2001

Insertional mutagenesis of the signal transduction pathway for low CO2 acclimation in Chlamydomonas reinhardtii

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Insertional mutagenesis of the signal transduction pathway for low CO₂ acclimation
in *Chlamydomonas reinhardtii*

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Physiology

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2001

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CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Acclimation to changed environmental conditions is a key of survival for every organism. Once organisms perceive environmental signals, they should show some specific adaptive changes like changing of important gene expression in order to survive. Many environmental conditions require adaptive changes, but CO$_2$ concentration is a major environmental signal in many photosynthetic eukaryotes and is the focus of my research.

*Chlamydomonas reinhardtii* and other microalgae show adaptive changes to limiting CO$_2$ conditions, such as, induction of a CO$_2$-concentrating mechanism (CCM) (Badger et al., 1980; for review, see Spalding, 1998), changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of periplasmic carbonic anhydrase 1 (pCA1) (*Cah1*) (Fujiwara et al., 1990; Fukuzawa et al., 1990; Ishida et al., 1993), mitochondrial carbonic anhydrase 1 and 2 (mtCA1 and mtCA2) (*Mca1* and *Mca2*) (Eriksson et al., 1996; Geraghty and Spalding, 1996), and the putative chloroplast carrier proteins 1 and 2 (Ccp1 and Ccp2) (*Ccp1* and *Ccp2*) (Geraghty et al., 1990; Ramazanov et al., 1993; Chen et al., 1997), and transient down-regulation in the synthesis of Rubisco (Coleman and Grossman, 1984; Winder et al., 1992).

Having a photosynthetic system similar to higher plants is one of the advantages of using *C. reinhardtii* as a model system to study regulation of gene...
expression by changes in CO₂ concentrations. Also, *C. reinhardtii* has a small genome, small size and short life cycle, which make manipulation and genetic analysis of this organism feasible. Recessive genes are expressed as a phenotype in the first generation because *C. reinhardtii* is haploid. Several methods of nuclear, chloroplast and mitochondrial transformations have been developed (Boynton et al., 1988; Kindle, 1990; Newman et al., 1991; Randolph-Anderson et al., 1993; Davies et al., 1994; Shimogawara et al., 1998), making mutant analysis even more valuable.

Each of the adaptive changes to limiting CO₂ conditions will be explained in detail. Although neither the signal for acclimation to limiting CO₂ in *C. reinhardtii* nor the pathway for transduction of this signal to changes in gene expression is identified, previously suggested signals and signal transduction pathways will be described, and approaches for dissecting the signal transduction pathway for low CO₂ acclimation will be discussed.

**LITERATURE REVIEW**

1. CO₂-Concentrating Mechanism (CCM)

The CCM is the most interesting adaptive change (for review, see Spalding, 1998) because characteristics resulting from the CCM include a high apparent affinity for CO₂, a low CO₂ compensation point, and reduced photorespiration due to a high ratio of CO₂ to O₂. The CCM requires at least two components. These components include active transport and accumulation of Cᵢ (Badger et al., 1980; Spalding et al., 1983b) and a thylakoid carbonic anhydrase (CA) (*Cah3*) to supply
CO₂ for Rubisco by dehydration of HCO₃⁻ (Spalding et al., 1983a; Funke et al., 1997; Karlsson et al., 1998).

A mutant lacking Cᵢ transport (pmp-1) that is unable to accumulate Cᵢ internally (Spalding et al., 1983b) has much lower affinity for CO₂ than wild-type high CO₂ grown cells. The identification of the disrupted gene of the pmp-1 mutant may tell more about functions of the Cᵢ-transport system. Although Cᵢ transporters are not identified yet, many researchers have confirmed that active Cᵢ transport occurs across both the plasmalemma and the chloroplast envelope (Badger et al., 1994; Palmqvist et al., 1994; Amoroso et al., 1998; for review, see Spalding, 1998). Low CO₂ grown cells are able to take up both CO₂ and HCO₃⁻ during steady-state photosynthesis (Amoroso et al., 1998), indicating the presence of a HCO₃⁻ transport system in intact cells (Badger et al., 1994; Palmqvist et al., 1994), even though CO₂ is the preferred Cᵢ species taken up by the cells (William and Turpin, 1987; Sütemeyer et al., 1989; Palmqvist et al., 1994). Whole cells and chloroplasts from high CO₂ grown cells have a HCO₃⁻ transport system and a functional CO₂ pump, respectively, although with much lower affinity in comparison with low CO₂ grown cells (Amoroso et al., 1998). The difference between high CO₂ and low CO₂ grown cells appears to be the increase in the affinity for uptake of both Cᵢ species without changing maximal activities.

The thylakoid lumen CA (C₉H₃) (Funke et al., 1997; Karlsson et al., 1998) is an essential component of CCM defined by ca-1 mutant analysis (Spalding et al., 1983a; Moroney et al., 1986; Suzuki and Spalding, 1989). Regardless of the Cᵢ species transported through the plasma membrane and the chloroplast envelope,
the accumulation of $C_i$ inside the chloroplast stroma appears to be as $HCO_3^-$ (Moroney et al., 1987; Sütemeyer et al., 1989; Badger et al., 1994; Palmqvist et al., 1994). Since Rubisco can use only $CO_2$ for fixing carbon, the accumulated $HCO_3^-$ must be converted to $CO_2$ by an intracellular CA. Without this thylakoid lumen CA, cells like the $ca-1$ mutant could not grow at air levels of $CO_2$ because the uncatalyzed conversion to $CO_2$ from $HCO_3^-$ in the stroma is too slow to support photosynthesis (Karlsson et al., 1998).

2. Induction of Specific Polypeptides

There are some specific polypeptides that are induced with the CCM, including the major periplasmic carbonic anhydrase (pCA1) (Coleman and Grossman, 1984; Bailly and Coleman, 1988), two 36 kD chloroplast carrier proteins (Ccp1 and Ccp2; products of $Ccp1$ and $Ccp2$) (Geraghty et al., 1990; Ramazanov et al., 1993; Chen et al., 1997), two 21 kD mitochondrial CA (mtCA1 and mtCA2; products of $Mca1$ and $Mca2$) (Eriksson et al., 1996) and two soluble polypeptides of 40-50 kD (Spalding and Jeffrey, 1989; Geraghty et al., 1990).

The $Cah1$ gene product, pCA1, is a zinc-containing metalloenzyme that catalyzes the interconversion of $CO_2$ and $HCO_3^-$ in the cell wall space of $C. reinhardtii$ (Badger and Price, 1994). There are two periplasmic CA isozymes. The major periplasmic CA, pCA1, is induced by limiting $CO_2$. The other periplasmic CA, pCA2, is the $Cah2$ gene product and is expressed only at very low abundance and only under elevated $CO_2$ (Fujiwara et al., 1990; Rawat and Moroney, 1991).
pCA1 has been considered a candidate for involvement in the CCM because it is the most abundant gene product induced by limiting CO$_2$ in *C. reinhardtii*. However, the characteristics of a *Cah1* structural gene mutant (*cah1-1*) demonstrate that pCA1 is not essential for function of the CO$_2$-concentrating mechanism or for growth of *C. reinhardtii* at limiting CO$_2$ concentrations, although the presence of pCA1 does apparently give some minor benefit at very low CO$_2$ concentrations (Van and Spalding, 1999). Both in spot tests and in liquid culture, cell growth rates for the *cah1-1* mutant were similar to those of wild-type over a pH range from 6 to 9, which argues against any essential role of pCA1 even at alkaline pH. Consistent with the lack of any effect on growth, the measured photosynthetic rates of the mutant and wild-type were very similar over a wide range of pH values as well. In addition, the $C_i$ response curve of the *cah1-1* mutant for photosynthetic O$_2$ evolution at pH 7 was very similar to that of wild-type, indicating that the lack of pCA1 had no major impact on photosynthetic rates over a range of $C_i$ concentrations or a range of pH conditions.

Ccp1 and Ccp2 are nearly identical and have been localized by cell fractionation to the chloroplast envelope (Chen et al., 1997). From analysis with deduced amino acid sequences, these proteins have very high similarity to the mitochondrial carrier protein superfamily and share common characteristics of established members of the mitochondrial carrier protein superfamily, such as three tandemly repeated sequences (Chen et al., 1997). Many proteins in the mitochondrial carrier superfamily are not mitochondrial proteins, such as Brittle 1 protein from maize amyloplasts (Sullivan et al., 1991) and the peroxisomal
membrane protein PMP47 (Jank et al., 1993). Chen et al., (1997) concluded that Ccp1 and Ccp2 are chloroplast carrier proteins because of localization and suggested they might be involved in Ci transport of the chloroplast envelope.

mtCA1 and mtCA2 have been localized immunocytochemically to mitochondria (Geraghty and Spalding, 1996) and also by cell fractionation (Eriksson et al., 1996). The amino acid sequences of the mature forms of these induced polypeptides are identical (Eriksson et al., 1996). The function of CA in mitochondria during acclimation to limiting CO2 is unclear. Two soluble polypeptides of 45-50 kD (Spalding and Jeffrey, 1989; Geraghty et al., 1990) are induced, but the corresponding genes have not been identified and their functions are not known.

3. Changes in Cell Organization

There are substantial structural changes in C. reinhardtii cells under limiting CO2 conditions. Mitochondrial relocation in cells adapting to limiting CO2 is an example (Geraghty and Spalding, 1996). Mitochondria move to peripheral positions from a central position within the cup of the chloroplast and are smaller and more numerous in limiting CO2 adapted cells. mtCA, which is one of the induced polypeptides, is specific to the relocated peripheral mitochondrial of air-grown cells (Geraghty and Spalding, 1996). These changes suggest mitochondria may play an important role in acclimation to limiting CO2.

The second example of cell organization during acclimation is an increased development of the starch sheath that surrounds the chloroplast pyrenoid (Ramazanov et al., 1994; Geraghty and Spalding, 1996). Because most of Rubisco
was localized in the pyrenoid (Lacoste-Royal and Gibbs, 1987; Borkhsonious et al., 1998), Badger and Price (1992) suggested that the starch sheath might act as a barrier to CO₂ diffusion. However, analysis of pyrenoid starch-less mutants did not support this idea (Plumed et al., 1996; Villarejo et al., 1996b). Even though they did not form a pyrenoid starch sheath under low CO₂, starch-less mutants from *Chlorrella* and *C. reinhardtii* did induce a fully active CCM and had the same high affinity for CO₂ as wild type 6 hr after transfer to limiting CO₂. These studies suggest that the pyrenoid starch sheath may not play a significant role in the CCM.

Also, *C. reinhardtii* had a 3-fold increase in vacuolization in both 24 hr-induced and air-grown cells relative to CO₂-enriched cells based on the percentage of cross-sectional area of the cell occupied by the vacuole (Geraghty and Spalding, 1996). A large, central vacuole was present in 24 hr-induced cells and air-grown cells had three to five, median size of vacuoles compared to smaller and numerous vacuoles in CO₂-enriched cells. This study speculated that the increased vacuolization might result from metabolic changes following transfer to limiting CO₂ (Geraghty and Spalding, 1996).

4. Other Changes in Low CO₂-associated Gene Regulation

Biosynthesis of both Rubisco subunits is also decreased transiently during acclimation to limiting CO₂ (Coleman and Grossman, 1984). The transient decrease is controlled by down-regulation at the translational level (Winder et al., 1992). Also, there are substantial increases in the activity of photorespiratory enzymes. The level of phosphoglycolate phosphatase (PGPase) is increased transiently (Marek and
Spalding, 1991), and the increased PGPase activity starts to decrease again at about the same time that cells are adapting to low CO₂. The levels of glycolate dehydrogenase and glutamine synthetase are also increased stably during acclimation to limiting CO₂ (Nelson and Tolbert, 1969; Marek and Spalding, 1991; Ramazanov and Cardenas, 1992, 1994).

5. Signals and Pathways for Limiting CO₂ acclimation

The signal for acclimation to limiting CO₂ in *C. reinhardtii* is unidentified. It is not known how they sense a change of CO₂ level, whether by CO₂ concentration directly or indirectly by a cellular process such as metabolism. In cyanobacteria, the cells acclimated to limiting CO₂ depending on the availability of C₅ species that they can utilize from medium (Badger, 1987; Kaplan et al., 1998). *Synechococcus* PCC7942 developed limiting CO₂ characteristics because cells recognized low CO₂ concentrations, even though the HCO₃⁻ concentration was high in high pH (Sültemeyer et al, 1998; Kaplan and Reinhold, 1999).

Since reduced O₂ levels or a low O₂/CO₂ concentration ratio reportedly decreased the induction (or derepression) of CA in *C. reinhardtii*, the signal for limiting CO₂ condition may be a photorespiratory metabolite (Spalding and Ogren, 1982). CA induction did not occur in the presence of aminooxyacetate, an inhibitor of the glyoxylate-serine aminotransferase (Ramazanov and Cardenas, 1992). This supports that idea a photorespiratory metabolite is involved in CA induction. On the other hand, the induction of the 36 kD low CO₂ induced polypeptides (Ccp1 and Ccp2) were not affected by aminooxyacetate, whereas the induction of pCA1 and
two 21 kD mitochondrial CA (mtCA1 and mtCA2) were eliminated by this glycolate pathway inhibitor (Villarejo et al., 1997). These observations suggest that there may be different signals involved in the induction of these three polypeptides.

Light and blue light might also be involved in the up-regulation of pCA1 expression (Dionisio et al., 1989b; Borodin et al., 1994). The light requirement probably reflects a requirement for photosynthesis (Spalding and Ogren, 1982; Spencer et al., 1983; Dionisio et al., 1989a, b; Dionisio-Sese et al., 1990), but the blue light effect is not related to photosynthesis. Cells transferred to low CO₂ conditions in the dark did not show any increase in pCA1 activity and the blue light has effect on the induction of pCA1 even with very low light intensities.

In contrast to the light requirement for pCA1 induction by Dionisio et al. (1989a, b) and Dionisio-Sese et al. (1990), some induction of Cah1 message was detected after exposure of CO₂-adapted cells to air levels of CO₂ in the dark (Bailly and Coleman, 1988). Even though the abundance of Cah1 message was increased significantly in the presence of light, this study indicated that light is not required for sensing CO₂ concentration. Mca1 and Mca2 messages also are induced by low CO₂ in the dark but only in synchronized cells (Eriksson et al., 1998). Using the promoter analysis of Mca1 and Mca2 fused to the arylsulphotase reporter gene, these genes also are induced by low CO₂ under continuous light with the levels of expression affected by light intensity and photosynthesis (Villand et al., 1997). In the same study, O₂ concentration did not give any impact on the promoter activity, suggesting the photorespiratory metabolites are not the signal for mtCA1 and mtCA2 induction. However, Villarejo et al., (1996a) reported that mtCA1 and mtCA2, and
Ccp1 and Ccp2 were induced only in the light while pCA1 was induced either in light, or in dark and in both synchronized and nonsynchronized cells. Once again, these observations suggest the three low CO2 induced polypeptides might be regulated differentially.

Obviously, many signals are involved for the signal transduction pathway for low CO2 acclimation. Like glucose repression in yeast, acetate utilized by C. reinhardtii as a reduced carbon source acts as a repressor of limiting CO2 acclimation, as indicated by repression of Cah1 expression (Fett and Coleman, 1994). The addition of acetate apparently mimics the high CO2 condition, so acetate grown cells showed reduced levels of Cah1 message abundance and lower pCA1 levels and activity. Also, acetate grown cells had much lower affinity for C1 compared with air grown cells (Moroney et al., 1987). It has been suggested that acetate repression of CA might be a response of increasing intracellular CO2 levels by elevated respiratory production (Fett and Coleman, 1994). However, either high CO2 or acetate may just represent rich carbon sources, which repress the expression of genes associated with acclimation to low CO2 through increases in common metabolic intermediates, such as sugars.

The limiting-CO2 signal must be transduced into the changes in gene expression observed during acclimation, such as expression of Cah1. However, the signal transduction pathway of the acclimation to limiting CO2 is uncharacterized. The gene expression changes during acclimation are regulated at different levels, so the signal transduction pathway of acclimation to limiting CO2 should be branched. The best way to identify the signals and components of the signal transduction
pathway for low CO$_2$ acclimation is the analysis and characterization of mutants, like the $cia$-5 mutant (Moroney et al., 1989). The $cia$-5 mutant does not have any of the low CO$_2$ induced polypeptides and also does not show $C_i$ accumulation, high affinity for CO$_2$, up-regulation of PGPase and glycolate dehydrogenase or down-regulation of Rubisco biosynthesis (Moroney et al., 1989; Marek and Spalding, 1991; Spalding et al., 1991; Burow et al., 1996). The $cia$-5 mutant can be used as an example for identification of other mutants defective in the signal transduction pathway for low CO$_2$ acclimation.

Recently, the Cia-5 gene was cloned (Xiang et al., manuscript submitted). The Cia-5 protein has two potential zinc finger domains and many potential sites for posttranslational modifications. The amino acid sequences and two zinc finger motifs of the Cia-5 protein suggest that this protein might be a transcription factor. The $cia$-5 mutant does not show any adaptive change for low CO$_2$ acclimation. So, one signal transduction pathway involving $cia$-5 regulates all low CO$_2$ responsive genes at different gene expression levels, such as transcriptional up-regulation of $Cah1$, $Mca1$, $Mca2$, $Ccp1$ and $Ccp2$, transcriptional down-regulation of $Cah2$, translational down-regulation of $rbcL$, $RbcS1$ and $RbcS2$ expression, transient up-regulation of phosphoglycolate phosphatase and long term up-regulation of glycolate dehydrogenase and glutamine synthetase, etc. This means the Cia-5 protein is involved in early steps in the signal transduction pathway for low CO$_2$ acclimation.
6. Approaches for Dissecting Signal Transduction Pathway for Low CO$_2$

Acclimation

Recent advances in transformation of *C. reinhardtii* may provide tools to help solve some important questions in cell biology and physiology. Although the frequencies of transformation are still fairly low, nuclear transformation in *C. reinhardtii* is used routinely (Kindle, 1990). Insertional mutagenesis by transformation provides a mechanism for genetic manipulation in *C. reinhardtii*, because this transformation occurs as a random insertion instead of homologous recombination. Insertional mutagenesis allows the defective genes in interesting mutants to be detected by gene tagging. The mutational analysis of acclimation to limiting CO$_2$ in *C. reinhardtii* should be a useful technique for investigating the signal transduction pathway, since tagged mutants will allow cloning of the disrupted genes.

**DISSERTATION ORGANIZATION**

The dissertation is written in the format to include a published paper (Chapter 2) and a manuscript (Chapter 3) for submission to Plant Physiology. The first paper demonstrates the characterization of periplasmic carbonic anhydrase structural gene (*Cah1*) mutant in *C. reinhardtii* and the second paper addresses newly isolated insertional mutants of *C. reinhardtii* that require elevated CO$_2$ for survival. One of the new mutants (HCR90) is discussed further in Chapter 4 and the dissertation is concluded with a general summary.
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CHAPTER 2. PERIPLASMIC CARBONIC ANHYDRASE

STRUCTURAL GENE (CAH1) MUTANT IN CHLAMYDOMONAS: REINHARDTII


Kyujung Van2 and Martin H. Spalding2

ABSTRACT

To survive in various conditions of CO2 availability, Chlamydomonas reinhardtii shows adaptive changes, such as induction of a CO2-concentrating mechanism, changes in cell organization and induction of several genes, including a periplasmic carbonic anhydrase (pCA1) encoded by Cah1. Among a collection of

1 Abbreviations: AMPSO, 3-(N-(1,1-dimethyl hydroxyethyl) amino)-2-hydroxypropane sulfonic acid; AZA, acetazolamide; CA, carbonic anhydrase; CCM, CO2-concentrating mechanism; Ccp, chloroplast carrier protein; Ci, inorganic carbon; HEPPS, 4-2(-hydroxyethyl)-1-piperazine propane sulfonic acid; MES, 2-(N-morpholino) ethanesulfonic acid; MOPS, 3-(N-morpholino) propane-sulfonic acid; mtCA, mitochondrial carbonic anhydrase; pCA1, periplasmic carbonic anhydrase 1; pCA2, periplasmic carbonic anhydrase 2.

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insertionally generated mutants, a mutant has been isolated that showed no pCA1 protein and no Cah1 mRNA. This mutant strain, designated cah1-1, has been confirmed to have a disruption in the Cah1 gene caused by a single Arg7 insert. The most interesting feature of cah1-1 is its lack of any significant growth phenotype. There is no major difference in growth or photosynthesis between wild-type and cah1-1 over a pH range from 5 to 9 even though this mutant apparently lacks Cah1 expression in air. Although the presence of pCA1 does apparently give some minor benefit at very low CO2 concentrations, the characteristics of this Cah1 null mutant demonstrate that pCA1 is not essential for function of the CO2-concentrating mechanism or for growth of C. reinhardtii at limiting CO2 concentrations.

INTRODUCTION

Aquatic and soil-borne photosynthetic organisms including Chlamydomonas reinhardtii live in quite variable conditions of CO2 availability. In order to survive in limiting CO2 conditions, C. reinhardtii and other microalgae show adaptive changes, such as induction of a CCM (Badger et al., 1980; for review, see Spalding, 1998), changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of pCA1 (Cah1) (Fujiwara et al., 1990; Fukuzawa et al., 1990; Ishida et al., 1993), mtCA (Mca1 and Mca2) (Eriksson et al., 1996; Geraghty and Spalding, 1996), and Ccp (Ccp1 and Ccp2) (Geraghty et al., 1990; Ramazanov et al., 1993; Chen et al., 1997),

Of these the CCM is the most studied adaptive change (for review, see Spalding, 1998), because it affects photosynthetic characteristics through increasing intracellular CO$_2$ concentration. So far, at least C$_i$ transport (Badger et al., 1980; Spalding et al., 1983b) and a thylakoid carbonic anhydrase (Cah3) to supply CO$_2$ for Rubisco by dehydration of HCO$_3^-$ (Spalding et al., 1983a; Funke et al., 1997; Karlsson et al., 1998) have been shown to be required for operation of the CCM. Characteristics resulting from the CCM include a high apparent affinity for CO$_2$, a low CO$_2$ compensation point, and reduced photorespiration due to a high ratio of CO$_2$ to O$_2$. It is not clear yet whether the induced genes (Cah1, Mca1, Mca2, Ccp1, Ccp2) are required for function of the CCM.

The Cah1 gene product, pCA1, is a zinc-containing metalloenzyme that catalyzes the interconversion of CO$_2$ and HCO$_3^-$ in the cell wall space of C. reinhardtii (Badger and Price, 1994). There are two periplasmic CA isozymes. The major periplasmic CA, pCA1, is induced by limiting CO$_2$. The other periplasmic CA, pCA2, is the Cah2 gene product and is expressed only at very low abundance and only under elevated CO$_2$ (Fujiwara et al., 1990; Rawat and Moroney, 1991). pCA1 has been considered a candidate for involvement in the CCM because it is the most abundant gene product induced by limiting CO$_2$ in C. reinhardtii. However, evidence for any function of pCA1 in the CCM has been contradictory, with Moroney et al. (1985) arguing that pCA1 is required to supply CO$_2$ from HCO$_3^-$ for rapid
photosynthesis at low CO₂ concentrations and alkaline pH and Williams and Turpin (1987) disagreeing. Among a collection of insertionally generated mutants, we found a mutant, named cah1-1, that showed no pCA1 protein and no Cah1 mRNA. Here we report on the photosynthetic characteristics of this Cah1 null mutant.

MATERIALS AND METHODS

Cell Strains, Culture Conditions and Mating

All C. reinhardtii strains (Table I) were grown as previously described (Geraghty et al., 1990). Cells were cultured on an orbital shaker under aeration with 5% CO₂ in air (CO₂-enriched cells) or no aeration (air-adapted cells). Cell cultures were switched from elevated CO₂ to limiting CO₂ (no aeration) for 1 d or 2 d induction. All matings were performed by crossing PCA57-61 with CC1068 (Table I) according to the protocol of Harris (1989).

Generation and Isolation of Mutants

Using glass bead transformation (Kindle, 1990; Davies et al., 1994), CC425 (Table I) was transformed with pArg7.8 (Debuchy et al., 1989) to generate a pool of insertional mutants on CO₂ minimal medium that lacked Arg. This plasmid has the structural gene (Arg7) for argininosuccinate lyase to complement the arg2 mutation. Each of more than 7000 colonies was suspended with air minimal medium in a 1.5 ml microcentrifuge tube and grown with shaking but no aeration for 2 d. After centrifugation the supernatant of each culture was screened for a lack of pCA1
expression using a slot blot and immunodetection as described for western blots. Mutants identified in this primary screen as having reduced pCA1 expression were screened again by western immunoblots following SDS-PAGE of extracellular protein from larger scale cultures.

**Protein Extracts, SDS-PAGE, and Western Immunoblots**

Cell cultures exposed to limiting CO$_2$ (no aeration) for 2 d were harvested by centrifugation (5,000 rpm, Sorvall GSA rotor, 15 min). The supernatants were precipitated with (NH$_4$)$_2$SO$_4$ overnight, and the precipitated proteins collected by centrifugation (10,000 rpm, Sorvall GSA rotor, 30 min), dissolved in 1X resolving gel buffer and then desalted using spin columns (1 ml disposable syringe, Becton Dickinson & Co., Franklin Lakes, NJ) of Sephadex G-25. Protein samples were separated by SDS-PAGE with 12% polyacrylamide using the buffer system of Laemmli (1970). Separated proteins were electrophoretically transferred to nitrocellulose with a Semi-Dry Blotting Unit (Fisher Biotech, Itasca, IL) and immunodetected using affinity-purified anti-pCA1 polyclonal antiserum as the primary antibody (Roberts and Spalding, 1995), horseradish peroxidase-linked second antibody, and aminoethylcarbazole as the chromogenic substrate (Harlow and Lane, 1988).

**Nucleic Acid Extractions and Analysis**

Genomic DNA was isolated using Elu-Quik DNA purification kits (Schleicher & Schuell, Keene, NH) and digested with appropriate restriction enzymes. Total RNA
was prepared (Chomczynski and Sacchi, 1987) from induced cells exposed to limiting CO₂ for 1 or 2 d. Nucleic acids (10 μg) were separated in 0.8% agarose gels for Southern analysis or in 0.66 M formaldehyde-1.5% agarose gels for northern analysis, and then blotted onto nylon transfer membranes (Micron Separations Inc., Westborough, MA). After UV cross-linking, membranes were hybridized with appropriate ³²P-labeled probes (Random Primers DNA Labeling System, Life Technologies, Gaithersburg, MD). Southern and northern blot analyses were performed by the method of Ausubel et al. (1987).

**Growth Tests and Photosynthetic O₂ Exchange**

For spot growth tests, active growing cells were suspended to similar cell densities with minimal medium, spotted (10 μl) onto agar plates with different pH and kept at air level of CO₂ for 10 d (Harris, 1989). Each medium was buffered with 25 mM MES-KOH for pH 6, 25 mM MOPS-KOH for pH 7, 25 mM HEPPS-KOH for pH 8 and 25 mM AMPSO-KOH for pH 9.

For growth curve tests, active, fully air-adapted cells were inoculated into different pH liquid minimal medium buffered as described above. The cell density was determined by using a hemacytometer (Reichert Scientific Instruments, Buffalo, NY) (Harris, 1986).

For O₂-exchange measurements, 1 d induced cells were harvested by centrifugation and resuspended in 25 mM Mops-KOH (pH 7) for analysis of the response to CO₂ concentration or in 25 mM of the appropriate buffer for analysis of the effects of pH. Suspended cells (1 ml) were added to a Rank Brothers O₂
electrode at 25 °C. The measurements were started by the addition of different concentrations of NaHCO₃ after confirmation by cessation of O₂ evolution of the depletion of inorganic carbon in medium under illumination (500 μmol photons m⁻² s⁻¹) provided by a slide projector. O₂ concentration data were collected every s, averaged, recorded every 5 s by a 21x datalogger (Campbell Scientific, Inc., Logan, UT) and transferred to an IBM computer. Smoothed data (Savitzky and Golay, 1964) were used for calculation of exchange rates. Maximum spontaneous CO₂ supply rates from uncatalyzed dehydration of bicarbonate were calculated as described by Miller and Colman (1980). Chlorophyll content was estimated after extraction with 96% (v/v) ethanol (Wintemans and De Mots, 1965).

RESULTS

Generation and Isolation of Mutants

A pool of more than 7000 transformants was generated by complementation transformation of an Arg-requiring mutant with the Arg7 gene. In an attempt to identify mutants failing to adapt to limiting CO₂, this pool of potential insertional mutants was screened immunochemically for the absence of pCA1, which was being used as a reporter for induction of genes involved in adaptation to limiting CO₂. Sixty-eight putative mutants were identified in the primary screen, and 18 were selected for further characterization based on the absence of or reduction in pCA1 expression in western blots. Among this collection of insertionally generated mutants, we found a mutant (PCA57-61) that showed no detectable pCA1 protein
(Fig. 1A) and no detectable Cah1 mRNA (Fig. 1B). Since the Cah1 cDNA probe used (Spalding et al., 1991) also detects the Cah2 message (Fujiwara et al., 1990) and the antibody used should cross react with pCA2, these blots also demonstrate that Cah2 message and pCA2 were undetectable as well. Expression of other proteins normally induced by limiting CO₂, mtCA (Mca1 and Mca2 genes) and Ccp (Ccp1 and Ccp2 genes), apparently was not affected by the mutation (data not shown).

Genomic Analysis of PCA57-61

PCA57-61 genomic DNA was compared with that of CC425 (Table I) by Southern blot analysis (Fig. 2). The presence of 1 insert in PCA57-61 was confirmed by probing with the 1.3 kb SalI fragment of Arg7 (no data shown). When the same blot was probed with the entire Cah1 cDNA, different patterns were observed for CC425 and PCA57-61 (Fig. 2A), indicating that the Cah1 structural gene had been disrupted. Some of the restriction fragments (indicated by arrows) apparently hybridized to both the Cah1 and the Arg7 probes (data not shown), indicating that the Arg7 insert is located near the Cah1 structural gene. Cah1 and Cah2 are arranged in tandem, separated by approximately 0.8 kb (Fig. 2B). The Cah1 cDNA probe hybridizes with both genes (Fujiwara et al., 1990), so bands from both Cah1 and Cah2 can be seen on the Southern analysis in Figure 2A. The Cah2 structural gene appears undisturbed. The 5.7 kb BglI fragment containing the 5' end of the Cah2 gene and the 3' end of the Cah1 gene appears intact, but the 6.8 kb KpnI fragment covering about the same area has been disrupted, along with all
restriction fragments 5' to the BglII site in Cah1. The simplest interpretation of these results is that the Cah1 gene 5' to the BglII site of Cah1 has been disrupted by an insertion, a deletion or a rearrangement. Since both the Arg7 probe and the Cah1 probe hybridized to the new EcoRI and KpnI fragments of PCA57-61 (indicated by arrows in Fig. 2A), it appears that this disruption has been caused by insertion of the Arg7 gene in or near the Cah1 structural gene.

Phenotypes and Genetic Analysis of PCA57-61

The most interesting feature of PCA57-61 was its lack of any significant growth phenotype even though this mutant apparently lacks any Cah1 expression in air. After PCA57-61 was crossed with CC1068 (Table I), progeny showed Mendelian 2:2 segregation of wild-type and Arg-requiring phenotypes (Table II). Both the absence of Cah1 mRNA accumulation and the restriction polymorphisms of genomic DNA co-segregated with the Arg7 insert, confirming that this insert was responsible for the absence of Cah1 mRNA in PCA57-61 (Table II). Progeny 57-61-612 was chosen for further physiological analysis because it had the same biochemical phenotype as PCA57-61 but had a normal cell wall.

Effects of pH on Growth

Because Moroney et al. (1985) and Williams and Turpin (1987) reported contradictory results for photosynthesis at alkaline pH using either CA inhibitors (Moroney et al., 1985) or washed, wall-less cells to eliminate pCA1 activity (Williams and Turpin, 1987), a Cah1 null mutant, such as PCA57-61, should help in resolving
the function of pCA1 in the CCM. In spot tests, growth of PCA57-61 and its walled progeny PCA57-61-612 was similar to that of two wild-types, one wall-less (CC400, Table I) and one walled (CC125, Table I), at all pHs in air (Fig. 3). However, other known mutants with defects in the CCM and related pathways, cia5, ca1-1, pmp1-1, and pgp1-1 (Table I), showed slow or no growth in air within the tested pH range. All cells grew poorly at pH 5 (data not shown). Both wild-types, PCA57-61 and PCA57-61-612 showed variable growth at pH 9, as illustrated by the poor growth of CC400 at pH 9 in Fig. 3. However, the slow growth usually caught up with that on the other pH plates. In liquid, the growth rate of PCA57-61-612 was similar to that of CC125 at pH 8 in air (Fig. 4) and at other pHs from 6 to 9 (data not shown). These growth tests demonstrated that there was no major difference in growth between wild-type and PCA57-61 over a pH range from 6 to 9, indicating the mutant grows normally under low CO₂ in this pH range even without detectable pCA1.

**Photosynthetic O₂ Exchange**

Photosynthetic O₂ evolution of PCA57-61-612 was investigated at different concentrations of NaHCO₃ and at different pHs. PCA57-61-612 showed a similar pattern of photosynthetic response to NaHCO₃ concentration as the CC125 wild-type (Fig. 5A), and there was no difference of photosynthesis rate between these strains at air levels of CO₂. At very low CO₂, however, PCA57-61-612 showed slightly lower photosynthesis rates than wild-type (Fig. 5B). The Kᵥ(CO₂) calculated from these data is higher for PCA57-61-612 (21 μM) than wild-type (11 μM). Since the O₂ evolution is measured in a closed system, these experiments used fairly low
cell densities (9–11 μg Chl ml⁻¹) to minimize problems with rapid Cᵢ depletion at low Cᵢ concentrations. At these relatively low cell densities, the rate of photosynthetic consumption in PCA57-61-612 was similar to the spontaneous CO₂ supply rate from uncatalyzed bicarbonate dehydration (Fig. 6A). However, this similarity is coincidental because at higher cell densities (35-38 μg Chl ml⁻¹) CO₂ response curves for both strains were similar to those obtained at lower cell densities (data not shown), and the photosynthesis rates of both strains clearly exceeded the spontaneous CO₂ supply rate at all bicarbonate concentrations up to 100μM (Fig. 6B). The apparently large difference between the calculated photosynthetic CO₂ consumption rates of PCA57-61-612 and CC125 in Figure 5A results only from the slightly lower cell density for the mutant because the rates are expressed as μmoles CO₂ L⁻¹ h⁻¹ to match the units used for the spontaneous CO₂ supply rate. The measured rates of O₂ evolution were more variable at the higher cell density, but PCA57-61-612 maintained a rate similar to that of wild-type even though the calculated maximum spontaneous CO₂ supply rate was only half of the photosynthetic rate.

Because it had been suggested that pCA1 was needed to supply CO₂ at alkaline pH (Moroney et al., 1985), photosynthetic O₂ evolution at different pHs also was investigated. Photosynthetic rates at two bicarbonate concentrations of wild-type CC125, and PCA57-61-612 decreased with increasing pH, but both showed approximately the same rate of photosynthetic O₂ evolution at the given concentration and pH (Table III). Although there was no significant difference between wild-type and PCA57-61-612 in photosynthetic O₂ evolution at any NaHCO₃
concentration or pH, small effects might be masked by the variation in the rate measurement. These results indicate that pCA1 is not required to supply CO₂ from HCO₃⁻ dehydration under the conditions used, but they do not exclude some minor benefit conferred by pCA1 at low Cᵢ concentrations.

**DISCUSSION**

Like C₄ plants, *C. reinhardtii* as well as many other microalgae and cyanobacteria have an active CCM that allows cells to assimilate inorganic carbon efficiently when they grow under limiting CO₂ conditions. The CCM results in increased internal CO₂ concentration, which increases the substrate for Rubisco carboxylation and favors the carboxylation reaction of Rubisco over the oxygenation reaction (Badger et al., 1980; Aizawa and Miyachi, 1986; Spalding, 1998). This mechanism is inducible after transfer from high (5% CO₂ in air) to low CO₂ (0.03% CO₂ in air). As previously described, *C. reinhardtii* shows adaptive changes to limiting CO₂ conditions other than the CCM, including the induction of several major genes (*Cah1, Mca1, Mca2, Ccp1, Ccp2*) (see Spalding, 1998 for review). However, it is not clear yet whether any of these induced genes are required for function of the CCM.

pCA1 has been considered a potential candidate for an essential CCM component because large amounts of this polypeptide accumulate under low CO₂ conditions, and affinity for inorganic carbon also is increased coincident with the induction of pCA1. For pCA1 to be essential, conversion between CO₂ and HCO₃⁻...
would have to be a critical step for *C. reinhardtii* in terms of adapting to low CO₂, but many studies have shown that *C. reinhardtii* can use both CO₂ and HCO₃⁻ (Williams and Turpin, 1987; Sülttemeyer et al., 1989; Palmqvist et al., 1994). On the other hand, the evidence does indicate that CO₂ is the preferred substrate (Moroney et al., 1985; Aizawa and Miyachi, 1986).

The function of pCA1 in the CCM has been controversial. Moroney et al. (1985) and Williams and Turpin (1987) arrived at contradictory conclusions regarding the need of pCA1 to supply CO₂ at alkaline pH. The photosynthetic rate of *C. reinhardtii* was significantly decreased in limiting CO₂ when pCA1 was inhibited by nominally non-permeant CA inhibitors, but only at alkaline pH (Moroney et al., 1985). The authors interpreted this to mean pCA1 was required to supply CO₂ from HCO₃⁻ for rapid photosynthesis at low CO₂ concentrations and alkaline pH, because the HCO₃⁻ concentration is higher than that of CO₂ under these conditions. However, Williams and Turpin (1987) were unable to demonstrate decreased photosynthetic rates at alkaline pH using washed wall-less cells that lack pCA1 activity rather than using CA inhibitors. These authors concluded that the pCA1 activity is not absolutely required for utilizing C₁ under alkaline pH, and suggested that, in the work of Moroney et al. (1985), nominally non-permeant CA inhibitors like AZA may have penetrated the cells and partially inhibited internal CA, which is essential for photosynthesis. Although Moroney et al. tried to control for secondary effects of the CA inhibitors based on the work reported here and by Williams and Turpin, it appears that they did have effects other than inhibition of extracellular CA.
We have identified a mutant (PCA57-61) that shows no pCA1 protein and no *Cah1* mRNA (Fig. 1) but apparently normal *Mca1, Mca2, Ccp1*, and *Ccp2* mRNAs and normal levels of the corresponding proteins. This insertional mutant was isolated after transformation of strain CC425 with a plasmid containing the argininosuccinate lyase gene (*Arg7*). Southern and genetic analyses have established the presence of 1 insert in this mutant that co-segregates with the lack of *Cah1* mRNA and with polymorphisms in the 5' region of *Cah1*. It is clear that insertion of this *Arg7* insert in the region of the *Cah1* and *Cah2* genes has disrupted the *Cah1* structural gene and that this disruption is responsible for lack of *Cah1* expression in this mutant. Thus, PCA57-61 is a *Cah1* structural gene mutant apparently null in pCA1 expression, which we have named *cah1-1*.

The most interesting feature of PCA57-61 is that it does not have a high CO₂-requiring phenotype even though it has no detectable pCA1. One might expect that the total absence of pCA1 would have a more significant effect on the growth rate of PCA57-61 at air level of CO₂, perhaps intermediate between wild-type and *cia5*, a mutant that lacks *Cah1* expression along with all other induced genes (Moroney, 1989). Both in spot tests and in liquid culture, cell growth rates for PCA57-61 and PCA57-61-612 without detectable pCA1 were similar to those of wild-type over a pH range from 6 to 9, which argues against any essential role of pCA1 even at alkaline pH.

Consistent with the lack of any effect on growth, the measured photosynthetic rates of the mutant and wild-type were very similar over a wide range of pH values as well (Table III). In addition, the *Cₙ* response curve of PCA57-61-612 for
photosynthetic O₂ evolution at pH 7 was very similar to that of wild-type CC125, indicating that the lack of pCA1 had no major impact on photosynthetic rates over a range of Cᵢ concentrations or a range of pH conditions. However, even though the photosynthetic rates of the two strains were indistinguishable at air levels of CO₂ (60 μM Cᵢ), the mutant was found to have a slightly elevated Kᵢ½ (Cᵢ) relative to wild-type (21 μM versus 11 μM) and slightly reduced photosynthetic O₂ evolution at Cᵢ concentrations lower than 50 μM. Although not nearly as extreme as the differences reported by Moroney et al. (1985), it appears that the abundant pCA1 activity may be of some benefit under very low Cᵢ concentrations.

The observations made here appear contradictory to the report by Moroney et al. (1985), based on inhibitor studies, that pCA1 was required to supply CO₂ from bicarbonate for rapid photosynthesis at low CO₂ concentrations, especially at alkaline pH. If pCA1 is essential to supply CO₂ through dehydration of bicarbonate, this should be most evident under conditions where the photosynthetic rate of wild-type clearly exceeds the calculated maximum spontaneous CO₂ supply rate. However, as demonstrated in Figure 5B, the Cah1 null mutant showed a photosynthetic rate similar to that of wild-type CC125 even though the spontaneous CO₂ supply rate was only half the rate of photosynthesis. This could possibly be explained by residual carbonic anhydrase activity from pCA2, but Cah2 expression should be repressed under these low CO₂ growth conditions (Fujiwara et al., 1990; Rawat and Moroney, 1991), as confirmed by our inability to detect either the Cah2 mRNA or the pCA2 protein. It seems more likely that, although C. reinhardtii apparently prefers CO₂ (Moroney et al., 1985; Aizawa and Miyachi, 1986), PCA57-
61-612 must be using bicarbonate directly from the medium. These results confirm those of others (Sütemeyer et al., 1989; Palmqvist et al., 1994) including Williams and Turpin (1987) who reported similar findings using wall-less *C. reinhardtii* washed free of pCA1.

We conclude that, although some benefit may be derived from the presence of pCA1 at very low $C_i$ concentrations, the benefit appears less substantial than that reported by Moroney et al., and this protein certainly does not appear to be essential either for function of the CCM or for growth of *C. reinhardtii* at limiting CO$_2$ concentrations. These conclusions beg the question, therefore, of why the expression level of pCA1 is so high if this protein provides only minimal benefit for photosynthesis and growth. It is possible that further work with this *Cah1* null mutant will allow the identification of conditions under which pCA1 provides more substantial benefits.

**LITERATURE CITED**


Miller AG, Colman B (1980) Evidence for HCO$_3^-$ transport by the blue-green alga (Cyanobacterium) _Coccochloris peniocystis_. Plant Physiol 65: 397-402


Sültemeyer DF, Miller AG, Espie GS, Fock HP, Canvin DT (1989) Active CO\textsubscript{2} transport by the green algae \textit{Chlamydomonas reinhardtii}. Plant Physiol \textbf{89}: 1213-1219


**FIGURE LEGENDS**

**Figure 1.** Western and northern blot analysis for wall-less "wild-type" *C. reinhardtii* (CC400 or CC425) and PCA57-61. Extracellular protein and total RNA (10 µg per lane) were isolated after adaptation of cells for 2 d in air. A, pCA1 protein detected with affinity-purified anti-pCA1 polyclonal antiserum. B, *Cah1* mRNA probed with 1.4 kb *BglII* and *Ncol* fragment of *Cah1* cDNA. The total RNA was probed with 25S and 5.8S rRNA. Northern blot was performed on the same membrane, but CC425 and PCA57-61 are selectively shown.

**Figure 2.** Southern blot analysis of wild-type CC425 and PCA57-61. A, Genomic DNA (10 µg per lane) was isolated, digested with different restriction enzymes and hybridized with the full length *Cah1* cDNA. Arrows indicate the band that hybridized both to the full length *Cah1* cDNA and the 1.3 kb *SalI* fragment of the *Arg7* probe. B, Restriction map of the *Cah1* and *Cah2* genomic region (adapted from Fujiwara et al., 1990). Restriction enzyme sites: B, *BglII*; E, *EcoRI*; H, *HindIII*; Hc, *HincII*; K, *KpnI*. The disrupted region is indicated by a bold arrow. Fragment sizes for each enzyme are indicated below the restriction map. Exons are indicated by filled boxes.

**Figure 3.** Spot test for growth response to different pH conditions of PCA57-61, wild-type strains (CC125, CC400) and four high-CO₂-requiring mutants (*cia5, ca1-1, pmp1-1, and pgp1-1*). All plates (except the high CO₂ plate) were kept at air level of CO₂ for 10 d.
Figure 4. Cell growth curve of CC125 (□) and PCA57-61-612 (●) at pH 8 in air levels of CO₂. The growth curves shown are from a single experiment but are representative of 3 independent growth experiments.

Figure 5. Photosynthetic response to NaHCO₃ concentration (pH 7) of wild-type CC125 (□), cia5 (▲) and PCA57-61-612 (●) A, 1 d air-adapted cells were used for all measurements. Chlorophyll concentrations were: CC125, 11.09 µg ml⁻¹; cia5, 10.12 µg ml⁻¹; PCA57-61-612, 9.14 µg ml⁻¹. B, Expansion of the data in A at NaHCO₃ concentrations up to 50 µM. Three independent measurements are averaged from three different cultures. SD is indicated by error bars.

Figure 6. Photosynthetic CO₂ consumption rate of wild-type CC125 (□) and PCA57-61-612 (●) calculated from O₂ evolution assuming 1:1 O₂:CO₂. The calculated maximum spontaneous CO₂ supply rate is indicated by the diagonal line. A, Chlorophyll concentrations were: CC125, 11.09 µg ml⁻¹; PCA57-61-612, 9.14 µg ml⁻¹. Three independent measurements are averaged from three different cultures. B, Chlorophyll concentrations were: CC125, 37.53 µg ml⁻¹; PCA57-61-612, 35.11 µg ml⁻¹. One sample was measured.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC125</td>
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<td>Harris (1989)</td>
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<td>CC2702</td>
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<td>ca1-1 mt&lt;sup&gt;+&lt;/sup&gt;</td>
<td>defective in Cah3, thylakoid lumen CA</td>
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<tr>
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<tr>
<td>CC2648</td>
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<td>deficient in phosphoglycolate phosphatase</td>
<td>Suzuki et al. (1990)</td>
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**Table II.** Genetic analysis of PCA57-61 progeny from a cross with CC1068

<table>
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<th>Strain</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cell Wall</th>
<th>Arg7</th>
<th>Cah1 Insert&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cah1 mRNA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cah1 Polymorphism</th>
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<td>-</td>
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<td>+</td>
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<td>10-1-4</td>
<td>wt, Arg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>-</td>
<td>1</td>
<td>-</td>
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</table>

<sup>a</sup> wt, wild-type (5% CO<sub>2</sub> concentration not required for photoautotrophic growth); Arg<sup>+</sup>, Arg not required for growth; Arg<sup>-</sup>, Arg required for growth.

<sup>b</sup> Arg7 insert detected by Southern analysis using 1.3 kb SalI fragment of Arg7 as probe.

<sup>c</sup> Total RNA was isolated from cells induced with limiting CO<sub>2</sub> for 1 d and probed with 1.4 kb BglII/NcoI fragment of Cah1 cDNA.
Table III. Effect of pH on the photosynthetic rate of wild-type CC125 and PCA57-61-612

<table>
<thead>
<tr>
<th>pH</th>
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<th>75 μM NaHCO₃</th>
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<td></td>
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<td>PCA57-61-612b</td>
</tr>
<tr>
<td>5</td>
<td>42.58 ± 9.24c</td>
<td>69.26 ± 34.16</td>
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<tr>
<td>6</td>
<td>56.88 ± 15.14</td>
<td>79.06 ± 32.08</td>
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<td>50.78 ± 12.40</td>
<td>33.33 ± 16.28</td>
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<td>16.82 ± 6.44</td>
<td>20.87 ± 17.66</td>
</tr>
<tr>
<td>9</td>
<td>8.21 ± 4.15</td>
<td>7.59 ± 7.01</td>
</tr>
</tbody>
</table>

a 1 d air-adapted cells were resuspended in either 25 mM citrate-KOH buffer (pH 5), 25 mM MES-KOH buffer (pH 6), 25 mM MOPS-KOH buffer (pH 7), 25 mM HEPPS-KOH buffer (pH 8), 25 mM AMPSO-KOH buffer (pH 9).

b Chlorophyll concentrations were: CC125, 8-38 μg ml⁻¹; PCA57-61-612, 8-48 μg ml⁻¹.

c Values are the means ± SD of four independent measurements from three different cultures.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
CHAPTER 3. INSERTIONAL MUTANTS OF CHLAMYDOMONAS REINHARDTII THAT REQUIRE ELEVATED CO₂ FOR SURVIVAL

A paper to be submitted to Plant Physiology

Kyujung Van, Yingjun Wang, Yoshiko Nakamura and Martin H. Spalding

ABSTRACT

Aquatic photosynthetic organisms live in quite variable conditions of CO₂ availability. In order to survive in limiting CO₂ conditions, Chlamydomonas reinhardtii and other microalgae show adaptive changes to limiting CO₂ conditions, such as, induction of a CO₂-concentrating mechanism, changes in cell organization, increased photorespiratory enzyme activity, induction of periplasmic carbonic anhydrase and specific polypeptides (mitochondrial carbonic anhydrases and putative chloroplast carrier proteins), and transient down-regulation in the synthesis of Rubisco. The signal for acclimation to limiting CO₂ in C. reinhardtii is unidentified, and it is not known how they sense a change of CO₂ level. The limiting CO₂ signals must be transduced into the changes in gene expression observed during acclimation, so mutational analyses should be helpful for investigating the signal transduction pathway for low CO₂ acclimation.

Eight independently isolated mutants of C. reinhardtii that require high CO₂ for photoautotrophic growth were tested by complementation group analysis. These
mutants are likely to be defective in some aspects of the acclimation to low CO₂, because they differ from wild type in their growth and in the expression patterns of five low CO₂ inducible genes (Cah1, Mca1, Mca2, Ccp1 and Ccp2). Two of the new mutants formed a single complementation group along with the previously described mutant cia-5, which appears to be defective in the signal transduction pathway for low CO₂ acclimation. The other mutations represent six additional, independent complementation groups.

INTRODUCTION

Acclimation to changed environmental conditions is a key to survival for all organisms. In response to perceived environmental signals, organisms may exhibit specific adaptive changes, such as changes in the expression of key genes in order to survive specific environmental changes. Because CO₂ can vary substantially in aquatic habitats and represents the major substrate for photosynthetic CO₂-fixation via the enzyme Rubisco, CO₂ concentration is an important environmental signal in aquatic photosynthetic organisms including cyanobacteria and Chlamydomonas reinhardtii.

Unlike terrestrial higher plants, aquatic photosynthetic organisms can face difficulties in acquiring CO₂. Since the CO₂ diffusion rate in water is much slower than that in air (Badger and Spalding, 2000), the CO₂ supply to Rubisco in these aquatic photosynthetic organisms can become limited. C. reinhardtii and other aquatic photosynthetic organisms have a genetic program to allow them to acclimate
to low CO$_2$. This acclimation includes induction of a CO$_2$-concentrating mechanism (CCM) that allows the cells to acquire CO$_2$ efficiently by increasing the CO$_2$ concentration around Rubisco under limiting CO$_2$ conditions (Badger et al., 1980; for review, see Spalding, 1998). The low CO$_2$ inducible CCM in *C. reinhardtii* has two essential components; active transport and accumulation of inorganic carbon (C$_i$) (Badger et al., 1980; Spalding et al., 1983b) and a thylakoid lumen CA (*Cah3*) to supply CO$_2$ to Rubisco by dehydration of accumulated HCO$_3^-$ (Spalding et al., 1983a; Funke et al., 1997; Karlsson et al., 1998).

Along with the induction of the CCM, *C. reinhardtii* shows adaptive changes to limiting CO$_2$ conditions, such as changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of the proteins pCA1 (*Cah1* gene) (Fujiwara et al., 1990; Fukuzawa et al., 1990; Ishida et al., 1993), mtCA (*Mca1* and *Mca2* genes) (Eriksson et al., 1996; Geraghty and Spalding, 1996), and Ccp (*Ccp1* and *Ccp2* genes) (Geraghty et al., 1990; Ramazanov et al., 1993; Chen et al., 1997), and transient down-regulation in the synthesis of Rubisco (Coleman and Grossman, 1984; Winder et al., 1992).

The signal for acclimation to limiting CO$_2$ in *C. reinhardtii* is unidentified. It is not known how they sense a change of CO$_2$ availability, whether by CO$_2$ concentration directly or indirectly via a cellular process such as carbohydrate metabolism. Acetate utilized by *C. reinhardtii* as a reduced carbon source also acts as a repressor of limiting CO$_2$ acclimation, as indicated by repression of *Cah1* expression (Fett and Coleman, 1994) and much lower affinity for C$_i$ in acetate grown cells compared with air grown cells (Moroney et al., 1987). This raises the possibility
that, like glucose repression in yeast (Gancedo, 1992), a sufficient supply of a preferred carbon source in *C. reinhardtii* may suppress alternative carbon acquisition strategies, such as the CCM.

In contrast to previous reports of a light requirement for pCA1 induction and other limiting-CO$_2$ acclimation responses (Spalding and Ogren, 1982; Spencer et al., 1983; Dionisio et al., 1989a, b; Dionisio-Sese et al., 1990, Borodin et al., 1994), some induction of *Cah1*, *Mca1* and *Mca2* messages have been reported in the dark (Bailly and Coleman, 1988; Fett and Coleman, 1994; Rawat and Moroney, 1995; Fujiwara et al., 1996; Villarejo et al., 1996; Eriksson et al., 1998) although pCA1, mtCA1, mtCA2, Ccp1 and Ccp2 reportedly were induced differentially depending on growth conditions (light vs. dark), cell conditions (synchronized vs. nonsynchronized), and presence of the photorespiratory inhibitor aminooxyacetate (Bailly and Coleman, 1988; Villarejo et al., 1996; Villarejo et al., 1997; Eriksson et al., 1998). These observations suggest that these low CO$_2$ induced genes might be regulated differentially and that multiple signals might be involved.

Whatever the limiting-CO$_2$ signal, it must be transduced into the changes in gene expression observed during acclimation, such as expression of *Cah1*. However, the signal transduction pathway for acclimation to limiting CO$_2$ is uncharacterized. A powerful way to identify components of the CCM and of the signal transduction pathway for low CO$_2$ acclimation is through the analysis and characterization of mutants specifically defective in growth in limiting CO$_2$, like the *cia-5* mutant (Moroney et al., 1989). The *cia-5* mutant exhibits no induction of the low CO$_2$ induced polypeptides and also does not show C$_i$ accumulation, high affinity
for CO₂, up-regulation of PGPase and glycolate dehydrogenase or down-regulation of Rubisco biosynthesis (Moroney et al., 1989; Marek and Spalding, 1991; Spalding et al., 1991; Burow et al., 1996). With the cia-5 mutant as a prototype, identification of similar mutants should help to dissect the CCM and the signal transduction pathway for acclimation to limiting CO₂. Using advances in nuclear transformation of C. reinhardtii (Kindle, 1990), a collection of insertionally generated HCR (high CO₂-requiring) mutants unable to grow in limiting CO₂ was obtained and is described here.

RESULTS

Generation and Isolation of Mutants

Using glass bead transformation (Kindle, 1990; Davies et al., 1994), CC425 (Table I) was complemented by transformation with pArg7.8 (Debuchy et al., 1989) to generate a pool of insertional mutants on CO₂ minimal medium. Cells from each of more than 7000 transformant colonies were suspended in air minimal medium and grown on plates in high CO₂ (5% CO₂ in air), normal air and low CO₂ (50-100 μL L⁻¹ CO₂). HCR mutants, defined as those showing little or no growth either in normal air or in low CO₂, should include mutants, like cia-5, that are defective in acclimation to limiting CO₂, as well as those with functional defects in the CCM. Sixteen putative HCR mutants were identified, and eight of those are described here (Table II).
General Characteristics of HCR mutants

The eight HCR mutants and their general characteristics are shown in Table II and Figure 1. When grown in high CO₂ on agar, all HCR mutants except HCR105 were indistinguishable from wild-type (Fig. 1). The eight HCR mutants could be divided into four groups based on their apparent high CO₂ requirement for photoautotrophic growth. The first group, including HCRP34, HCR209 and HCR90, showed a leaky HCR phenotype in air but a stringent phenotype in low CO₂. The second group, including HCR86 and HCR105, showed a stringent HCR phenotype both in air and in low CO₂. HCR89 and HCR95, comprising the third group, had a leaky HCR phenotype both in air and in low CO₂. HCR3510 lacked a significant growth phenotype in air but had a stringent phenotype in low CO₂.

Genetic Characteristics of HCR mutants

Seven of the eight HCR mutants were found by Southern analysis (data not shown) to contain only one copy of the Arg7 insert, and the presence of vector sequences was confirmed in six mutants (Table II). The presence of vector sequences provides an opportunity for the cloning of sequences flanking the insert by plasmid rescue (Quarmby and Hartzell, 1994).

Selected random progeny and/or tetrads from HCR mutants have been tested in crosses with another arg2 mutant (CC1068, Table I) for linkage of the Arg insert with Arg⁺ and HCR phenotypes (Table II). Five of the eight mutants show co-segregation of the single Arg insert with the HCR phenotype, suggesting that the Arg insert is responsible for the HCR phenotype in these five mutants. In two of the
mutants, HCR95 and HCR105, the inserts did not co-segregate with the HCR phenotype, indicating that insertion of the Arg plasmid was not directly responsible for the HCR phenotype in these two mutants. In HCR209, which has two inserts, co-segregation crosses were not conclusive, but other evidence (see below) suggests the two inserts are tandemly arranged and are responsible for the phenotype.

Heterozygous vegetative diploids, generated in crosses with CC1068 and selected by their resistance to both kanamycin and streptomycin, were used to determine the dominant/recessive nature of the HCR phenotype of each mutant. Based on growth tests of the heterozygous diploids, the mutant phenotype of all eight HCR mutants was judged to be recessive.

**Complementation Group Analysis**

Complementation between recessive nuclear mutations with similar phenotypes is easily assessed in *C. reinhardtii* in vegetative diploid cells, so diploids can be used to establish complementation group among a collection of mutants (Harris, 1989). Crossing with the various known mutants such as, *cia-5, ca-1, pmp-1* and *pgp-1*, should help identify new alleles of previously characterized mutants. If any wild-type colonies appear under low CO2 conditions (50-100 μL L⁻¹ CO₂) after mating with HCR mutants, this indicates they are not allelic to each other, because these known mutants show HCR phenotypes, too.

Both complementation analysis in vegetative diploids and rapid allelism tests were used to place the various HCR mutants into different complementation groups. Complementation analysis was tested with eight HCR mutants along with *cia-5, ca-
1, \textit{pmp-1} and \textit{pgp-1} (Table III). Six new complementation groups were established and \textit{cia-5}, HCRP34 and HCR209 were placed in the same complementation group. In crosses amongst the eight new mutants and four previously described mutants, only crosses between \textit{cia-5} x HCRP34, \textit{cia-5} x HCR209 and HCRP34 x HCR209 failed to generate wild-type colonies. HCRP34 and HCR209 have been confirmed as defective in the same locus as \textit{cia-5} by comparison of the sequence of the DNA flanking the inserts with a cloned \textit{cia-5} gene (Xiang et al., manuscript submitted) and by complementation with a cloned \textit{cia-5} gene (manuscript in preparation).

\textbf{Liquid Growth Experiments}

Growth experiments showed patterns of high CO\textsubscript{2} requirement for photoautotrophic growth consistent with those seen in spot tests (Fig. 1). Active, 1 d air-adapted cells were inoculated into liquid minimal medium with similar starting cell densities (5x10\textsuperscript{4} cells ml\textsuperscript{-1}), grown with no aeration, and the cell densities measured daily at the same time of day for 10 d. HCRP34 and HCR209, judged to be allelic to \textit{cia-5}, grew very similar to \textit{cia-5} in air (Fig. 2A). The growth rates of HCR86 and HCR90 also were only slightly better than that of \textit{cia-5} in air (Fig. 2B), but the growth rates of HCR89 and HCR95 were intermediate between wild-type (ars301; see Table I) and \textit{cia-5} (Fig. 2C). HCR105 was able to grow slightly in air but bleached within a few days (Fig. 2B). HCR3510, which showed a wild-type phenotype in air on agar, also grew as well as wild-type (ars301) in air in liquid culture (Fig. 2C). Chlorophyll content also was measured in these cultures along with cell density, and
the growth curves based on chlorophyll content showed the same pattern as those of cell density (data not shown).

**Accumulation of Low CO₂ Inducible Transcripts**

Since the expression of low CO₂ inducible polypeptides (pCA1, mtCA1, mtCA2, Ccp1 and Ccp2) has been reported to change differentially during acclimation to limiting CO₂ (Villarejo et al., 1996; Villarejo et al., 1997; Eriksson et al., 1998), accumulation of these three transcripts also was analyzed. The *cia*-5-like mutants, HCRP34 and HCR209, showed no detectable *Cah1* mRNA, *Mca1* and *Mca2* mRNA and *Ccp1* and *Ccp2* mRNA (Fig. 3A; data shown only for HCRP34). HCR90, which showed a leaky HCR phenotype in air but a stringent phenotype in low CO₂, had reduced expression only of *Mca1* and *Mca2* mRNA (Fig. 3B). In separate, long-term experiments, the expression of the other genes was somewhat variable, but only *Mca1* and *Mca2* showed reproducibly decreased message abundance (data not shown). HCR3510, which showed a wild-type phenotype in air but a stringent HCR phenotype in low CO₂, had normal expression of these genes compared with wild-type (CC849; see Table I) (Fig. 3A). However, HCR95 showed a much different pattern of expression for these three genes. From cells exposed for 2 h to air, *Cah1* mRNA of HCR95 was detected at normal levels, whereas much reduced levels of *Mca1* and *Mca2* mRNA and *Ccp1* and *Ccp2* mRNA were detected relative to wild type (Fig. 3B). After 6 h, wild type showed the same or increased levels of these three mRNAs, but expression of all three mRNA in HCR95 was dramatically reduced (Fig. 3B), suggesting only a transient induction of their
expression in this mutant. In separate, long-term experiments, this apparent transient induction in HCR95 also was confirmed up to 24 h (data not shown). The other HCR mutants (HCR86, HCR89, HCR90 and HCR105) did not show reproducibly different patterns of expression for the three low CO₂ inducible transcripts relative to wild type (data not shown).

**DISCUSSION**

HCR mutants have been useful for investigation of various processes, both in algae and in higher plants. HCR mutants with defects in several of the enzymes of the photorespiratory pathway have been isolated in the C₃ plants *Arabidopsis thaliana* (Somerville and Ogren, 1982) and barley (Joy et al., 1992; Leegood et al., 1996; Wingler et al., 1999). These photorespiratory mutants exhibited lethality (HCR phenotype) in air levels of CO₂ for various reasons, including accumulation of toxic intermediates during photorespiration and depletion of exchangeable nitrogen in photorespiratory intermediates. In *C. reinhardtii*, the photorespiratory mutant *pgp-1* (lacks PGPase), has a HCR phenotype, indicating that the oxygenase activity of Rubisco was not completely suppressed by operation of the CCM and that photorespiratory mutants in *C. reinhardtii* also are lethal in air levels of CO₂ (Suzuki et al., 1990; Spalding, 1998).

Mutants defective in functional components of the CCM also exhibit a HCR phenotype in *C. reinhardtii* (Spalding et al., 1983a; Spalding et al., 1983b; Moroney et al., 1986; Suzuki and Spalding, 1989; Funke et al., 1997; Karlsson et al., 1998)
and cyanobacteria (Price and Badger, 1989; Ogawa, 1991; Ogawa, 1992; Marco et al., 1993; Ohkawa et al., 1998; Price et al., 1998). Isolation and characterization of the C. reinhardtii mutants, ca-1 and pmp-1, demonstrated the requirement for active transport and accumulation of Ci (Badger et al., 1980; Spalding et al., 1983b) and for a thylakoid lumen CA (Spalding et al., 1983a; Funke et al., 1997; Karlsson et al., 1998) for function of the CCM. Another C. reinhardtii HCR mutant, cia-5, exhibits no apparent low CO₂ acclimation responses, such as induction of CCM, up-regulation of low CO₂ inducible polypeptides, up-regulation of photorespiratory enzymes or down-regulation of Rubisco biosynthesis, (Moroney et al., 1989; Marek and Spalding, 1991; Spalding et al., 1991; Burow et al., 1996). This mutant is thought to be defective in the signal transduction pathway for acclimation to limiting CO₂.

Since many changes involved in acclimation to limiting CO₂ conditions appear to be controlled at different gene expression levels, it is possible that mutations in several different loci might yield signal transduction mutants like cia-5 with HCR phenotypes. Thus, the HCR phenotype should be a good indicator of nonacclimation to low CO₂ as well as for a dysfunctional CCM, so isolation of HCR mutants should be helpful for identification of loci required for either function of the CCM or for signal transduction leading to low CO₂ acclimation.

Among the eight new HCR mutants described here, six represent new complementation groups and the other two represent new alleles of the previously described cia-5 locus. The patterns of growth and of low CO₂ inducible transcript accumulation for HCRP34 and HCR209 were similar to those of cia-5, and complementation group analyses confirmed that the three are allelic (Table III). As
new alleles of *cia-5*, HCRP34 and HCR209 should prove valuable in understanding the function of the gene product from this important locus.

Other than for HCRP34 and HCR209, the growth responses to air and low CO$_2$ varied among these new HCR mutants, as did the pattern of accumulation of limiting-CO$_2$ inducible genes. HCR90, which showed a stringent HCR phenotype in low CO$_2$ and grew only slightly better than *cia-5* in air (Fig. 2B), had reproducibly reduced expression of only one pair of the limiting-CO$_2$ inducible transcripts, *Mca1* and *Mca2*. No disruption of the structural gene for either *Mca1* or *Mca2* was found in genomic Southern blots probed with the *Mca1* and *Mca2* promoter region (data not shown), so HCR90 may be defective in a regulatory component that preferentially affects expression of *Mca1* and *Mca2*. HCR86, which has a growth phenotype very similar to HCR90, showed limiting-CO$_2$ inducible transcripts accumulations that were not reproducibly different from those of CC849 (data not shown). The leaky phenotype in low CO$_2$ of HCR89 and HCR95 was supported by their growth patterns (Fig. 2C), but only HCR95 reproducibly showed reduced level of low CO$_2$ inducible transcripts (Fig. 3B).

Originally, HCR86, HCR90 and HCR95 were identified as putative signal transduction mutants based on immunochemical screens for reduced level of pCA1 and western blot analyses of the expression levels of four other low CO$_2$ inducible polypeptides (mtCA1, mtCA2, Ccp1 and Ccp2) (data not shown). However, it is difficult to conclude that HCR86, HCR90 and HCR95 are signal transduction mutants based on low CO$_2$ inducible transcript accumulation. Cloning of the disrupted genes in HCR86, HCR90 and HCR95 would help to clarify these
discrepancies and to demonstrate the defective functions responsible for their HCR phenotypes.

The defect in HCR89 has been identified as the disruption of a novel glycolate dehydrogenase (Nakamura et al., manuscript in preparation) putatively involved in the photorespiratory glycolate pathway of *C. reinhardtii* (for review see Spalding, 1998). Thus, this mutant represents the second identified photorespiratory mutant in *C. reinhardtii*, so it apparently is defective neither in a functional component of the CCM nor in the signal transduction pathway for adaptation to limiting CO$_2$.

HCR3510 showed no significant differences from wild type in terms of low CO$_2$ inducible transcript accumulation, suggesting it is unlikely to be defective in the limiting-CO$_2$ responsive signal transduction pathway. The growth phenotype of this mutant, near wild-type growth in normal air but a stringent phenotype in low CO$_2$, suggests a defect in a functional component of the CCM (or another pathway required for acclimation to limiting CO$_2$) that is essential in very low CO$_2$ but not in air levels of CO$_2$.

The advantage of using insertional mutagenesis to generate mutants lies in the use of the inserted DNA as a "tag" to clone the disrupted gene, but, of course, this only works if the insert co-segregates with the mutant phenotype, i.e., if the insert is responsible for the mutation. As judged by the Arg$^+$ phenotype, the Arg7 inserts in mutants HCRP34, HCR3510, HCR86, HCR89 and HCR90 co-segregate with the HCR phenotype (Table II), suggesting the insert caused the mutation in each of these strains. As indicated above, both HCRP34 and HCR209 are allelic to *cia*-5 and the insert in each has been confirmed to disrupt the *cia*-5 gene. Thus we
know the defect in both these mutants, even though co-segregation of the Arg$^+$ and HCR phenotypes has not been demonstrated for HCR209. As indicated above, the insertional tag in HCR89 was used to identify the disruption of a glycolate dehydrogenase gene by the Arg7 insert in this mutant (Nakamura et al., manuscript in preparation).

Unfortunately, the inserts in mutants HCR95 and HCR105 do not co-segregate with the HCR phenotype, so identification of the disrupted gene responsible for the HCR phenotype in these mutants will have to be accomplished without the aid of an insertional tag. The three remaining tagged mutants (HCR3510, HCR86 and HCR 90) remain as viable candidates for identification of novel genes essential for acclimation of *C. reinhardtii* to limiting CO$_2$. Cloning of the disrupted genes in these three HCR mutants is in progress.

**MATERIALS AND METHODS**

**Cell Strains and Culture Conditions**

All *C. reinhardtii* strains (Table I) were grown as previously described (Geraghty et al., 1990). Cells were cultured on an orbital shaker under aeration with 5% CO$_2$ in air (high CO$_2$-grown cells) or no aeration (air-adapted cells). For experiments monitoring the accumulation of low CO$_2$ inducible transcripts, cell cultures were switched from aeration with 5% CO$_2$ to aeration with normal air for 2 h to 6 h. For growth on solid media, cells were maintained under 5% CO$_2$ in air (high CO$_2$), normal air or 50-100 µL L$^{-1}$ CO$_2$ (low CO$_2$).
Generation and Isolation of Mutants

Glass bead transformations were performed as described previously (Van and Spalding, 1999). To generate a pool of insertional mutants on CO2 minimal medium, CC425 (Table I) was transformed with linearized pArg7.8 (Debuchy et al., 1989) containing the structural gene (Arg7) for argininosuccinate lyase to complement the arg2 mutation. Each of more than 7000 colonies was screened by spot tests to identify high CO2 requiring (HCR) mutants. After replica plates with transformants were made, each plate was placed in high CO2 and air or high CO2 and low CO2. Mutants identified in this primary screen as having HCR phenotype were screened again by western immunoblots of extracellular protein to identify mutants in which pCA1 expression was decreased or absent (Van and Spalding, 1999).

Spot Growth Tests and Growth Experiments

For spot growth tests, actively growing cells were suspended to similar cell densities in minimal medium, spotted (10 µl) onto minimal agar plates and grown in different concentrations of CO2 for 10 d (Harris, 1989).

For liquid growth experiments, active, 1 d air-adapted cells were inoculated into liquid minimal medium at similar cell densities (5x10^4 cells ml^-1). The cultures were grown on an orbital shaker without aeration for the next 10 d. The cell density was determined using a hemacytometer (Reichert Scientific Instruments, Buffalo,
NY) (Harris, 1989). Chlorophyll content was estimated after extraction with 96% (v/v) ethanol (Wintermans and De Mots, 1965).

**DNA and RNA Blot Analysis**

Southern and northern blot analyses were performed as described by Van and Spalding (1999), with little revision. Total RNA was purified with TRizol reagent (Life Technologies, Gaithersburg, MD) from air-induced cells exposed to limiting CO₂ (aeration with normal air) and Hybond-N⁺ nylon transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used for blotting. After phosphorimager analysis of each northern blot (Molecular Dynamics, Piscataway, NJ), total RNA amounts were normalized to hybridization with 25S and 5.8S rRNA (Marco and Rochaix, 1980) using ImageQuaNT (Molecular Dynamics).

**Genetic Analyses**

All matings were performed by crossing insertionally generated mutants with various strains (Table I) according to the protocol of Harris (1989). To isolate vegetative diploids, gametes from HCR mutants (sr-u-2-60) and CC1068 (nr-u-2-1) were induced under nitrogen stress, mated and the mating mixture spread onto kanamycin containing medium to select for expression of the plastid-encoded kanamycin resistance (nr-u-2-1) transmitted from the mating-type minus parent. Putative diploids (surviving colonies) were verified by selection for simultaneous expression of the plastid-encoded streptomycin resistance (sr-u-2-60) from the
mating-type plus parent and by DNA quantity in flow cytometry (performed at the Iowa State University Cell Facility).

Complementation group analyses required construction of mating type minus strains of each HCR mutant (both new and previously described mutants). Mating type minus strains of *cia-5, ca-1, pmp-1* and *pgp-1* were generated by crossing with CC124 (Table I). CC1068 (Table I) was used for generating mating type minus strains from all new HCR mutants, except HCR95 and HCR105. After crossing each of the seven new HCR mutants and the four known mutants with each other, the progeny from each cross were tested for photoautotrophic growth in low CO$_2$ (50-100 µL L$^{-1}$). Since all HCR mutants required elevated CO$_2$ for survival, wild-type colonies were observed in low CO$_2$ only if the cross generated wild-type recombinant progeny and/or complementation in vegetative diploids.

**LITERATURE CITED**


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Geraghty AM, Anderson JC, Spalding MH (1990) A 36 kilodalton limiting-CO$_2$ induced polypeptide of *Chlamydomonas* is distinct from the 37 kilodalton periplasmic carbonic anhydrase. Plant Physiol 93: 116-121


**FIGURE LEGENDS**

Figure 1. Spot tests for growth response to different CO$_2$ concentrations for wild-type strains (CC849, ars301), four previously described HCR mutants (cia-5, ca-1, pmp-1, pgp-1) and eight new HCR mutants. Plates were kept either at high CO$_2$ (5% CO$_2$), at air level of CO$_2$ or at low CO$_2$ (50-100 μL L$^{-1}$) for 10 d.

Figure 2. Liquid cell growth curves for wild type (ars301), cia-5 and HCR mutants grown at pH 7 on an orbital shaker without aeration. A, HCRP34 and HCR209. B, HCR86, HCR90 and HCR105. C, HCR89, HCR95 and HCR3510. The growth curves shown are averages of three independent growth experiments.

Figure 3. Northern blot analyses for wild-type (CC849) and HCR mutants. A, HCRP34 and HCR3510. B, HCR90 and HCR95. Total RNA (10 μg per lane) was isolated 2 h to 6 h after transfer of cells to air levels of CO$_2$ from high CO$_2$. Cah1 mRNA was probed with the 1.4 kb BgIII and Ncol fragment of Cah1 cDNA (Van and Spalding, 1999). Mca1 and Mca2 mRNA was probed with the full length Mca2 cDNA (Eriksson et al., 1996; Eriksson et al., 1998). Ccp1 and Ccp2 mRNA was probed with the 1.2 kb EcoRI and HindIII fragment of Ccp1 G1 (Chen et al., 1997). The rRNA was probed with 25S and 5.8S rDNA (Marco and Rochaix, 1980).
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<td>ars301</td>
<td>cw15 sr-u-2-60 mt⁺</td>
<td>generated by transformation of CC425 with Arg7; used as wild-type in liquid growth experiments</td>
<td>Davies, JP (personal communication)</td>
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<td>CC124</td>
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<td>wild-type (137C)</td>
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<td>arg2 cw15 sr-u-2-60 mt⁺</td>
<td>cell wall-less and Arg-requiring and streptomycin resistant mutant</td>
<td>Harris (1989)</td>
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<td>spr1-1 mt⁻</td>
<td>spectinomycin resistant mutant</td>
<td>Harris (1989)</td>
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<td>cell wall-less mutant</td>
<td>Harris (1989)</td>
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<td>Arg-requiring and kanamycin resistant mutant</td>
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<td></td>
<td>ca1-1 mt$^-$</td>
<td>generated by CC1219 x CC801</td>
<td>Suzuki, K (personal communication)</td>
</tr>
<tr>
<td>CC1860</td>
<td>pmp1-1 mt$^+$</td>
<td>deficient in C$_i$ transport</td>
<td>Spalding et al. (1983b)</td>
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<td>pmp1-1 mt$^-$</td>
<td>generated by CC1860 x CC124</td>
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<td>CC2648</td>
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<td>deficient in phosphoglycolate phosphatase</td>
<td>Suzuki et al. (1990)</td>
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<td>HCRP34 mt$^-$</td>
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<td></td>
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<td>generated by HCR209 x CC1068</td>
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<td></td>
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<td>HCR89 mt$^-$</td>
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<td></td>
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Table II. Characteristics of HCR mutants

<table>
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<tr>
<th>Mutants</th>
<th>High CO₂ requirement&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Arg7</th>
<th>Vector sequence</th>
<th>Co-segregation with</th>
<th>Diploid</th>
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<td>Air</td>
<td>Low CO₂</td>
<td>Insert&lt;sup&gt;b&lt;/sup&gt;</td>
<td>present</td>
<td>Arg&lt;sup&gt;+&lt;/sup&gt; phenotype and Arg7 insert</td>
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<tr>
<td>cia-5</td>
<td>leaky&lt;sup&gt;c&lt;/sup&gt;</td>
<td>stringent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>NA</td>
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<td>stringent</td>
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<td>+&lt;sup&gt;o&lt;/sup&gt;</td>
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<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
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<td>1</td>
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<sup>a</sup> Growth phenotype in low CO₂ was determined by spot test on agar in high CO₂ (5% CO₂ in air) versus either air (300-500 μL L⁻¹ CO₂) or low CO₂ (50-100 μL L⁻¹ CO₂).

<sup>b</sup> Arg7 insert detected by Southern analysis using 1.3 kb SalI fragment of Arg7 as probe.

<sup>c</sup> Leaky, cells grow very slowly; stringent, cells did not grow at all.

<sup>d</sup> NA = not applicable.

<sup>e</sup> +/- indicate the presence (+) or absence (-) of vector sequence.

<sup>f</sup> ND = not determined.
### Table III. Results of complementation analysis

<table>
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<tr>
<th></th>
<th>ca-1</th>
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<th>HCR86</th>
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\( ^a \) +/- indicate exhibition of complementation as judged by the presence (+) or absence (-) of wild-type colonies.
Figure 1.
Figure 2.
Figure 3.
<table>
<thead>
<tr>
<th>B</th>
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<th>HCR90</th>
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<td>Cah1</td>
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<td></td>
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<td>Mca1 &amp; Mca2</td>
<td></td>
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<td>Ccp1 &amp; Ccp2</td>
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Figure 3.
Aquatic photosynthetic organisms live in quite variable conditions of CO$_2$ availability. In order to survive in limiting CO$_2$ conditions, *Chlamydomonas reinhardtii* shows adaptive changes to limiting CO$_2$ conditions, including induction of a CO$_2$-concentrating mechanism (CCM), changes in cell organization, increased photorespiratory enzyme activity, induction of at least five specific genes (*Cah1, Mca1, Mca2, Ccp1, Ccp2*), and transient down-regulation in the synthesis of Rubisco. We are trying to identify mutants defective in the acclimation response to limiting CO$_2$. Nuclear transformation to complement an Arg$^+$ phenotype was used to generate insertional mutants, and *Cah1* expression and/or high CO$_2$-requiring phenotype (HCR) was used as a reporter for induction of genes involved in acclimation to limiting CO$_2$. From approximately 7000 transformants screened for lack of *Cah1* expression, HCR90 was identified. This mutant shows a recessive HCR phenotype that co-segregates with the Arg$^+$ phenotype and presence of 1 Arg7 insert. HCR90 does not show any allelism to the known HCR mutants, *cia-5, ca-1, pmp-1* or *pgp-1*. The K$_m$(C$_i$) of HCR90 (64 μM) was found to be higher than that of wild type (11 μM), suggesting HCR90 lacks a functional CCM. After 5' and 3' sequences flanking the Arg insert were obtained, genomic clones from a C.
*reinhardtii* BAC library were identified by using flanking DNA from both sides of the insert as probes. The 5' and 3' flanking sequences identified non-overlapping sets of BACs, indicating a rather large disruption. Since HCR90 grows poorly in low CO₂ (50-100 μL L⁻¹ CO₂), complementation of the mutation should be possible under direct selection in low CO₂, but complementation experiments were unsuccessful with each of six different BAC clones. Transformation with a cDNA expression plasmid library may represent an alternative for complementing HCR90. These cDNA complementation experiments are in progress.

**INTRODUCTION**

Like C₄ plants, *C. reinhardtii* and many other microalgae and cyanobacteria have an active CO₂-concentrating mechanism (CCM) that allows cells to assimilate inorganic carbon efficiently when they grow under limiting CO₂ conditions. The CCM results in an increased internal CO₂ concentration, which increases the substrate for Rubisco carboxylation and favors the carboxylation reaction of Rubisco over the oxygenation reaction (Badger et al., 1980; Aizawa and Miyachi, 1986; Spalding, 1998). This CCM is inducible after transfer from high (5% CO₂ in air) to limiting CO₂ (0.03% CO₂ in air) and requires at least two components, including active Cᵢ transport (Badger et al., 1980; Spalding et al., 1983b) and a thylakoid carbonic anhydrase (CA) (*Cah₃*) to supply CO₂ for Rubisco by dehydration of HCO₃⁻ (Spalding et al., 1983a; Funke et al., 1997; Karlsson et al., 1998).
Along with induction of the CCM, *C. reinhardtii* exhibits a range of adaptive changes to limiting CO\(_2\) conditions, including changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of the proteins pCA1 (*Cah1*) (Fujiwara et al., 1990; Fukuzawa et al., 1990; Ishida et al., 1993), mtCA (*Mca1* and *Mca2*) (Eriksson et al., 1996; Geraghty and Spalding, 1996), and Ccp (*Ccp1* and *Ccp2*) (Geraghty et al., 1990; Ramazanov et al., 1993; Chen et al., 1997), and transient down-regulation in the synthesis of Rubisco (Coleman and Grossman, 1984; Winder et al., 1992).

The signal for limiting CO\(_2\) must be transduced into the changes in gene expression observed during acclimation, such as expression of *Cah1*, but neither the signal for acclimation to limiting CO\(_2\) in *C. reinhardtii* nor the pathway for transduction of this signal to changes in gene expression is identified. Since reduced O\(_2\) levels or low O\(_2\)/CO\(_2\) concentration ratio decreased the induction (or derepression) of CA in *C. reinhardtii*, the signal for limiting CO\(_2\) condition may be a photorespiratory metabolite (Spalding and Ogren, 1982). If so, the differential effect of the glyoxylate-serine aminotransferase inhibitor, aminooxyacetate, on induction of pCA1, mtCA1, mtCA2, Ccp1 and Ccp2 (Ramazanov and Cardenas, 1992; Villarejo et al., 1997) may suggest different signals are involved in the induction of these three polypeptides. Both light for photosynthesis and non-photosynthetic blue light might also be involved in the upregulation of pCA1 expression (Spalding and Ogren, 1982; Spencer et al., 1983; Dionisio et al., 1989a, b; Dionisio-Sese et al., 1990; Borodin et al., 1994). In contrast to these apparent light requirements for pCA1 induction, some induction of *Cah1*, *Mca1* and *Mca2* messages and pCA1, mtCA1,
mtCA2, Ccp1 and Ccp2 reportedly occurred in the dark but were expressed differently depending on growth conditions (synchronized vs. nonsynchronized) (Bailly and Coleman, 1988; Villarejo et al., 1996; Eriksson et al., 1998). Once again, these observations suggest the three limiting CO₂ induced polypeptides might be regulated differentially. Like glucose repression in yeast, acetate utilized by \textit{C. reinhardtii} as a reduced carbon source acts as a repressor of limiting CO₂ acclimation (Fett and Coleman, 1994; Moroney et al., 1987).

Mutational analysis should be helpful for identifying the signals and dissecting the signal transduction pathway for low CO₂ acclimation. The \textit{cia-5} mutant (Moroney et al., 1989) does not show any response to limiting CO₂, so serves as an example for identification of other mutants defective in the signal transduction pathway for limiting CO₂ acclimation. Using advances in nuclear transformation of \textit{C. reinhardtii} (Kindle, 1990), a collection of insertionally generated mutants putatively defective in CO₂ signal transduction was obtained (Van & Spalding, 1999; Van, Chapter 3 this thesis). Here, we describe a high CO₂-requiring (HCR) mutant (HCR90) with deficient responses to limiting CO₂ conditions.

**MATERIALS AND METHODS**

**Cell Strains, Culture Conditions and Mating**

All \textit{C. reinhardtii} strains were grown as previously described (Geraghty et al., 1990). Cells were cultured on an orbital shaker under aeration with 5% CO₂ in air (high CO₂-grown cells) or with air (air-adapted cells). Cell cultures were switched
from elevated CO₂ to air for 1 d or 2 d induction. For solid media, cells were
maintained under 5% CO₂ in air (high CO₂), air or 50-100 μL L⁻¹ CO₂ (low CO₂). All
matings were performed by crossing insertionally generated mutants with various
strains according to the protocol of Harris (1989).

**Generation and Isolation of Mutants**

Glass bead transformations were performed as described previously (Van
and Spalding, 1999). To generate a pool of insertional mutants on CO₂ minimal
medium, CC425 (*arg2 cw15 sr-u-2-60 mt*) was transformed with linearized pArg7.8
(Debuchy et al., 1989) containing the structural gene (*Arg7*) for argininosuccinate
lyase to complement the *arg2* mutation. Each of more than 7000 colonies was
screened by spot tests for identifying low CO₂ nonacclimating mutants. After replica
plating the transformants, each plate was grown in high CO₂ and low CO₂. Mutants
identified in this primary screen as having high CO₂-requiring (HCR) phenotype were
screened again by western immunoblots following SDS-PAGE of extracellular
protein.

**Protein, DNA and RNA Blot Analysis**

Protein extraction and protein blot analysis were performed as described
previously (Van & Spalding, 1999). Southern and northern blot analyses also were
performed as described by Van and Spalding (1999), except that total RNA was
purified with TRIzol reagent (Life Technologies, Gaithersburg, MD) from 1 d limiting
CO₂-induced cells and Hybond-N⁺ nylon transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used for blotting.

**Growth Tests and Photosynthetic O₂ Exchange**

For spot growth tests, actively growing cells were suspended to similar cell densities in minimal medium and spotted (10 μl) onto agar plates for growth with different concentrations of CO₂ for 5 d (Harris, 1989).

For O₂-exchange measurements, 1 d air-induced cells were harvested by centrifugation and resuspended in 25 mM Mops-KOH (pH 7) for analysis of the photosynthetic response to CO₂ concentration, which was measured as described by Van and Spalding (1999).

**Construction and Screening of Genomic Libraries**

Using CsCl-purified genomic DNA of the HCR90 mutant (Sambrook et al., 1989), both BamHI and EcoRI complete digestion genomic libraries of the mutant were constructed in λ DASH II (Stratagene, La Jolla, CA). Plaque lifts were made onto Hybond-N⁺ nylon transfer membrane (Amersham Pharmacia Biotech Inc.), hybridized with appropriate ³²P-labeled probes (Random Primers DNA Labeling System, Life Technologies) and washed at high stringency (Ausubel et al., 1989). After three rounds of screening, phage DNA was purified from a positive plaque (Sambrook et al., 1989). Subcloned DNA flanking regions in pBluescript II SK⁺ (Stratagene) were sequenced at Iowa State University DNA Sequencing and Synthesis Facility.
The *Chlamydomonas* genomic BAC library filter and positive BAC clones were obtained from Genome Systems Inc. (St. Louis, MO). Hybridization and washing methods were performed according to protocols from the company.

**Construction of a cDNA Expression Plasmid Library and Complementation Experiments**

A cDNA expression library was constructed in the vector pJD405, containing the promoter, terminator, two exons and two introns from *rbcS2*. The library was constructed with *C. reinhardtii* poly (A+) RNA combined from cells grown in TAP, TAP+dark, high salt (HS) and HS+CO$_2$ conditions (Sueoka, 1960; Gorman and Levine, 1966; Fig. 1). Following the addition of EcoRI adaptors and NotI digestion of the synthesized cDNAs, they were inserted into EcoRI/NotI sites of the pJD405 vector (Fig. 1B) to construct a library. After isolation of the expression plasmid (Qiagen Plasmid Maxi-Prep Kit; Qiagen, Valencia, CA), plasmids linearized with Swal (New England Biolabs, Beverly, MA) were used for complementation experiments. Complementation experiments, using either purified lambda DNA, BAC DNA or linearized plasmid DNA, were performed basically by the same method as that used to generate insertional mutants. Selection for complements was on plates in low CO$_2$ (50-100 µL L$^{-1}$).
RESULTS

Generation and Isolation of Mutants

We are trying to identify components of this signal transduction pathway through analysis of mutants defective in the acclimation response to limiting CO₂. Using insertional mutagenesis, we generated a pool of more than 7000 transformants by complementation of an Arg-requiring mutant (CC425) with the Arg7 gene (Van & Spalding, 1999; Van, Chapter 3 this thesis). To identify mutants failing to acclimate to limiting CO₂, this pool was screened immunochemically for the absence of pCA1 (encoded by Cah1), used as a reporter for acclimation to limiting CO₂, and also screened for high CO₂-requiring (HCR) mutants unable to acclimate to limiting CO₂. HCR90 was identified among this collection of insertionally generated mutants as showing both a HCR phenotype (Fig. 2) and decreased pCA1 abundance (data not shown). Low CO₂ growth was determined by spot tests on agar in high CO₂ (5 % CO₂ in air) versus air or low CO₂ (50 - 100 μL L⁻¹ CO₂). HCR90 grew poorly or not at all in low CO₂ (Fig. 2) and poorly in air (data not shown). In preliminary screen, HCR90 also was found to have reduced levels of mtCA1 and mtCA2, but Ccp1 and Ccp2 were undetectable (data not shown). After normalization by 25S and 5.8S rRNAs, only levels of Mca1 and Mca2 mRNAs were found to be reduced in HCR90 (Fig. 3). This reduced expression was reproducible in separate, short-term experiments (data not shown).
Genomic Analysis of HCR90

When HCR90 genomic DNA was compared with that of CC425 by Southern analysis, the presence of one insert with an intact vector sequence was confirmed (data not shown). The HCR phenotype of HCR90 co-segregates with the insert and the Arg\(^{+}\) phenotype (Table I). Vegetative diploids, generated in the cross with CC1068 and selected as described previously (Chapter 3 this thesis), demonstrated that the phenotype of HCR90 is recessive. Crosses with previously described HCR mutants such as cia-5 (Moroney et al., 1989), ca-1 (Spalding et al., 1983a), pmp-1 (Spalding et al., 1983b) and pgp-1 (Suzuki et al., 1990) demonstrated that HCR90 is not allelic to any of the known HCR mutants. Thus, a single Arg\(^{7}\) insert is responsible for a novel and recessive HCR mutation in HCR90.

Photosynthetic Characterizations for a HCR90

The presence of a functional CCM of this mutant was evaluated by measuring the photosynthetic rate at various CO\(_2\) concentrations. HCR90-4-1 (Table I) was chosen for physiological analysis because it has the same biochemical phenotype as HCR90 but has a normal cell wall. The K\(_{m}(C_i)\) of HCR90-4-1 (64.42 \(\mu\)M) was substantially higher than that of wild type (11.16 \(\mu\)M) and similar to that of cia5 (113.12 \(\mu\)M) (Fig. 4), indicating that HCR90 lacks a functional CCM.

Cloning of Disrupted Genes and Complementation Experiments

In order to identify the disrupted gene responsible for the HCR phenotype in HCR90, the sequences flanking the inserted DNA were first cloned by screening
BamHI and EcoRI λ DASH II (Stratagene) genomic libraries of HCR90 using Arg7.8 vector fragments (2.2 kb of HinClI and SaI and/or 340 bp of BamHI and HindIII) as probes. A 14.3 kb λ clone (flanking 3' end of insert) from the EcoRI library and a 13.9 kb λ clone (flanking 5' end of insert) from the BamHI library were identified, and the restriction map of the genomic region of HCR90, represented by these λ clones, is shown by the second bar in Figure 5. Fragments of 1.9 kb (3') and 0.9 kb (5') from both flanking regions were obtained from these clones as BamHI/EcoRI fragments subcloned in pBluescript SK+. Southern blots of HCR90 probed with the subcloned flanking regions showed polymorphisms relative to wild type (data not shown). Using the 1.9 Kb Stul/EcoRl probe, four different positive clones were identified in screens of Sau3A partial-digest λ DASH II genomic libraries (provided by Dr. Yoshiko Nakamura and Jeff McDermott).

Since HCR90 grows poorly in low CO₂ (50-100 μL L⁻¹ CO₂), complementation of the mutation should be possible under direct selection in low CO₂. Complementation experiments were performed with four different positive λ clones (data not shown) along with positive and negative controls. Complementation of mutations was not achieved with any of these four λ clones.

Since large deletions or gene rearrangement at the site of insert integration can occur by insertional mutagenesis (Tam and Lefebvre, 1993; Van and Spalding, 1999), both flanking DNAs were used as probes to identify genomic clones from a BAC library (constructed by Dr. Lefebvre, Genome Systems, Inc.) with insert sizes in the range of 50 to over 240 kb. Six different BAC clones were identified (Fig. 5), but
none of these BAC clones hybridized to both flanking regions, indicating very large rearrangement of genomic DNA in HCR90. Also, none of these six BAC clones complemented HCR90 (Fig. 5). Finding overlapping BAC clones may be the key to complement for the observed HCR90 phenotype. So, the second round of BAC library screens was performed with sequences from 26j05, 34j10, 39c13 and 36j09 ends. Again, no overlapping BAC clones found to be hybridized to these ends sequences. These BAC clones have not been used for complementation experiments.

As an alternative strategy, a cDNA expression plasmid library has been constructed in pJD405 (Fig. 1) for use in cloning the disrupted gene in HCR90 by complementation of its HCR phenotype. Complementation experiments with selection in low CO₂ have been unsuccessful so far. If a complemented transformant is confirmed by northern and Southern analyses, sequences of cDNA can be obtained easily by PCR because regions of two primers that are not compatible with other regions of genomic DNA in *C. reinhardtii* flank the cDNA inserts in pJD405.

**DISCUSSION**

Since CO₂ availability is a major challenge to aquatic photosynthetic organisms like *C. reinhardtii*, they have developed special genetic programs to acclimate to limiting CO₂. Cells need to recognize they are in limiting CO₂ via some
signal(s) and transmit the signals through a signal transduction pathway to activate adaptive changes, such as induction of the CCM, derepression or induction of low CO₂ inducible polypeptides, etc. However, the signal for acclimation to limiting CO₂ in *C. reinhardtii* is unidentified. It is not known how they sense a change of CO₂ level, whether by CO₂ concentration directly or by an indirect cellular process such as metabolism.

The limiting-CO₂ signal must be transduced into the changes in gene expression observed during acclimation, such as expression of *Cah1*. During low CO₂ acclimation in *C. reinhardtii*, *Cah1*, *Mca1*, *Mca2*, *Ccp1* and *Ccp2* mRNA abundance are up-regulated at the level of transcript abundance (Dionisio-Sese et al., 1990; Fujiwara et al., 1990; Geraghty et al., 1990; Spalding et al., 1991; Eriksson et al., 1996, 1998; Chen et al., 1997), whereas expression of *rbcL*, *RbcS1* and *Rbcs2* are down-regulated at the translational level (Winder et al., 1992). Up-regulation of photorespiratory enzymes, glycolate dehydrogenase (long term) and phosphoglycolate phosphatase (transient) also are observed (Marek and Spalding, 1991).

One way to identify the signals and components of the signal transduction pathway for low CO₂ acclimation is through analysis and characterization of mutants defective in limiting-CO₂ acclimation, like the *cia*-5 mutant generated by UV mutagenesis (Moroney et al., 1989). The *cia*-5 mutant does not have any of the low CO₂ induced polypeptides and also does not show Cᵢ accumulation, high affinity for CO₂, upregulation of PGPase and glycolate dehydrogenase or downregulation of Rubisco biosynthesis (Moroney et al., 1989; Marek and Spalding, 1991; Spalding et
al., 1991; Burow et al., 1996). The cia-5 mutant can be used as an example for identification of other mutants defective in the signal transduction pathway for low CO₂ acclimation. Cloning of the defective gene in cia-5 would be helpful for dissecting components of this signal transduction pathway. Recently, the Cia-5 gene was cloned and tentatively identified as a transcription factor (Xiang et al., manuscript submitted). The Cia-5 protein has two potential zinc finger domains and many potential sites for posttranslational modifications. Since the cia-5 mutant does not show any adaptive change for low CO₂ acclimation, it seems that cia-5 controls all low CO₂ responsive genes at different gene expression levels. This means the Cia-5 protein is involved in early steps in the signal transduction pathway for low CO₂ acclimation.

Nuclear transformation was used to generate insertional mutants for investigating the signal transduction pathway for low CO₂ acclimation. HCR90 was identified in screens for reduced Cah1 expression and a HCR phenotype. HCR90 shows a recessive HCR phenotype that co-segregates with the insert and does not show allelism to any known HCR mutants. The high Kₘ(Cᵢ) of HCR90 (64 μM) indicates it lacks a functional CCM. Since the message abundance of only Mca1 and Mca2, but not Cah1, CCP1 and CCP2, was reduced in HCR90, a genomic Southern analysis of CC425 and HCR90 was performed to determine whether the mtCA1 and/or mtCA2 structural genes were disrupted. Since no disruption of either Mca1 or Mca2 was found, it is possible that HCR90 is defective in regulation of Mca1 and Mca2 expression.
Using DNA flanking both ends of the Arg7 insert, six genomic clones from a *C. reinhardtii* BAC library were identified. However, the 5' and 3' flanking sequences identified non-overlapping sets of BACs, indicating a rather large disruption. Complementation experiments were not successful with any of these six BAC clones. The lack of complementation by these BAC clones and the clear indication of a large disruption means it may be difficult to identify the disruption responsible for the HCR phenotype using the strategy of walking through the disruption with BAC clones.

Transformation with cDNA expression plasmid library may be helpful for complementing of HCR90 phenotype. Complementation experiments of HCR90 with cDNA expression plasmid library are in progress. If a positive cDNA clone obtained in complementation experiments or a genomic clone obtained via BAC walking is confirmed by northern and Southern analyses, sequence comparisons may help identify possible functions of identified genes.

Although preliminary indicators suggested that HCR90 might be defective in the signal transduction pathway for acclimation to limiting CO$_2$, decreased induction of limiting-CO$_2$ induced genes has not been consistently reproducible in this mutant. Therefore it is not clear whether HCR90 is defective in signal transduction, but it clearly is defective in a gene whose expression is required for growth in limiting CO$_2$. Cloning of the disrupted gene in HCR90 may be helpful for dissecting additional components in the signal transduction pathway for low CO$_2$ acclimation.
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Xiang Y, Zhang J, Weeks DP (submitted) The *Cia5* gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii*. 
**FIGURE LEGENDS**

**Figure 1.** Construction of a plasmid library for expression of *C. reinhardtii* cDNAs in *Escherichia coli.* A cDNA with EcoRI and NotI sites at each end was inserted into the pJD405 vector to create a cDNA expression plasmid library. pJD405 contains the promoter region and exon I, intron I, exon II, intron II and terminator region of *rbsS2,* with a cloning site in exon II that eliminates the *rbcS2* translation start site.

**Figure 2.** High CO$_2$-Requiring (HCR) phenotype of HCR90. Spot tests on replica plates in high CO$_2$ (5% CO$_2$) and low CO$_2$ (50-100 µL L$^{-1}$ CO$_2$) were used for identifying low CO$_2$ nonacclimating mutants. CC400 and *cia-5* were used as wild type and a known low CO$_2$ nonacclimating mutant, respectively.

**Figure 3.** Northern blot analyses for wild-type (CC425) and HCR90. Total RNA (10 µg per lane) was isolated 24 h after transfer of cells to air levels of CO$_2$ from high CO$_2$. *Cah1* mRNA was probed with the 1.4 kb *Bgl*II and *NcoI* fragment of *Cah1* cDNA (Van and Spalding, 1999). *Mca1* and *Mca2* mRNA was probed with the full length *Mca2* cDNA (Eriksson et al, 1996; Eriksson et al., 1998). *Ccp1* and *Ccp2* mRNA was probed with the 1.2 kb *EcoRI* and *HindII* fragment of *Ccp1* G1 (Chen et al., 1997). The rRNA was probed with 25S and 5.8S rDNA (Marco and Rochaix, 1980).

**Figure 4.** Photosynthetic response to NaHCO$_3$ concentration (pH 7) of wild-type CC125, *cia-5,* and HCR90-4-1. Cells acclimated for 1 d in air were used for all measurements. Chlorophyll concentrations were: CC125, 11.09 µg ml$^{-1}$; *cia-5,* 10.12 µg ml$^{-1}$.
μg ml⁻¹; HCR90-4-1, 10.61 μg ml⁻¹. Three independent measurements were averaged from three different cultures.

**Figure 5.** Complementation of the mutation by transformation with BAC clones identified by hybridization with the 0.9 kb BamHI/BglI and the 1.9 kb Stul/EcoRI flanking regions. The restriction map of the genomic region of CC425 (host strain) is shown in the upper bar. The second bar represents a restriction map of the genomic region of HCR90 including the Arg7 insert. Restriction enzyme sites: B, BamHI; Bg, BglI; E, EcoRI; Sa, SalI; Ss, SstI; St, Stul.
Table I. Genetic analysis of HCR90 progeny from a cross with CC1068 (arg2 nr-u-2-1 mt<sup>+</sup>)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Arg7 Insert&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Strain</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Arg7 Insert&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>HCR90</td>
<td>HCR, Arg&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<td>HCR, Arg&lt;sup&gt;+&lt;/sup&gt;</td>
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<sup>a</sup>HCR, high CO<sub>2</sub>-requiring for growth; wt, wild-type (5% CO<sub>2</sub> concentration not required for photoautotrophic growth); Arg<sup>+</sup>, Arg not required for growth; Arg<sup>-</sup>, Arg required for growth.

<sup>b</sup>Arg7 insert detected by Southern analysis using 1.3 kb SalI fragment of Arg7 as probe.
Figure 1.
High CO$_2$ (5% CO$_2$)

Low CO$_2$ (50-100 μL L$^{-1}$ CO$_2$)

Figure 2.
Figure 3.
Figure 4.
Figure 5.
CHAPTER 5. GENERAL SUMMARY

GENERAL CONCLUSIONS

Aquatic photosynthetic organisms live in quite variable conditions of CO₂ availability. In order to survive in limiting CO₂ conditions, *Chlamydomonas reinhardtii* shows adaptive changes to limiting CO₂ conditions, including induction of a CO₂-concentrating mechanism (CCM) (Badger et al., 1980; for review, see Spalding, 1998), changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of at least five specific genes (*Cah1, Mca1, Mca2, Ccp1, Ccp2*) (Fujiwara et al., 1990; Fukuzawa et al., 1990; Geraghty et al., 1990; Ishida et al., 1993; Ramazanov et al., 1993; Eriksson et al., 1996; Geraghty and Spalding, 1996; Chen et al., 1997), and transient down-regulation in the synthesis of Rubisco (Coleman and Grossman, 1984; Winder et al., 1992). We are trying to identify mutants defective in the acclimation response to limiting CO₂. Nuclear transformation to complement an Arg⁺ phenotype was used to generate insertional mutants, and decreased *Cah1* expression and a high CO₂-requiring (HCR) phenotype were used as reporters for defective acclimation to limiting CO₂.

Among a collection of insertionally generated mutants, a mutant has been isolated that showed no pCA1 protein and no *Cah1* mRNA (Van and Spalding, 1999). This mutant strain, designated *cah1-1*, has been confirmed to have a disruption in the *Cah1* gene caused by a single Arg7 insert. The most interesting
feature of \textit{cah1-1} is its lack of any significant growth phenotype. There is no major
difference in growth or photosynthesis between wild-type and \textit{cah1-1} over a pH
range from 5 to 9 even though this mutant apparently lacks \textit{Cah1} expression in air.
Although the presence of pCA1 does apparently give some minor benefit at very low
CO$_2$ concentrations, the characteristics of this \textit{Cah1} null mutant demonstrate that
pCA1 is not essential for function of the CO$_2$-concentrating mechanism or for growth
of \textit{C. reinhardtii} at limiting CO$_2$ concentrations.

Eight independently isolated mutants of \textit{C. reinhardtii} that require high CO$_2$
for photoautotrophic growth were tested by complementation group analysis. Some
of these mutants are likely to be defective in some aspects of the acclimation to low
CO$_2$, because they differ from wild type in their growth and in the expression
patterns of five low CO$_2$ inducible genes (\textit{Cah1}, \textit{Mca1}, \textit{Mca2}, \textit{Ccp1} and \textit{Ccp2}). Two
of the new mutants formed a single complementation group along with the
previously described mutant \textit{cia-5} (Moroney et al., 1989), which appears to be
defective in the signal transduction pathway for low CO$_2$ acclimation. The other
mutations represent six additional, independent complementation groups.

One of the HCR mutants, HCR90, which preliminary results suggested might
be defective in signal transduction for limiting-CO$_2$ acclimation, was investigated
further. This mutant shows a recessive HCR phenotype that co-segregates with the
Arg$^+$ phenotype and presence of 1 Arg insert. HCR90 does not show any allelism to
the known HCR mutants, \textit{cia-5} (Moroney et al., 1989), \textit{ca-1} (Spalding et al., 1983a),
\textit{pmp-1} (Spalding et al., 1983b) and \textit{pgp-1} (Suzuki et al., 1990). K$_m$ (C$_i$) of HCR90
(64 $\mu$M) was found to be higher than that of wild type (11 $\mu$M), suggesting HCR90
lacks a functional CCM. After 5' and 3' sequences flanking the Arg insert were obtained, genomic clones from a *C. reinhardtii* BAC library were identified by using both flanking DNA as a probe. The 5' and 3' flanking sequences identified non-overlapping sets of BACs, indicating a rather large disruption. Since HCR90 grows poorly in low CO₂ (50-100 µL L⁻¹ CO₂), complementation of the mutation should be possible under direct selection in low CO₂. Complementation experiments were unsuccessful with each of six different BAC clones. Transformation with a cDNA expression plasmid library may be helpful for complementing of HCR90 phenotype. These cDNA complementation experiments are in progress. Even though it is no longer evident whether HCR90 is defective in signal transduction, it is clear HCR phenotype means the disrupted gene responsible for the phenotype is of high interest regardless.

**FUTURE RESEARCH WITH HCR90**

**Identification of the disrupted gene responsible for the HCR phenotype in HCR90**

The strategy currently being applied is to identify the disrupted gene responsible for the HCR phenotype in HCR90 by complementation with the cDNA expression library described above. However, there are several possible reasons why this strategy might not succeed, including a poor (or absent) representation of the target cDNA in the library if its mRNA abundance is very low, or if it has a very large message. If complementation with a cDNA expression plasmid library does
not work, other options would include resumption of the "walk" through the disrupted region using overlapping BAC clones. The size of the disruption is not known, so it is difficult to assess the difficulty of this approach or its chance for success. Other options include the use of a cosmid library for complementation, using either a random (Purton and Rochaix, 1994) or an indexed (Zhang et al., 1994) cosmid library. Genomic cosmid libraries have been used successfully both for "shotgun" style complementation (Purton and Rochaix, 1994) and for systematic screening-type complementation with an indexed library. This second approach has been used successfully to complement the ca-1-1 mutant (Cah3 gene; Funke et al., 1997) and the cia5 mutant (Cia5 gene; Xiang et al., manuscript submitted), two genes involved in the acclimation to limiting CO₂ in C. reinhardtii. Rapid screening methods with a cosmid library have been done, and even genomic complementation of ca-1 (Spalding et al., 1983a) was demonstrated by using an indexed cosmid library (Funke et al., 1997).

If the phenotype of HCR90 is rescued by complementation with the plasmid cDNA expression library described above, the sequence of the complementing cDNA can be obtained easily by PCR using unique primer regions flanking the cDNA inserts in pJD405. If HCR90 is rescued by complementation with an indexed cosmid library, deletion analysis of the complementing genomic clones should be performed to identify the minimum sequence necessary for complementation. The identified minimum genomic sequence will be used to clone a corresponding cDNA by screening one or more of several cDNA libraries available in our lab.
Function of Cloned Genes

Using probes from a sequenced cDNA, it should be tested whether wild type and HCR90 show polymorphisms on genomic Southern blots. Using the same probes, it should also be determined whether HCR90 shows any expression of the identified gene. Once a sequenced cDNA is confirmed by northern and southern analyses, sequence comparisons may tell something about possible functions of the identified gene. A possible function of this identified gene might be confirmed by the expression in *E. coli*, either by complementing an *E. coli* mutant or by assay for a specific enzymatic activity and in yeast by complementing a yeast mutant. This type of expression also might allow the generation of antibody for the identified gene, and this antibody production will help further investigation of the function and location of the gene product.

After a potential protein is expressed in *E. coli* or yeast, the yeast two-hybrid system might help to identify interacting proteins. The yeast two-hybrid system is a sensitive in vivo assay and allows identification of genes encoding proteins that have protein-protein interactions with a target protein. If an identified gene does not have any homology with, e.g., transcriptional factors, DNA binding proteins, protein kinases, or protein phosphatases, etc., the yeast two-hybrid system might help identify proteins that interact with the gene product in question. If the gene does have homology with protein kinases or protein phosphates, this yeast two-hybrid system also might help to additional signal transduction components that interact.
Physiological and Biochemical Characterization of HCR90

If further analysis, including identification of the gene responsible for the mutant phenotype, confirms that HCR90 is defective in the signal transduction pathway for limiting-CO$_2$ acclimation, the differences in acclimation between HCR90 and wild type might help establish the position of the defect in the signal transduction pathway.

HCR90 has reproducibly reduced expression of only $Mca1$ and $Mca2$ mRNA, but expression of $Cah1$, $Ccp1$ and $Ccp2$ mRNA appeared normal. Since no disruption of $Mca1$ and $Mca2$ was found, HCR90 might be defective in regulation of expression of $Mca1$ and $Mca2$. Thus this pattern of expression for five genes in HCR90 indicates that the genes may be differentially regulated, so the signal transduction pathway for low CO$_2$ acclimation might branch, not only prior to control of expression of these five genes, but also prior to other adaptive changes, such as induction of a functional CCM, changes in cell organization, and increased photorespiratory enzyme activity.

Although expression of only $Mca1$ and $Mca2$ appears to be affected in HCR90, only five genes have been investigated so far. Expression of additional CO$_2$-regulated genes will be investigated, including two cDNA clones ($Lci1$ and $Att1$) identified by differential screening of a cDNA library (Burow et al., 1995), the glycolate dehydrogenase gene ($Gdh1$; Nakamura, manuscript in preparation), the glutamine synthetase gene ($Gs2$; Chen and Silflow, 1996), $rbcL$, $RbcS1$ and $RbcS2$ (Coleman and Grossman, 1984; Winder et al., 1992). $Lci1$ might encode a transmembrane protein based on amino acid sequence, and $Att1$ encodes
alanine:α-ketoglutarate aminotransferase (Chen et al., 1996). Expression data for these additional cDNA clones may help position the mutation in the signal transduction pathway.

Since HCR90 lacks a functional CCM, functional evaluations of the CCM, including measurement of internal C₄ accumulation, and product labeling, will be performed further in order to determine whether the functional defect in the CCM can be localized.

In addition to analysis of changes in CCM function, changes in the level of photorespiratory enzymes (Marek and Spalding, 1991), such as phosphoglycolate phosphatase (PGPase), glycolate dehydrogenase (Nakamura, et al., in preparation) and glutamine synthase (Chen and Silflow, 1996), might help establish the position of defects in the signal transduction pathway. HCR90 also may exhibit defects in organizational changes associated with acclimation, such as characteristic changes in starch organization, mitochondrial distribution, and vacuolization, depending on the position of its defects in the signal transduction pathway.

Neither the signals nor the signal transduction pathway for low CO₂ acclimation in C. reinhardtii are known. Since different adaptive changes to limiting CO₂ are observed depending on light, CO₂ concentration and acetate (see Chapter 1), much research needs to be done in terms not only in dissecting components of the signal transduction pathway for low CO₂ acclimation but also in exploring the interaction of multiple signals. If we characterize more of the signal transduction mutants other than HCR90, determining relationships between gene expression, physiological, biochemical or structural changes and acclimation to limiting CO₂ with
HCR90 would also help order the mutations. Construction of double mutants with combinations of signal transduction mutants, including the cia-5 mutant (Moroney et al., 1989), would be helpful to distinguish whether individual mutations lie on parallel pathways or the same pathways. All this information will be most useful if other mutants also can be positioned in the signal transduction pathway along with HCR90 and the cia-5 mutant (Moroney et al., 1989).

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ACKNOWLEDGEMENTS

First of all, I would like to express my full gratitude to my major professor, Dr. Martin H. Spalding. His suggestions and guidance throughout my Ph.D. program were tremendous. Also, his 100% support and encouragement kept me going and completing my study. THANK YOU!!!

My sincere appreciation goes to my committee members: Drs. Jim T. Colbert, David J. Hannapel, David J. Oliver and Steve R. Rodermel for their guidance and critical review of my thesis. I thank Dr. John P. Davies, Dr. Kensaku Suzuki and Jeff McDermott for providing strains, plasmids and libraries used in my research.

I would like to appreciate to my current and previous lab personnel (Andrea Flack, Dr. Sarada Kanakagiri, Dr. Yoshiko Nakamura, Dr. Carl Schlagnhaufer, Peter Vance, Yingjun Wang and many undergraduate helpers) for their encouragement and discussion. Many friends in IPPM along with Botany 4th floor people made me have fun in lab life.

Finally, I would like to thank my parents (Dr. Byung Gil Van and Jung Ae Yoon) and sisters (Juwon and Gail) for their love and support during my study. At this moment, my father is probably sitting in his office preparing a lecture or writing his book. Because of his example of hard work and dedication, I plan on following in his footsteps.