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Molecular control of carbon concentrating mechanism in *Chlamydomonas reinhardtii*: CCM1(CIA5), a regulatory factor and CO_2-responsive genes

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Photosynthetic organisms sense environmental changes *e.g.* light, temperature and various nutrient availabilities to modulate and optimize photosynthetic activities. A number of aquatic photosynthetic organisms are able to concentrate dissolved inorganic carbon (DIC) intracellularly, allowing rapid growth despite low-CO_2 availability externally. This carbon concentrating mechanism (CCM) shows acclimation to external DIC to optimize CO_2 fixation efficiency (Badger 1980, Kaplan 1999). During acclimation they induce the expression of a set of genes required for various aspects of the CCM. In *Chlamydomonas reinhardtii*, several genes are regulated in response to changes in external CO_2 concentration, including *Cah1* coding periplasmic carbonic anhydrase (CA) (Fukuzawa 1990, Kucho 1999), *Mca* for mitochondrial CA (Eriksson 1996) and *Ccp1* a chloroplast envelope protein LIP-36 (Chen 1997). This acclimation to CO_2-limiting conditions suggests the existence of sensory mechanisms by which cells perceive the shortage of CO_2 and pathways by which the signal is transduced into the specific gene regulation. However, the regulation of gene expression during this acclimation in eukaryotic organisms is still poorly understood. We have cloned a nuclear regulatory gene, *Ccm1*, which complements the mutation in the pleiotropic high-CO_2 requiring C16 (Fukuzawa 2001). A similar mutant *cia5* (Moroney 1989) was also complemented by the same genomic DNA fragment encoding *Ccm1*. Based on the structure and expression of the *Ccm1* gene we discuss the function and importance of the CCM1 in the CO_2-signal transduction pathways in the eukaryotic photosynthetic

organism, *C. reinhardtii*. And we have isolated several CCM1-regulated genes using cDNA macroarray membranes generated from EST clones (Asamizu et al. 1999, Asamizu et al. 2000).

Materials and Methods

***Chlamydomonas* Cells and transformation.** *C. reinhardtii* strain 5D (*nit1-305, cw15*), which is the wild type (WT) cells for photosynthesis, and high-CO_2 requiring mutants, C16 (Fukuzawa 1998) and *cia5* (Moroney 1989) were cultured in a modified 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). *C. reinhardtii* was transformed with 20 μg plasmid DNA by electroporation (Fukuzawa 2001).

Molecular and biochemical techniques. cDNA was synthesized with oligo-dT nucleotides using Superscript II (Life Technologies) in the presence of 0.6 M trehalose at 60^{o}C. To generate cDNA macroarray membranes, cDNA fragments were amplified by PCR from EST clones and spotted on Biodyne nylon membranes (0.2mm, Pall) using Biomek2000. Poly(A)+-RNA was labeled with ^{32}P-dCTP using reverse Superscript II and used for hybridization.

Physiological Measurements. The rates of photosynthesis were measured in a Clark-type O_2 electrode (Chloroview 1, Hansatech Instruments Ltd., U.K.) and the intracellular DIC concentration was measured by the silicon oil centrifugation method (Fukuzawa 1998).

Results

Isolation of a *Ccm1* gene, which complements the C16 phenotype. It was previously shown that the high-CO_2 requiring mutant, C16, is defective in the ability to induce various aspects of the CCM, including induction of the periplasmic carbonic anhydrase gene, *Cah1* (Fukuzawa 1998). The mutant contains the *Nia1* gene encoding nitrate reductase as a single copy tag in its genome, and the pleiotropic mutation was named as *ccm1*. By using the *Nia1* tag and the corresponding genomic clones were isolated. One of genomic clones, pKI4, harboring 38-kb insert DNA successfully restored the growth and inducibility of the *Cah1* under low-CO_2 conditions. Subsequent subcloning and

transformation into C16 delimited *Ccm1* into 5.1-kb *Xho*I-*Apa*I fragment (Fig. 1). A 5,128-bp cDNA (Accession no. AB052695) corresponding to this genomic region was isolated. This cDNA consists of a 2,097-bp ORF encoding 699-aa hydrophilic protein. This ORF complements the C16 mutation, and named as *Ccm1*. Sequence analysis of the 8,474-bp region (accession number AB052694) in the 12-kb *Xho*I genomic DNA in pKI4Xh, which complements the C16 mutation revealed that the *Ccm1* mRNA is encoded in the 12-kb *Xho*I genomic region (Fig. 1). The *Nia1* was inserted into the coding region of the 4th exon of *Ccm1*. The transgenic strain C16::pKI4XA grown under low-CO_2 conditions showed a high affinity against DIC for photosynthesis with Km(HCO_3^-) value of 122 M, which is comparable to 145 M of the WT. In addition, although the C16 mutant was defective in DIC accumulation and carbon fixation, C16::pKI4XA accumulated DIC and fixed carbon similarly to WT cells. These results demonstrate that a single nuclear mutation at the *ccm1* locus resulted in multiple deficiencies in components of the CCM, which are needed to acclimate to CO_2-limiting conditions. Induction of the CCM under low-CO_2 stress is accompanied by a transcriptional activation of a set of genes *e.g. Cah1*, *Mca*, *Ccp2*, *Lci1* and *Att1* encoding the alanine aminotransferase. Northern blot analyses using gene specific probes indicated that activation of these 5 genes in low-CO_2 conditions was recovered in C16::pKI4XA (Fukuzawa 2001). In addition, the mRNA transcripts of *Cah3* encoding a chloroplastic CA (Karlsson 1998) and *Cyp1* encoding the cyclophylin (Somanchi & Moroney 1999), which are reported to be up-regulated under low-CO_2 conditions in WT cells, were not detected in C16 but detected in the C16::pKI4XA under low-CO_2 conditions. Therefore, it is now clear that the *Ccm1* controls the expression of at least 7 genes and modulates physiological properties and pyrenoid-development in response to CO_2 availability. During course of the isolation of cDNA for CCM1, namely CCM1-A, 3-bp shorter cDNA of 5,125 bp encoding another 698-aa CCM1 protein, named as CCM1-B (accession number AB052696), was also isolated from *Chlamydomonas* cells. Alternative splicing between the 3rd and 4th exons causing a three-nucleotide deletion generated the shorter cDNA. CCM1-B has a substitution of Glu-183 instead of Asp-183 in CCM1-A and a deletion of Arg-184 present in CCM1-A. Influence of this heterogeneity in the CCM1 proteins on their regulatory functions is to be elucidated. The amino acid sequence of CCM1-B was identical to that reported as CIA5 (Xiang *et al.* 2001, accession no. AF317732).

***Ccm1* is constitutively expressed in wild type cells both in high- and low-CO_2 conditions**. In northern blots 5.1-kb *Ccm1* mRNA was detected both in high- and low-CO_2 conditions in WT (Fukuzawa 2001). In vivo labeling and immunoprecipitation with anti-CCM1 antibody revealed that the CCM1 protein was detected as a single band with a molecular mass at 76 kDa in WT cells both in high- and low-CO_2 conditions (Fukuzawa 2001). These results indicate that the CCM1 protein abundance was not affected by change of CO_2 levels, as was the case of the mRNA transcripts.

His-54 of CCM1 is replaced by Tyr in the high-CO_2 requiring mutant *cia5*. When the pKI4XA plasmid was transformed into another high-CO_2 requiring mutant *cia5* (Moroney 1989), one of transformants, *cia5*::pKI4XA, restored the inducibility of CCM-related genes under low-CO_2 conditions as was the case for C16. Determined the nucleotide sequence of the 8,474-bp *cia5* genomic region corresponding to that of *Ccm1* revealed that the *cia5* mutant had a single point mutation from T to C in the 2nd exon of *Ccm1*. As a consequence of this base substitution, His-54 in the putative zinc-finger motif of the CCM1 protein was replaced by Tyr in the *cia5* mutant (Fig. 2). This indicates that the His-54 in the CCM1 plays a critical role in the regulation of CCM.

CCM1 Shares Significant Sequence Similarities to Zinc Finger Domains. Although the deduced amino acid sequence of CCM1 did not show high levels of similarity overall were detected, three characteristic sequence stretches were identified. First, a 27-aa sequence stretch **FxCxxxxCxRxFxxxxxLxxHxxxxxD** from Phe-34 to Asp-60 showing significant similarities with those of C_2H_2-type zinc-finger motifs in other organisms, that functions in protein-DNA or protein-protein interactions (Mackay 1998) (Fig. 2); *e.g.* ZNF76 from *Homo sapiens*, TFIIIa-2 from *Xenopus laevis*, ZFH2 and Krüppel from *Drosophila melanogaster*, EPF1-1 from *Petunia hybrida*, and WZF1 from *Triticum aestivum*. Second, a 14-meric Gln-repeat typical to several transcription factors was found in the middle of the protein spanning from Gln-317 to Gln-330. Third, a Gly-rich domain was at the C-terminal region spanning from Gly-555 to Gly-628. Therefore, CCM1 is possibly one of the C_2H_2-type zinc-finger transcription factors although the Asp-60 in CCM1, corresponding to the second His in the C_2H_2 motif, is not conserved (Fig. 2). Since Asp serves as a zinc-ligand in other cellular components such as adenylate kinase from *Bacillus subtilis* (Perrier 1994), it is possible that Asp ligands to zinc to form finger-like secondary structure as predicted in other transcriptional factors with C_2H_2-type zinc-finger motifs (Pabo and Sauer 1992). Moreover, other

amino acid residues such as Phe-34, Arg-43, Phe-45 and Leu-51, and β-sheet structures in this domain of CCM1 are highly conserved with other zinc-containing transcription factors (Pabo and Sauer 1992). Identification of a mutation site in *cia5* revealed that the His-54 in the putative zinc-finger motif in CCM1 is essential to transduce a low-CO_2 signal into regulatory systems for CCM-related genes. Taken together with the fact that CCM1 also contains a Gln-repeat stretch, which is reported to be necessary for the regulatory functions of several eukaryotic transcription factors (Courey and Tjian 1988), CCM1 may directly interact with DNA or regulatory proteins through putative zinc-finger domain(s) and activate the transcription of CCM-related genes under low-CO_2 conditions. CCM1 has 14 putative phosphorylation sites, 3 putative glycosylation sites, and putative myristylation sites. The level of CCM1 protein accumulation does not change during acclimation to low-CO_2. These findings suggest that modifications of CCM1 protein under specific conditions, such as high- or low-CO_2 stress conditions might occur post-translationally in response to CO_2 availability.

cDNA array is a powerful tool to identify target genes, which are regulated by a master regulator CCM1. To isolate target genes which are controlled by CCM1, we have developed cDNA macroarray membrane filters containing 10,368 different cDNA fragments generated form EST clones by PCR (Asamizu 1999, Asamizu 2000, Fukuzawa manuscript in preparation). At first, we have compared signal patterns between autoradiograms obtained by macroarray membranes and those of ordinary northern blots (Fig. 3). RNA samples from wild type cells cultured in high-CO_2 or low-CO_2 and also from low-CO_2-acclimated C16 were labeled with ^{32}P-dCTP by reverse transcription and hybridized with cDNA array membranes (Fig. 3A). cDNA probe for *Cah1* was double-spotted on the specific positions and gave positive signals only when a RNA sample from low-CO_2 grown wild type cells was used as target RNA. On the other hand, no signal was detected when RNA samples from wild type cells grown in high-CO_2 or from low-CO_2 grown C16. These expression patterns in cDNA array membranes gave good agreement with those shown in northern analysis (Fig. 3B). Therefore, it is now possible to isolate CCM1-regulated gene by using these cDNA array membranes. Preliminary hybridization experiments using this cDNA array, we have detected more than 50 EST clones, which are regulated by CCM1, meaning that those EST clones are not induced in C16 mutant.

Discussion

In this paper we have identified a *Ccm1* gene encoding a hydrophilic protein, which is shown to be essential to regulate CCM induction in the eukaryote *C. reinhardtii* by sensing CO_2 availability. The gene product, CCM1, appears to control the expression of at least seven CCM-related genes; *Cah1, Mca, Ccp2, Lci1, Att1, Cah3, Cyp1*, as well as induction of CCM. Since *Ccm1* appears to be at a higher position in gene hierarchy and 10,368 EST groups have been obtained in this organism (Asamizu 2000), it is possible that some of other unidentified genes could be also regulated by *Ccm1*. In those *Ccm1*-dependent genes, there should be genes encoding CCM-components such as CO_2- or bicarbonate-transporters. Since other pleiotropic mutants have been isolated (Fukuzawa unpublished data), other signal components would be isolated from *Chlamydomonas* cells. Biochemical characterization of *Chlamydomonas* CCM1 protein would lead better understanding of CO_2-sensing and regulatory control of the CCM in eukaryotic photosynthetic organisms. This discovery may also open the door to a greater understanding of a cross-talk between signal transduction cascades that are involved in acclimation of eukaryotic photosynthetic organisms to environmental stresses such as nutrient shortage.

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References

Asamizu E et al. (1999) *DNA Res.* **6**: 369-373.

Asamizu E et al. (2000) *DNA Res.* **7,** 305-307.

Badger MR, Kaplan A, Berry JA (1980) *Plant Physiology* **66,** 407-413.

Burow MD et al. (1996) *Plant Mol. Biol.* **31,** 443-448.

Chen ZY et al. (1997) *Plant Physiology* **114,** 265-273.

Courey, A. J. & Tjian, R. (1988) *Cell* **55,** 887-898.

Eriksson M et al. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12031-12034.

Fukuzawa H et al. (1998) *Canadian Journal of Botany* **76,** 1092-1097.

Fukuzawa H et al. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4383-4387.

Fukuzawa H et al. (2001) *Proc. Natl. Acad. Sci. USA* **98**: 5347-5352.

Kaplan A, Reinhold L. (1999) *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 539-70.

Karlsson J et al. (1998) *EMBO Journal* **17,** 1208-1216.

Kucho K, Ohyama K, Fukuzawa H (1999) *Plant Physiology* **121,** 1329-1337.

Moroney J et al. (1989) *Plant Physiology* **89,** 897-903.

Somanchi A, Moroney JV (1999) *Plant Molecular Biology* **40,** 1055-62.

Perrier V et al. (1994) *Biochemistry* **33,** 9960-9967.

Pabo CO, Sauer RT (1992) *Annual Review of Biochemistry* **61,** 1053-95.

Xiang Y, Zhang J, Weeks DP (2001) *Proc. Natl. Acad. Sci. USA* 98: 5341-5346.

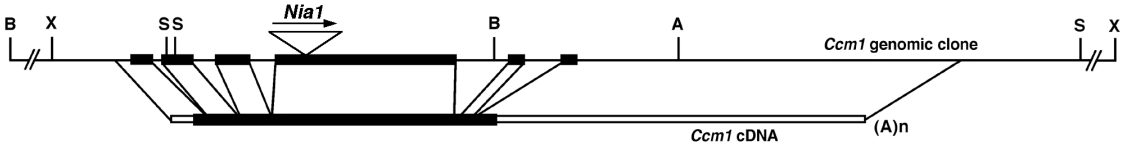

Fig. 1. Gene organization of *Chlamydomonas Ccm1*. The solid rectangles indicate the position of the protein coding regions. The insertion site of the *Nia1* gene in the C16 mutant is depicted by an open triangle with an arrow. A, *Apa*I; B, *Bam*HI; S, *Sac*I; X, *Xho*I; (A)n, a poly(A) tail.

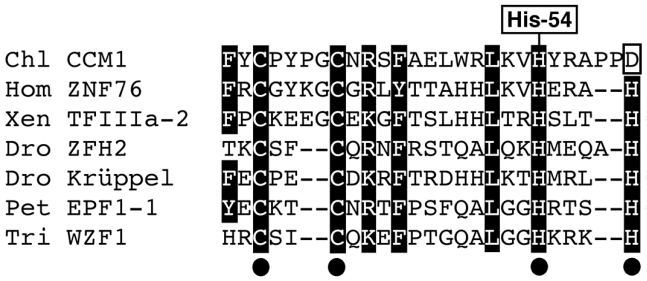

Fig. 2. Sequence comparison of the zinc-finger motif of CCM1 with those in other organisms; *Homo sapiens* (Hom) ZNF76 (NM_003427), *Xenopus laevis* (Xen) TFIIIa2 (K02938), *Drosophila melanogaster* (Dro) ZFH2 (P28167), and Krüppel (X03414), *Petunia hybrida* (Pet) EPF1-1 (X60700), and *Triticum aestivum* (Tri) WZF1 (D16415). Closed circles denote zinc-liganding amino acids. Conserved amino acids are highlighted. The 54th His residue, which is changed into Tyr in the *cia5* mutant, is depicted as His-54. The 60th Asp residue, which is substituted by a Val residue by means of site-directed mutagenesis, is boxed.

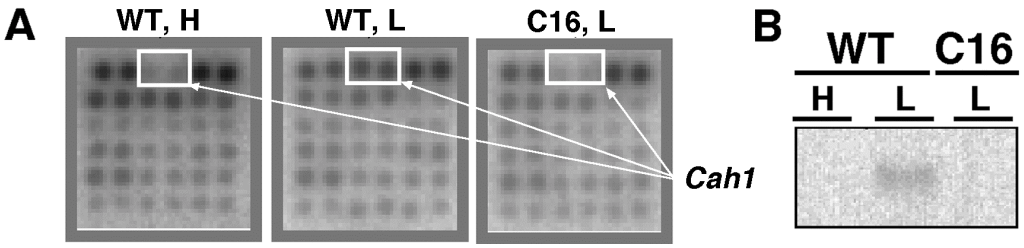

Fig. 3. Comparison of autoradiograms obtained by cDNA macroarray analysis and northern blot analysis of *Cah1*. A, three macroarrays containing *Cah1* cDNA probes were hybridized with target cDNAs derived from high- and low-CO_2 grown wild type cells and low-CO_2 grown C16 mutant cells. B, northern blot analysis of *Cah1* were performed with corresponding samples of macroarray analysis.