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Isolation and characterization of regulatory mutants defective in CO_2-signal transduction in *Chlamydomonas reinhardtii*

Hideya Fukuzawa[1], Kenji Miura[1], Tsutomu Kohinata[1], Satoshi Yoshioka[1], Fumiya Taniguchi[1], Takeshi Inoue[1], Tastuaki Saito[2], Ken-ichi Kucho[1], Kanji Ohyama[1]

[1]*Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan. fukuzawa@lif.kyoto-u.ac.jp*

[2]*Faculty of Science, Okayama University of Science,Ridai-cho, Okayama 700-0005, Japan.*

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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 CO_2-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-CO_2 availability (Kaplan 1999). Taken with the facts that *Ccm1* controls expression of these CO_2-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 CO_2-signaling mechanisms, by which cells perceive changes in the levels of environmental CO_2 and the signal indicative of the shortage of CO_2 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 CO_2-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-CO_2 conditions, cells were cultured in modified HSM medium (Sueoka, 1960), supplemented with 20 mM MOPS (pH 7.2) and 0.4 mM $MgSO_4$ (HSM+S) under aeration with air enriched with 5% (v/v) CO_2. For low-CO_2 conditions, cultures were bubbled with ordinary air containing 0.04% (v/v) CO_2.

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(NO_3) agar plates containing Zeocin at 8 μg/ml or TAP(NO_3) agar plates, respectively.

Quantification of arylsulfatase activity.

Cell cultures were assayed for arylsulfatase activity by adding 8 mM N-SO_4 (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-CO_2 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*+ transformatns 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-CO_2 condition.

Three mutants, C22, C44, and C55, were further characterized on the growth rate under the high- and low-CO_2 conditions (Fig.2). The growth of the C22 and C55 mutants was dependent on the supplied CO_2 level, suggesting that these mutants show a moderate high-CO_2 requiring characteristics. On the other hand, the C44 strain showed almost the same growth rate both under high- and low-CO_2 conditions, as in the case of the host strains. The mRNA levels of low-CO_2 inducible genes including *Cah1*, *Mca* and *Ccp2* under the high- and low-CO_2 conditions were also examined (summarized in Table 1). As in the case of ARS activities, the *Cah1* mRNA was not induced under the low-CO_2 conditions in the three mutants. Two of them, C22 and C44, induced two genes such as *Mca* and *Ccp2* under the low-CO_2 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-CO_2 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-CO_2 conditions, although other CO_2 responsive genes such as *Mca* and *Ccp2* are induced in low-CO_2 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-CO_2 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 CO_2 availability. Especially cDNA array generated from EST clones should be most powerful tool to identify genes defective in

the mutants.

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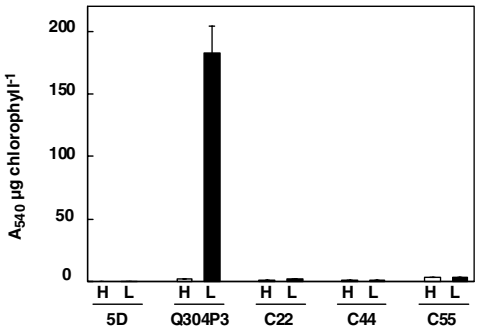

Fig.1. Quantification of arylsulfatase activity in individual transformants. Cells were cultured under high-CO_2 (H), and low-CO_2 (L). Both cultures were illuminated at 150 µmol m^{-2} s^{-1}. Arylsulfatase activity was measured by a 2-h enzyme reaction, as descrived in Materials and Methods. Average of three determinations, with SD is represented by the bars above the graph.

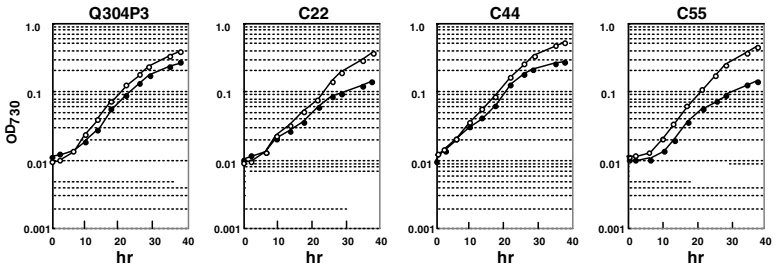

Fig.2. Growth curves of the host and the mutant *Chlamydomonas* strains under high-CO_2 or low-CO_2 conditions with continuous illumination (100 µmol m^{-2} s^{-1}) at 28˚C. Until early log phase, cells were cultured in a HSM minimum medium with bubbling of 5% CO_2 enriched air (high CO_2). Cultures 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.

Strains	Q304P3		C22		C44		C55	
Genes	H	L	H	L	H	L	H	L
Cah1	-	+	-	-	-	-	-	-
Mca	-	+	-	+	-	+	-	-
Ccp2	-	+	-	+	-	+	-	-

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 µmol 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.