Isolation and characterization of regulatory mutants defective in CO2-signal transduction in *Chlamydomonas reinhardtii*

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Introduction

Photosynthetic organisms sense environmental changes e.g. light, temperature and various nutrient availability to modulate photosynthetic activities. During acclimation to CO2-limiting conditions, a set of genes for carbon concentrating mechanism (CCM) e.g. *Cah1* encoding periplasmic carbonic anhydrase (Fukuzawa 1990), *Mca* for mitochondrial carbonic anhydrase (Eriksson 1996), and *Ccp* for chloroplast envelope protein LIP-36 (Chen 1997) in *Chlamydomonas reinhardtii* are induced to allow rapid growth despite low-CO2 availability (Kaplan 1999). Taken with the facts that *Ccm1* controls expression of these CO2-responsive genes (Fukuzawa 2001) and that *Cah1* and *Mca* are regulated by specific cis-elements (Kucho 1999, Villand 1997), the induction of CCM is assumed to be regulated by some CO2-signaling mechanisms, by which cells perceive changes in the levels of environmental CO2 and the signal indicative of the shortage of CO2 is transduced into the induction of specific genes. However, this gene regulation during the acclimation in eukaryotic organisms is still poorly understood because of the paucity of mutants impaired in induction processes.

To identify other regulatory genes essential to the CO2-sensing mechanism, we isolated three regulatory mutants by using a chimeric construct between the *Cah1* promoter and arylsulfatase structural gene (*Ars*) as a marker gene. In this study, we report the characterization of these regulatory mutants.

Materials and methods

**Strains and culture conditions.**

*Chlamydomonas reinhardtii* Q304 and its progeny Q304P3 were used as host strains. These strains contain an exogenous arylsulfatase structural gene fused with 5'-upstream region, spanning from -651 to +41, of *Cah1* and show the wild type photosynthetic characteristics (Kucho 1999). For the high-CO2 conditions, cells were cultured in modified HSM medium (Sueoka, 1960), supplemented with 20 mM MOPS (pH 7.2) and 0.4 mM MgSO4 (HSM+S) under aeration with air enriched with 5% (v/v) CO2. For low-CO2 conditions, cultures were bubbled with ordinary air containing 0.04% (v/v) CO2.

**Transformation of Chlamydomonas cells.**

The Q304 strain or its progeny Q304P3 containing an additional nit1-305 mutation, were transformed with
pSP109 harboring bleomycin-resistant gene (Stevens 1996) or modified pMN24 harboring Nia1 gene (Kindle 1990), respectively. Zeocin resistant colonies or nia+ colonies were selected on TAP(NO3) agar plates containing Zeocin at 8 µg/ml or TAP(NO3) agar plates, respectively.

**Quantification of arylsulfatase activity.**

Cell cultures were assayed for arylsulfatase activity by adding 8 mM N-SO4 (Ohresser 1997). The mixture was incubated at 37°C and the reaction was stopped by adding equal volume of a solution containing 4% (w/v) SDS and 0.4 M sodium acetate (pH 4.8). The absorbance was measured at 540 nm immediately after addition of 1mg/ml tetrazotized-o-dianisidine, and the value was normalized by dividing with the chlorophyll content of the culture.

**Northern blot analyses**

RNA isolation from *Chlamydomonas* cells and Northern blot hybridization were performed as described (Kucho 1999).

**Results and Discussion**

The *Chlamydomonas* strains, Q304 and Q304P3, harboring a fusion construct between Cah1 promoter and the Ars gene, showed the arylsulfatase activity only under low-CO2 conditions as in the case of Cah1 (Fig.1). After transformation of a pSP109 harboring the ble gene or pMN24E containing Nia1 gene encoding nitrate reductase for a selectable marker, approximately 2,000 Zeocin-resistant transformants or 25,000 nia+ transformants were screened by using the arylsulfatase activity. One regulatory mutant named as C22 obtained from Zeocin-resistant transformants, and 7 nia+ regulatory mutants, as C44 and C55, did not show any arylsulfatase activities even under low-CO2 condition.

Three mutants, C22, C44, and C55, were further characterized on the growth rate under the high- and low-CO2 conditions (Fig.2). The growth of the C22 and C55 mutants was dependent on the supplied CO2 level, suggesting that these mutants show a moderate high-CO2 requiring characteristics. On the other hand, the C44 strain showed almost the same growth rate both under high- and low-CO2 conditions, as in the case of the host strains. The mRNA levels of low-CO2 inducible genes including Cah1, Mca and Ccp2 under the high- and low-CO2 conditions were also examined (summarized in Table 1). As in the case of ARS activities, the Cah1 mRNA was not induced under the low-CO2 conditions in the three mutants. Two of them, C22 and C44, induced two genes such as Mca and Ccp2 under the low-CO2 condition as in the case of photosynthetically wild type host strain Q304P3. However, the C55 mutant did not induce none of three genes under low-CO2 conditions.

In the mutants, C22 and C44, both the exogenous arylsulfatase gene fused with Cah1 promoter and the endogenous Cah1 gene were suppressed under low-CO2 conditions, although other CO2 responsive genes such as Mca and Ccp2 are induced in low-CO2 condition. These results strongly suggest that the lesions impaired in these two mutants, could encode transcriptional regulators, which specifically act with the Cah1 promoter. On the other hand, the mutant C55 was impaired in the induction of at least three genes, Cah1, Mca and Ccp2, as in the case of the previously characterized high-CO2 requiring mutant C16 (Fukuzawa 1998, 2001), that is cia-5 (Moroney 1989, Xiang 2001).

Further genetic and molecular characterization of this mutant C55 would clarify some other parts of the signal transduction pathways, which regulate CCM in *Chlamydomonas* cells by sensing CO2 availability. Especially cDNA array generated from EST clones should be most powerful tool to identify genes defective in
the mutants.

Acknowledgements

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References

Fig. 1. Quantification of arylsulfatase activity in individual transformants. Cells were cultured under high-CO$_2$ (H), and low-CO$_2$ (L). Both cultures were illuminated at 150 $\mu$mol m$^{-2}$ s$^{-1}$. Arylsulfatase activity was measured by a 2-h enzyme reaction, as described in Materials and Methods. Average of three determinations, with SD is represented by the bars above the graph.

Fig. 2. Growth curves of the host and the mutant *Chlamydomonas* strains under high-CO$_2$ or low-CO$_2$ conditions with continuous illumination (100 $\mu$mol m$^{-2}$ s$^{-1}$) at 28°C. Until early log phase, cells were cultured in a HSM minimum medium with bubbling of 5% CO$_2$ enriched air (high CO$_2$). Cultures were then divided into two tubes at hour 0. Half of the cells were continuously bubbled with CO$_2$-enriched air (5%) as indicated by open circles. The other cultures were bubbling with ordinary air (low CO$_2$) as shown in closed circles. Cell growth was estimated from optical density measurements at 730 nm.

**Table 1.** Inducibility of CO$_2$-responsive genes.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Q304P3</th>
<th>C22</th>
<th>C44</th>
<th>C55</th>
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<tr>
<td>Genes</td>
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<td>H</td>
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<td>Cah1</td>
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<td>Mca</td>
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<td>Ccp2</td>
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The mRNA levels were determined by RNA blot analyses. Total RNA was isolated from cells under high-CO$_2$ (H) or low-CO$_2$ (L). Cells were illuminated at 150 $\mu$mol m$^{-2}$ s$^{-1}$. RNA blots were hybridized with $^{32}$P-labelled gene specific oligonucleotide probes. Presence and absence of mRNA are indicated by plus and minus, respectively.