2)	*Rpl3*	50S ribosomal protein L3	Arabidopsis	NP_181831	–
035a04 (2)	BP096510	3.1 (±1.0)	1.8 (±0.3)	*Cpn60 α*	Chaperonin 60 alpha chain	*C. reinhardtii*	L27472	–
110a08	AV388533	3.0 (±0.6)	2.0 (±0.1)	–	–	–	–	No hit
007f10	AV638595	2.8 (±1.1)	1.9 (±0.7)	*Psb2*	Oxygen-evolving enhancer protein 2	*C. reinhardtii*	M15187	–
159g03	BP088024	2.7 (±0.1)	2.1 (±0.7)	*Alad*	Delta-aminolevulinic acid dehydratase	*C. reinhardtii*	U19876	–
002b05	AV395046	2.6 (±0.3)	0.4 (±0.1)	*MetC*	Cobalamin-independent methionine synthase	*C. reinhardtii*	U36197	–
019f08	AV620136	0.8 (±0.2)	22 (±7.7)	–	–	–	–	No hit
136a01 (2)	AV620380	1.0 (±0.1)	5.5 (±1.6)	*H3-I*	Histone H3	*C. reinhardtii*	S59581	–
167g10	AV619315	1.8 (±0.2)	5.4 (±2.4)	*Gbp1*	G-strand telomere binding protein 1	*C. reinhardtii*	S46234	–
036b09 (2)	BP097895	1.7 (±0.5)	5.2 (±1.7)	–	–	–	–	No hit
139d05	AV623043	0.4 (±0.2)	4.4 (±2.5)	–	–	–	–	No hit
029h01	AV631148	1.2 (±0.2)	4.2 (±0.8)	*Ftr1*	Plasma membrane iron transporter	*C. reinhardtii*	AAM45938	–
115d09	AV392433	1.5 (±0.5)	3.9 (±1.4)	*Bip*	Luminal binding protein, BiP	*Scherffelia dubia*	CAC37635	–
019b08 (4)	AV619634	1.4 (±0.4)	3.9 (±1.1)	*Heph*	Hephaestin; multicopper ferroxidase	*Mus musculus*	NP_034547	–
034h03	BP096342	1.2 (±0.5)	3.9 (±1.0)	–	–	–	–	No hit
030f10	BP086097	1.8 (±0.7)	3.8 (±1.1)	–	–	–	–	No hit
114b09	AV391439	1.7 (±0.8)	3.8 (±0.9)	*DnaK*	DnaK-type molecular chaperone	*O. sativa*	T03581	–
143f07	AV625849	0.8 (±0.1)	3.8 (±0.7)	–	–	–	–	No hit

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Array ID[a]	Clone ID[b]	WT-HC/ WT-LC	C16-LC/ WT-LC	Gene[c]	Putative Function[c]	Organism[c]	GenBank Hit[d]	Motif(pfam)[e]
004f08 (2)	AV387133	1.7 (±0.4)	3.7 (±0.9)	–	–	–	–	No hit
033g07 (5)	BP094894	1.2 (±0.1)	3.7 (±1.4)	*Hsp22*	22 kD Heat shock protein	*C. reinhardtii*	P12811	–
033b07	BP094124	2.2 (±1.2)	3.6 (±1.5)	–	–	–	–	No hit
159h04	BP088169	2.1 (±0.3)	3.2 (±0.4)	–	–	–	–	No hit
101a04	AV393298	1.0 (±0.2)	3.1 (±0.8)	–	–	–	–	No hit
106g04	AV386548	1.0 (±0.2)	3.1 (±0.8)	–	–	–	–	No hit
017a08 (2)	AV642841	1.3 (±0.1)	3.0 (±0.5)	–	–	–	–	No hit
140d07	AV623827	1.8 (±1.0)	2.9 (±0.5)	–	–	–	–	No hit
028f11	AV630205	1.6 (±0.3)	2.9 (±0.3)	*Mbf1*	Multiprotein bridging factor 1	*Solanum tuberosum*	AAF81108	–
158h12	BP087717	1.4 (±0.1)	2.8 (±0.3)	*Thi1*	Thiazole (thiamine) biosynthetic enzyme	*Citrus sinensis*	O23787	–
120g09	AV636430	2.3 (±0.8)	2.5 (±0.2)	*PetO*	Cytochrome b_6/f-associated phosphoprotein	*C. reinhardtii*	AF222893	–

Genes whose expression ratios were in excess of 2.5-fold are underlined. Dashes indicate not applicable. [a]Representative array IDs are shown in the first column. Numbers in parentheses indicate numbers of array IDs grouped into the same contigs, after sequencing the 3′ end of the cDNA. [b]GenBank accession numbers for the EST clones. [c]Genes with e-values $<e^{-10}$ were annotated. [d]Best or most informative GenBank sequence alignments. [e]Motifs were annotated by the conserved domain database using RPS-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). [f]Two expression ratios out of 4 did not exceed 2.5-fold.

Induction of Most Genes under Low-CO_2 Conditions Was Impaired by the *ccm1* Mutation

Furthermore, to evaluate the role of *Ccm1*, which encodes a protein containing a zinc-finger motif, in the regulatory network for acclimation to low-CO_2 stress conditions, the DNA array was used to obtain expression profiles of a *ccm1* mutant, C16, which does not induce the CCM and its related genes during acclimation to low-CO_2 stress (Fukuzawa et al., 2001). After culturing the C16 cells in high-CO_2 conditions, cells were transferred into low-CO_2 conditions for 1 h. This change in CO_2 levels does not lead to increased photosynthetic affinity for inorganic carbon since the *ccm1* mutant C16 does not induce the CCM (Fukuzawa et al., 2001). The poly(A)$^+$-RNA was isolated from the C16 cells and used for hybridization with the membrane arrays to monitor global gene expression after 1 h of low-CO_2 stress conditions. Genes, whose expression failed to be induced in the *ccm1* mutant C16 under low-CO_2 conditions, were selected and grouped by the same strategy as for selecting low-CO_2 inducible genes described above. Of 51 low-CO_2 inducible genes, the expression ratios of 47 genes were significantly reduced in the *ccm1* mutant. Since expression levels of the other 4 genes, 171b12 (2.1-fold), 021d12 (2.4-fold), 010b02 (2.3-fold), and 160c11 (2.0-fold), decreased relatively by the *ccm1* mutation (Table I), it was shown that CCM1 controls almost all the low-CO_2 inducible genes in wild-type cells. In addition, eleven genes, which were slightly induced under low-CO_2 conditions in the wild-type cells, were not induced by the *ccm1* mutation (Table I). Of these genes, *Cah3*, coding for a chloroplast CA, which is essential to the CCM, was slightly upregulated in low-CO_2 conditions (Karlsson et al., 1998) and is controlled by CCM1 as shown previously (Fukuzawa et al., 2001). Some of the EST clones showed significant sequence similarities to known genes; e.g., 023c07 product was similar to a putative heparan sulfate proteoglycan of *Ovis aies*, 019h09 to a putative Tyr phophatase gene from *Oryza sativa*, 022a05 to the voltage-dependent anion channel gene from *O. sativa*, 138h02 to a putative crystalline J1C of *Tripedalia cystophora*. *Chlamydomonas Mdh1*, coding for NADP-malate dehydrogenase, which plays a role in the malate valve, was not induced in the *ccm1* mutant.

Suppression of Expression of Several Genes under Low-CO_2 Conditions Was Impaired by the *ccm1* Mutation

Suppression of 15 genes including *Gsa*, *HemC*, *Lil3*, *Cpx1*, *Crt* coding for Calreticulin, *Psb3*, *H43*, *lpmdh*, *Lhchm9*, *LhcII-3*, *Ubc1* for ubiquitin-conjugating enzyme, and *Pgk*, was impaired by the *ccm1* mutation under low-CO_2 conditions. In addition, 23 genes were expressed irrespective to CO_2 levels in the wild type, but expression levels of these genes were higher in the *ccm1* mutant compared with those in wild type. *H3-I* coding for the histone H3 and *Gbp1* coding for a telomere-binding protein, possibly involved in changes of the chromosomal organization, and *PetO* coding for a cytochrome b6/f-associated phosphoprotein were not repressed in the *ccm1* mutant C16. Expression levels of 4 other genes, *Chlamydomonas Ftr1* coding for a plasma membrane iron transporter, 019b08 similar to *Mus Heph* for multicopper ferroxidase, 028f11 similar to *Solanum Mbf1* for a multiprotein-bridging factor, and 158h12 similar to *Citrrus Thi1* for thiamin biosynthetic enzyme were also higher in the *ccm1* mutant under low-CO_2 conditions. Whether these 23 genes are actually regulated by CCM1 or by a secondary effect is not clear; some of these genes may play roles in the regulation of CO_2-responsive genes. Interestingly, expression of housekeeping genes such as *H3-1*, *Gbp1*, and 114b09 for a *DnaK*-homolog was repressed under

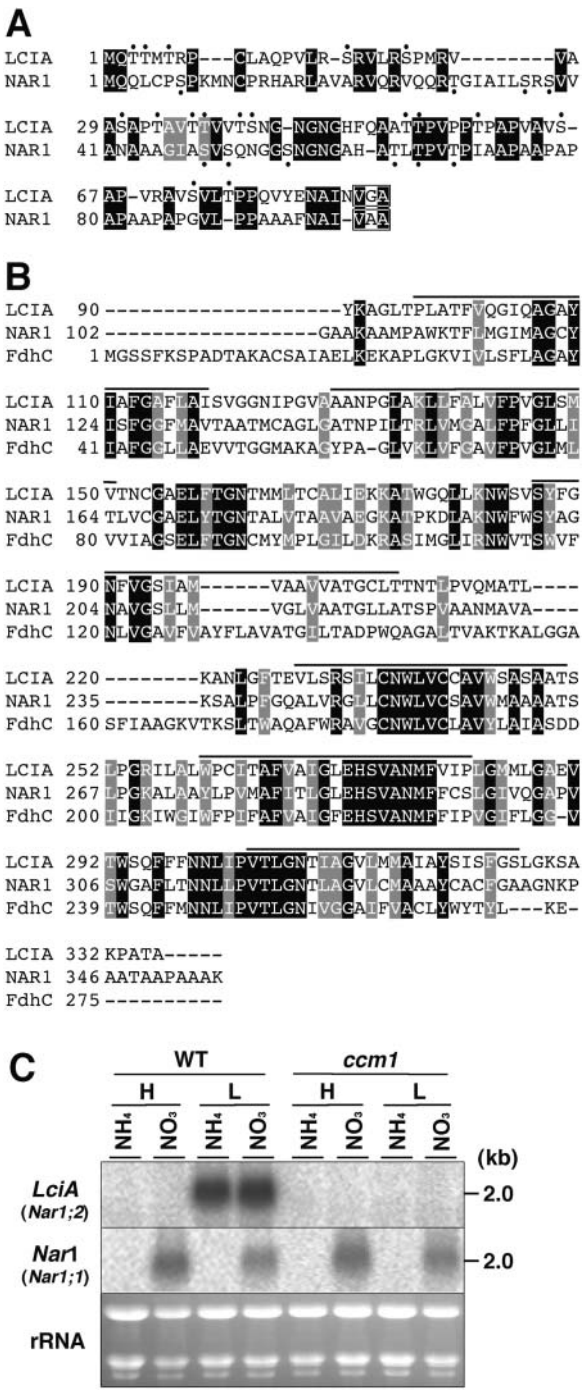

Figure 2. Amino acid sequence comparison of LCIA (AB168092) with anion-transporters and mRNA expression of the *LciA* gene. A, Amino acid sequence alignment of the putative chloroplast transit peptide of LCIA with that of NAR1 (AF149738). Artificially introduced gaps to allow optimal alignment are indicated by hyphens. Hydroxylated amino acid residues are depicted by dots. The putative signal cleavage sequences (V/I-X-A) are boxed. B, Sequence alignment of LCIA with the mature NAR1 protein and the formate transporter, FdhC (Q50568), from *Methanothermobacter thermautotrophicus*. Bars indicate transmembrane regions. Identical and similar amino acids in the three proteins are indicated by black and gray boxes, respectively. C, Northern-blot analyses of *LciA* and *Nar1* in response to different carbon and nitrogen sources. Cells were cultured in media containing ammonium salts (NH_4) or nitrate (NO_3) as a nitrogen source. Total RNA samples isolated from wild-type and the *ccm1* mutant C16 cells grown under high-CO_2 (H) or acclimated to low-CO_2 for 2 h (L) were hybridized with ^{32}P-labeled gene specific probes for *LciA* and *Nar1*. To show that identical amounts of RNA were loaded, an image of the ethidium bromide-stained gel is shown.

CO_2-limiting conditions, possibly by the secondary effect of the growth arrest of the *ccm1* mutant in the low-CO_2 environment.

Putative Transporters Encoded by Low-CO_2 Induced Genes

Although CO_2 uptake systems have not been elucidated in eukaryotes including *C. reinhardtii*, several candidate genes responsible for Ci uptake and Ci-transport systems were included in low-CO_2 inducible genes. One of them is *Ccp*, encoding LIP-36, which has sequence similarity with the mitochondrial-carrier-protein superfamily. Since this protein has six transmembrane domains and is located in the chloroplast envelope (Chen et al., 1997), it is assumed that *Ccp* is involved in Ci-transport systems. Other genes related to transporters are *Mcp*, which is similar to the mitochondrial carrier protein, and 148a10, which is similar to the chromate resistance protein. Although 022a05 was not induced under low-CO_2 conditions, this gene shows similarity with voltage-dependent anion channels, termed porins, of several plant species (Elekeles et al., 1995).

Of other low-CO_2 inducible genes, expression ratios in low- to high-CO_2 conditions of the two unknown genes, 018h08 and 012g07, designated as *LciA* and *LciB*, respectively, were typically high among the low-CO_2 inducible genes. To predict the possible functions of these gene products, amino acid sequences of *LciA* and *LciB* were deduced from the cDNA sequences (Figs. 2 and 3). By comparison with the protein sequence databases, *LciA* is predicted to encode a putative polypeptide of 336 amino acid residues with significant similarities with 2 anion transporters, the chloroplast nitrite transporter NAR1 in Chlamydomonas (CrNAR1;1) (Rexach et al., 2000) and the formate transporter, FdhC, from *Methanothermobacter thermautotrophicus* (Nölling and Reeve, 1997; Fig. 2B).

To examine the regulation of *LciA* with respect to the previously identified *Nar1*, cells cultured in high CO_2 conditions were transferred into low-CO_2 conditions in medium containing ammonia or nitrate as a sole nitrogen source (Fig. 2C). The mRNA transcript of *Nar1* was detected only when cells were grown in nitrate-containing culture medium but not when grown in ammonium-containing medium, as reported previously (Rexach et al., 2000). The *Nar1* expression was largely unaffected by the change in CO_2 levels and by the *ccm1* mutation (Fig. 2C). In contrast, the expression of *LciA* corresponding to *Nar1;2* (Galván et al., 2002) was induced in low-CO_2 conditions irrespective of the nitrogen source, and the *ccm1* muta-

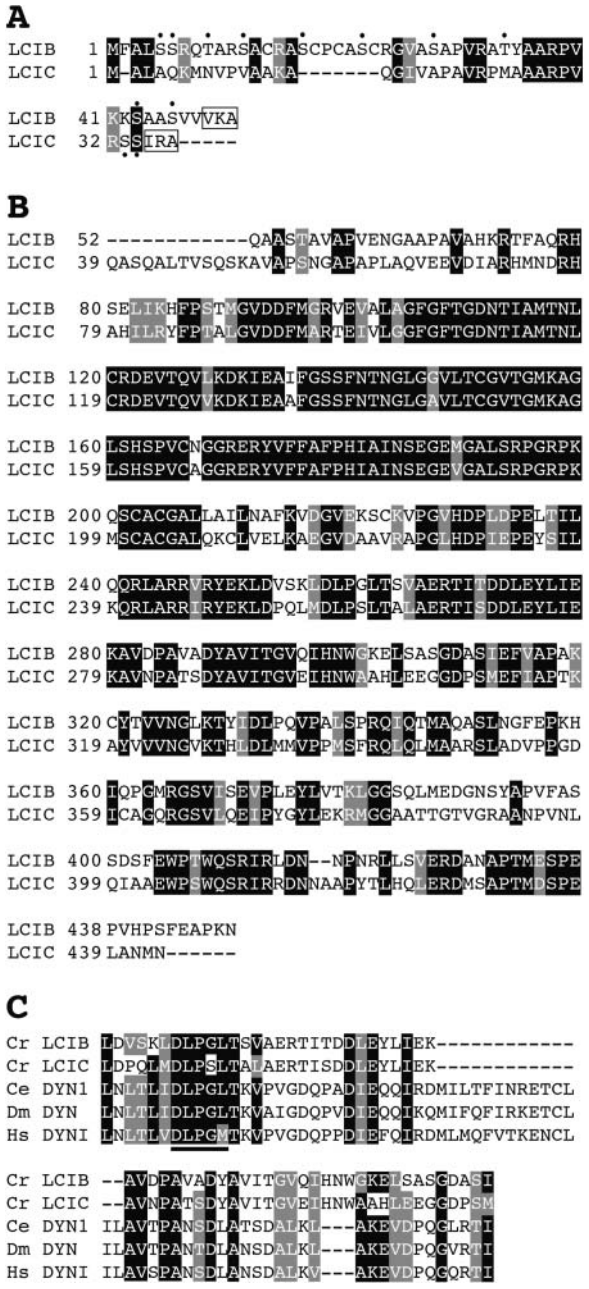

Figure 3. Amino acid sequence alignment of the Chlamydomonas LCIB (AB168093) and LCIC (AB168094). A, Putative chloroplast transit peptides of LCIB and LCIC. Hydroxylated amino acid residues are depicted by dots. The putative signal peptide cleavage sequences (V/I-X-A) are boxed. B, Sequence alignment of the mature LCIB and LCIC proteins. C, Sequence comparison of a portion of the dynamin domains (pfam00350): *Chlamydomonas reinhardtii* LCIB and LCIC, *Caenorhabditis elegans* DYN1 (P39055), *Drosophila melanogaster* DYN (P27619), and *Homo sapiens* DYNI (Q0519). The GTP-binding domain is underlined. Identical and conserved amino acids are indicated by black and gray boxes, respectively.

tion completely abolished *LciA* expression (Fig. 2C). The computer programs PSORT (http://psort.nibb.ac.jp/form.html) and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) predicted that LCIA would localize in the thylakoid or chloroplast envelope membranes, since LCIA has a putative chloroplast transit peptide containing hydroxylated amino acid residues, which tend to be high in many chloroplast transit peptides (Heijne et al., 1989; Fig. 2A). The mature polypeptide of LCIA has 6 transmembrane domains. These results strongly suggest that LCIA functions under low-CO_2 stress conditions but not for nitrite transport. On the other hand, the LCIB protein, consisting of 448 amino acid residues, did not show any sequence similarities over the entire sequence except with 028e01, designated as LCIC, which is one of the low-CO_2 induced genes (Table I; Fig. 3). The amino acid sequence of LCIC was 59.9% identical to that of LCIB. By using the RPS-BLAST program, a portion of LCIB (positions 252–312) was shown to have significant sequence similarity with the dynamin families (pfam00350), e.g. DYN1 derived from *Caenorhabditis elegans*, DYN from *Drosophila melanogaster*, and DYNI from *Homo sapiens* (Fig. 3C). Although dynamin proteins have 3 GTPase domains, LCIB has only 1 putative GTP-binding domain. The signature sequence of the GTPase domain, DLPGL, was conserved in the LICB protein, but it was replaced by the sequence DLPSL in the LCIC protein (Fig. 3C). LCIB and LCIC were predicted to localize in the chloroplast stroma, and any transmembrane domains were not detected by the SOSUI program. These transit peptides also have several hydroxylated amino acid residues (Fig. 3A) as found in LCIA.

A high-CO_2 requiring mutant, *pmp1-1*, defective in the Ci transport and accumulation, has been isolated (Spalding et al., 1983). The lesion in this strain may be associated with a gene involved in Ci uptake. To determine whether these two genes are involved in *Pmp1* function, we examined *LciA* and *LciB* mRNA levels in the *pmp1* mutant background. The mRNA accumulation of *LciA* and *LciB* was not detected in a high-CO_2 requiring mutant *pmp1* as well as in the *ccm1* mutant (Fig. 4). In addition, one of the putative ATP-binding cassette-type transporters, *Mrp1* (=*Hla3*), shows low-CO_2 inducibility and *Ccm1* dependency. Although significant induction of the *Mrp1* was not detected in the array analysis due to the low level of signal intensity using our criteria (normalized value of $S_A > 25$; see ''Materials and Methods''), the mRNA levels of the *Mrp1* reached significant levels in wild-type cells 2 h after transfer to low-CO_2 conditions. This induction of the *Mrp1* was impaired by both the *ccm1* and *pmp1* mutations.

DISCUSSION

In this study, we have identified 83 CO_2-responsive genes including 51 low-CO_2 inducible genes and

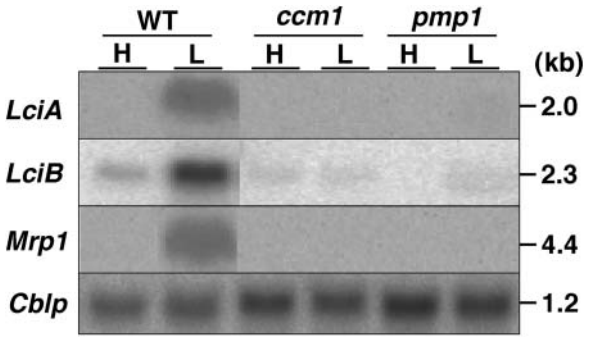

Figure 4. Northern-blot analyses of low-CO_2-inducible genes, *LciA*, *LciB*, and *Mrp1*. For detection of *LciA* and *LciB* mRNA, 30 μgs of total RNA isolated from wild-type (WT), *ccm1*, or *pmp1-1* cells grown under high-CO_2 (H) or 1 h of low-CO_2 (L) were hybridized with gene specific ^{32}P-labeled probes. For detection of *Mrp1* mRNA, 50 μgs of total RNA from cells grown in high-CO_2 (H) or 2 h of low-CO_2 (L) conditions. A ^{32}P-labeled cDNA probe for *Cblp* encoding the G-protein β-subunit, which is expressed constitutively, was used as a loading control.

32 genes repressed under low-CO_2 conditions by global expression analyses using a cDNA macroarray in the eukaryotic photosynthetic alga, *C. reinhardtii*. Previously known CO_2-responsive genes in Chlamydomonas—CA genes (*Mca* and *Cah1*), a chloroplast membrane protein gene (*Ccp1*), unknown low-CO_2 inducible genes (*Lci1* and *Lci5*), and photorespiratory genes (*Aat1* and *Pgp1*)—were identified as CO_2-responsive using the macroarray with the exception of a few such as *Mrp1*, encoding a putative ATP-binding cassette-type transporter (Fig. 4). Since we used stringent criteria to identify low-CO_2 responsive genes and used cells only adapted for 1 h to low-CO_2 conditions, several CO_2-responsive genes have not been identified by this macroarray analysis. Indeed, *Mrp1* was significantly induced after 2 h exposure to low-CO_2 stress conditions (Fig. 4).

In addition, CCM1-regulated genes were selected by comparing the transcript profiles of the wild-type cells and the *ccm1* mutant C16 cultured under CO_2-limiting conditions. Since the *ccm1* mutant C16 exhibits a high-CO_2 requiring phenotype (Fukuzawa et al., 1998), some of the genes whose expression was impaired in the C16 mutant should also be essential to the operation of the CCM, in addition to the *Ccm1* gene itself. Since almost all genes induced by low-CO_2 conditions (47 out of 51) failed to be induced in the *ccm1* mutant C16 and the other 4 genes were affected 2.0-fold to 2.4-fold in C16 (Table I), CCM1 is probably a master regulatory factor of low-CO_2 inducible genes (Fig. 5). Furthermore, expression profiles of approximately one-half of the genes repressed by low-CO_2 conditions (15 out of 32) were also highly altered in the *ccm1* mutant C16. Therefore, it is likely that CCM1 is at the higher position in both the high-CO_2- and the low-CO_2-signal transduction cascades but preferentially functions as a positive regulator. Considering that CCM1 regulates the expression of genes for CA isozymes, chlorophyll-related proteins, putative Ci uptake, photorespiratory activity, and development of the pyrenoid structure, CCM1 must play a substantial role not only in the regulation of the CCM but also in the modulation of photosynthesis. Some of the low-CO_2 inducible genes may be induced by secondary effects of the induction of regulatory genes in low-CO_2. By the array analyses, 34 genes were shown to be impaired in the *ccm1* mutant, which were not so affected by changes in CO_2 levels (11 genes in Table I and 23 in Table II). Some of 34 CCM1-dependent genes may not be regulated by CCM1, because differences in the expression ratios might be caused by differences in genetic background between the wild-type strain C9 and the *ccm1* mutant C16.

The photorespiratory carbon oxidation cycle of *C. reinhardtii* is different from that of higher plants, because these microalgae have no peroxisomes (Spalding, 1989). The photorespiratory cycle of *C. reinhardtii* is thought to exist in the mitochondria. The CCM elevates the internal levels of dissolved inorganic carbon and facilitates the carboxylation reaction of Rubisco over the competitive oxygenation reaction (Kaplan and Reinhold, 1999). Therefore, it has been suggested that photorespiratory activity is greatly suppressed by the CCM in air-adapted cells even under CO_2-limiting conditions. However, our macroarray analysis indicated that seven genes encoding enzymes, which function in the photorespiratory cycle, were induced by lowering CO_2 concentration. In fact, activities of photorespiratory enzymes such as phosphoglycolate phosphatase and glycolate dehydrogenase were increased in air-adapted cells (Marek and Spalding, 1991), and a *pgp1-1* mutant, impaired in the phosphoglycolate phosphatase activity, showed a high-CO_2 requiring phenotype (Suzuki et al., 1990).

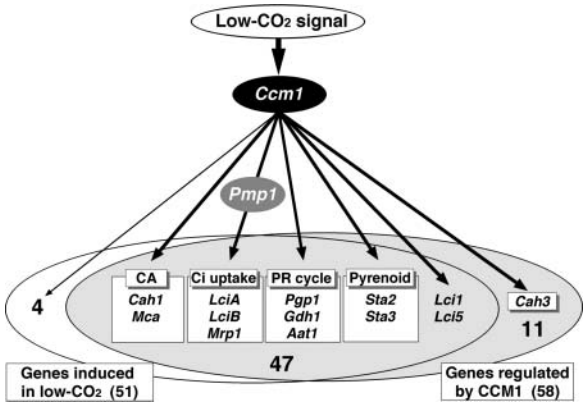

Figure 5. Schematic illustration of a model for the relationship among *Ccm1*, *Pmp1*, 51 low-CO_2 inducible genes, and 58 *Ccm1*-regulated genes. Numbers of the genes are indicated in each group. Of these genes, *Cah1* and *Mca* encoding carbonic anhydrases (CA); *LciA*, *LciB*, and *Mrp1* possibly involved in Ci uptake; *Pgp1*, *Gdh1*, and *Aat1* for the photorespiratory cycle (PR cycle); *Sta2* and *Sta3* possibly involved in the pyrenoid development, are highlighted. Previously known low-CO_2 inducible genes (*Lci1* and *Lci5*) and chloroplast CA gene (*Cah3*) are also indicated.

Considering the fact that mRNA levels of other photorespiratory genes such as *Sgat* and *GcsP* were induced under low-CO_2 conditions, it is possible that the photorespiratory cycle is transcriptionally activated during or before induction of the CCM in acclimation steps to CO_2-limiting conditions via regulation by CCM1. In addition, one of the photorespiratory genes, *Shmt*, is induced also under high light conditions (Im and Grossman, 2002). Considering that other photorespiratory genes are not induced under high light conditions (Im et al., 2003), it is possible that *Shmt* has additional functions besides photorespiration.

In a cyanobacterium *Synechocystis* sp. PCC6803, two types of functionally distinct NDH-1 complexes have been identified with the aid of mutants impaired in one or more subunits of NDH-1 (Ohkawa et al., 2000a). One is for photosystem-I cyclic electron flow, another is for CO_2, not bicarbonate, uptake (Ohkawa et al., 2000b; Shibata et al., 2001, 2002a). Although the *Ndh* gene encoding a type-2 NADH dehydrogenase was detected as a low-CO_2 inducible gene in our array analysis, it is not known whether this type of NADH dehydrogenase functions in Ci-uptake systems. Taken with the fact that at least four systems for Ci acquisition are present in cyanobacteria (Shibata et al., 2002a), it is likely that there could be several Ci-uptake systems in the eukaryotic alga Chlamydomonas. Although the necessity of the Ci-uptake system in the CCM is clear and the system is assigned to localize in chloroplast envelope (Kaplan and Reinhold, 1999; Badger and Spalding, 2000), no molecular components for Ci-uptake systems in photosynthetic eukaryotes have been identified yet. Taken with the fact that the *pmp1-1* and the *ccm1* mutants show high-CO_2 requiring phenotypes (Spalding et al., 1983; Fukuzawa et al., 1998), expression levels of genes involved in the Ci-uptake system should be decreased in these mutants. By the global expression analyses using DNA arrays and northern-blot analyses, two new candidates, *LciA* and *LciB*, for the inorganic carbon transporters were isolated in this study. *LciA* shows sequence similarity with *Nar1*, and five *Nar1*-related genes, *Nar1;1* to *Nar1;5*, were identified in *C. reinhardtii* (Galván et al., 2002). Of these genes, *Nar1;1* corresponds to *Nar1* encoding a nitrite transporter, and *Nar1;2* is identical to *LciA*. The fact that a bicarbonate transporter shows homology to a nitrate transporter in cyanobacteria (Omata et al., 1999) supports our assumption that LCIA functions as an anion transporter to take up bicarbonate into chloroplasts. Our array data showed that the other three *Nar1*-related genes were not induced in low-CO_2 conditions (*Nar1;3* [array ID, 028e05; expression ratio of signal intensity in wild type cells (WT) under low-CO_2 (LC) to that in WT under high-CO_2 (HC), 1.1 ± 0.3], *Nar1;4* [149a12, 0.8 ± 0.2], and *Nar1;5* [127g03, 0.8 ± 0.0]), suggesting that low-CO_2 inducible *LciA* (=*Nar1;2*) may encode a bicarbonate-transporter. This prediction is supported by a finding that LCIA has a putative chloroplast-targeting sequence in its N-terminal region, since Ci is accumulated in the intact chloroplasts isolated from Chlamydomonas grown in low-CO_2 conditions but not in high-CO_2 conditions (Moroney et al., 1987). *LciB* failed to be induced in the *ccm1* and *pmp1-1* mutants. This gene product does not have any sequence similarities with known proteins except for LCIC (Fig. 3). LCIB is probably localized in chloroplast stroma because of a lack of transmembrane domains. Previous work indicates two soluble proteins with molecular masses of 46 and 44 kD are not induced in the *pmp1-1* mutant (Manuel and Moroney, 1988). Since the molecular mass of mature LCIB is predicted to be 44 kD, this 44-kD protein missing in the *pmp1-1* mutant could be the LICB protein. Motif searches show that LCIB has a GTP-binding domain. We also identified an *LciB*-related gene, *LciC*, which failed to be induced in the *ccm1* mutant C16. Since the signature sequence of GTPase domains (DLPGL) was not conserved in LCIC (Fig. 3C), this protein may not have GTPase activity. Determination of subcellular localization and further biochemical analyses would reveal the functions of LCIA, LCIB, and LCIC.

The *Chlamydomonas pmp1-1* mutant was isolated as lacking an active Ci-uptake system (Spalding et al., 1983). This mutation in *Pmp1* caused a severe requirement of high levels of CO_2 for its photosynthetic growth in various pH ranges (Van and Spalding, 1999). In cyanobacteria, four Ci-uptake systems exist and a deletion mutant of one system does not show severe a high-CO_2 requiring phenotype (Shibata et al., 2002a). Considering the above two facts, it is more likely that *Pmp1* is involved in regulation of several Ci-uptake systems. Our analyses revealed that *Pmp1* regulates *LciA*, *LciB*, and *Mrp1*, but not *Cah1*, transcriptionally (Fig. 4; data not shown). Since the *ccm1* mutant is impaired in Ci accumulation (Fukuzawa et al., 2001), it is possible that some genes for Ci-uptake systems other than *LciA*, *LciB*, and *Mrp1* could also be regulated by *Pmp1* (Fig. 5).

Large-scale analyses in this study extend our knowledge of acclimation to CO_2-limiting conditions and CO_2-signal transduction through CCM1. Since photosynthetic electron flow is required for the CCM (Badger and Spalding, 2000), expression of some genes would be regulated by not only CO_2 availability but also light intensities as in the case of *Shmt* (Im and Grossman, 2002; Im et al., 2003). For the control of these genes, it is likely that the redox state of the plastoquinone pool is involved. Accumulation of further genome-wide transcriptome information on various acclimation processes and conditions should enable us to identify the genes involved in many adaptation processes to different environmental stress conditions, which would lead to a better understanding of the complicated network of regulatory interactions (Kucho et al., 2003; Yoshioka et al., 2004). Identification of the functions of CO_2-responsive genes and/or CCM1-regulated genes would enhance our understanding of the molecular mechanisms of acclimation to CO_2-limiting conditions.

MATERIALS AND METHODS

Cells and Growth Conditions

Chlamydomonas reinhardtii Dangeard wild-type cells C9 (mt^-) strain and the high-CO_2 requiring mutant, C16 (*ccm1*), have been described previously (Fukuzawa et al., 1998, 2001) and another high-CO_2 requiring mutant, *pmp1-1*, was kindly provided by Dr. J.V. Moroney. Cells were cultured in buffered high salt (HS) medium supplemented with 20 mM MOPS (pH 7.2) under aeration with ordinary air containing 0.04% CO_2 (low-CO_2) or air enriched with 5% CO_2 (high-CO_2) as described previously (Kucho et al., 1999).

Preparation of DNA Macroarrays

To construct the Chlamydomonas cDNA membrane array, three groups of cDNA libraries derived from cells in photoautotrophic growth (Asamizu et al., 1999), from high-CO_2 and low-CO_2 grown cells (Asamizu et al., 2000), and from cells grown under 23 different kinds of stress conditions to generate 12,842 5′-ESTs (E. Asamizu, Y. Nakamura, K. Miura, H. Fukuzawa, S. Fujiwara, M. Hirono, K. Iwamoto, Y. Matsuda, J. Minagawa, K. Shimogawara, Y. Takahashi, and S. Tabata, unpublished data). A total of 50,832 EST sequences were clustered by BLAST followed by PHRAP programs as described previously (Asamizu et al., 1999). *Esherichia coli* cells containing EST clones were cultured and used for PCR in the mixture containing 1 × buffer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 5% (v/v) dimethyl sulfoxide, 1.75 units Ex Taq DNA polymerase (Takara, Kyoto) and the primers T7-25-kyoto (5′-CGCGTAATACGACTCACTATAG-GGC-3′) and T3-25-kyoto (5′-AGCGCGCAATTAACCCTCACTAAAG-3′). After confirmation of PCR amplification, one-sixth volume of dye solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25% (w/v) bromo-phenol blue, and 60% (v/v) glycerol was added to the PCR products. These PCR products were spotted on Biodyne-A nylon membranes (0.2 μm, Pall Biosupport, Port Washington, NY) using a spotting device, Biomek2000 (Beckman Coulter, Roissy, France). One membrane can contain 1,728 cDNAs spotted in duplicate. To spot 10,368 cDNAs, we used 6 membranes per set. The spotted membranes were treated twice with UV light using a UV Stratalinker 2400 (Stratagene, La Jolla, CA), and baked at 80°C for 2 h.

Preparation of Probes and Hybridization

The ^{32}P-labeled target DNA samples were prepared from poly(A)$^+$ RNAs by incorporation of [α-^{32}P]dCTP during first-strand cDNA synthesis. Each reaction consisted of 1 μg of poly(A)$^+$ RNAs, 2 μg random primers pd $(N)_9$, 1 mM each of dATP, dGTP, and dTTP, 15 μM dCTP, 0.1mCi [α-^{32}P]dCTP (6000 Ci/mmol, NEN Life Science Products, Boston), 60 units RNase inhibitor (RNaseOUT, Invitrogen, Carlsbad, CA), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.6 M trehalose, and 400 units Superscript II reverse transcriptase (Invitrogen). The reverse transcription reaction was performed at 37°C for 15 min and at 50°C for 5 min followed by incubation for 90 min at 60°C. The labeled cDNA products were used for hybridization with ExpressHyb Hybridization Solution (CLONTECH, Palo Alto, CA) at 68°C for 12 to 16 h. The membranes were washed with 0.1 × SSC and 0.5% SDS, and with 0.08 × SSC and 0.5% SDS, and then exposed to imaging plates (Fuji Photo Film, Tokyo).

Data Analysis

Radioactive images were obtained using a scanner, FLA-2000 (Fuji Photo Film), and quantification of the signal intensity was performed using the program ArrayVision (Amersham Pharmacia Biotech, Little Chalfont, UK). Raw values were 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 SD 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 (c). To reduce area-specific effects, mean normalization was adapted. A trimmed mean (μ_{mem}) was calculated for each membrane by using 80% of the 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 2 normalized values. This estimated relative signal intensity is called the expression ratio (S_A/S_B). The expression ratios (WT-LC/WT-HC or WT-LC/C16-LC) of the duplicated spots were averaged. Data were obtained from 2 independent cultures and hybridizations for each experiment. If the correlation coefficient between these two experimental data was more than 0.90, these were used for further analyses. Only ESTs whose averaged expression ratios were more than 2.5 and normalized values of numerators (S_A) were more than 25, corresponding to 0.1% of the total signal, were assigned to be significantly expressed in the cells. It was confirmed that normalized values of numerators (S_A) were at least 2-fold higher than the average background (plus 2 × SD of background). Using 4 expression ratio data per EST clone, the means and their SD were calculated. Since each EST clone has 4 expression ratios, if 3 of 4 expression ratios were more than 2.5-fold, the EST clone was selected as a significantly differentially expressed gene for further analysis. After grouping, the mean and SD of the expression ratios of each gene were calculated as the arithmetic average by using values from the 4 signal intensities of each EST clone.

Northern-Blot Analyses

Northern-blot analyses were performed as described previously (Kucho et al., 1999) using probes generated by PCR in the presence of [α-^{32}P]dCTP with primers T7-25- kyoto and T3-25-kyoto and EST clones as follows: CM035d02 for *Cah1*, LCL016g10 for *Aat1*, LC048g05 for *Pgp1*, CM085d07 for *Cblp*, LC010h12 for *LciB*, and HCL059f06 for *Mrp1*. To detect the *LciA* gene, the EST clone, LC055f02, was amplified with the primers LciA-f (5′-GCTCTAGAGCTAGCATGCAGACCACTATGA-3′) and ds-LciA-r (5′-CTAGATCTATGCATGCGGAAACAGCGACGG-3′). To detect the *Nar1* gene, wild-type genomic DNA was used as a template for amplification with the primers Nar1;1_5UTR-f (5′-GAAACGGGTTGGTTGAAGAGAATTCAACCT-3′) and Nar1;1_5UTR-r (5′-TTTCATCCCTTGTGGCGCGATGATG-3′).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purpose.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank data libraries under accession numbers AB168092 (LCIA), AB168093 (LCIB), and AB168094 (LCIC).

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