Insertional Mutants of *Chlamydomonas reinhardtii* That Require Elevated CO₂ for Survival

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Aquatic photosynthetic organisms live in quite variable conditions of CO₂ availability. To survive in limiting CO₂ conditions, *Chlamydomonas reinhardtii* and other microalgae show adaptive changes, 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.

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 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. Because 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 CO₂. This acclimation includes induction of a CO₂-concentrating mechanism (CCM) that allows the cells to acquire CO₂ efficiently by increasing the CO₂ concentration around Rubisco under limiting CO₂ conditions (Badger et al., 1980; for review, see Spalding, 1998; Kaplan and Reinhold, 1999).

Along with the induction of the CCM, *C. reinhardtii* shows adaptive changes to limiting CO₂ conditions, such as changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of periplasmic carbonic anhydrase (CA) (*pCA1*, encoded by the *Cah1* gene; Fujiwara et al., 1990; Fukuwaza et al., 1990; Ishida et al., 1993), mitochondrial CA (*mtCA*, encoded by the *Mca1* and *Mca2* genes; Eriksson et al., 1996; Geraghty and Spalding, 1996), and putative chloroplast carrier protein (*Ccp*, encoded by the *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 CO₂ in *C. reinhardtii* is unidentified. It is not known how they sense a change of CO₂ availability, whether by CO₂ concentration directly or indirectly via a cellular process such as carbohydrate metabolism. Whatever the limiting-CO₂ signal, it must be transduced into the changes in gene expression observed during acclimation, such as expression of *Cah1*. A powerful way to identify components of the CCM and of the signal transduction pathway for low CO₂ acclimation is through the analysis and characterization of mutants specifically defective in growth in limiting CO₂, like the *ca-1*, *pmp-1*, and *cia*-5 mutants (Spalding et al., 1983a; 1983b; Moroney et al., 1989). Using advances in nuclear transformation of *C. reinhardtii* (Kindle,
Van et al.

1990), a collection of insertionally generated high CO2-requiring (HCR) 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 p-Arg7.8 (Debuchy et al., 1989) to generate a pool of insertional mutants on CO2-minimal medium. Cells from each of more than 7,000 transformant colonies were suspended in air-minimal medium and grown on plates in high CO2 (5% [v/v] 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 cir-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 the 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CC1068</td>
<td>arg2</td>
<td>Arg-requiring and kanamycin-resistant mutant</td>
<td>Harris (1989)</td>
</tr>
<tr>
<td>CC2702</td>
<td>cia-5</td>
<td>No acclimation to limiting CO2</td>
<td>Moroney et al. (1989); Spalding et al. (1991)</td>
</tr>
<tr>
<td>CC1219</td>
<td>ca1-1</td>
<td>Defective in Cah3, thylakoid lumen CA</td>
<td>Spalding et al. (1983a); Funke et al. (1997); Karlsson et al. (1998)</td>
</tr>
<tr>
<td>CC1860</td>
<td>pmp1-1</td>
<td>Deficient in C, transport</td>
<td>Spalding et al. (1983b)</td>
</tr>
<tr>
<td>CC2648</td>
<td>ppg1-1</td>
<td>Deficient in phosphoglycolate phosphatase</td>
<td>Suzuki et al. (1990)</td>
</tr>
<tr>
<td>HCRP34</td>
<td></td>
<td>Generated by transformation of CC425 with Arg7; used as wild type in liquid growth experiments</td>
<td>Harris (1989)</td>
</tr>
<tr>
<td>HCR209</td>
<td></td>
<td>Cell wall-less, Arg-requiring, and streptomycin-resistant mutant</td>
<td>Harris (1989)</td>
</tr>
<tr>
<td>HCR3510</td>
<td></td>
<td>Generated by CC2648</td>
<td>Provided by John P. Davies (Exelixis, Inc., South San Francisco)</td>
</tr>
<tr>
<td>HCR86</td>
<td></td>
<td>Generated by CC2702 × CC124</td>
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<tr>
<td>HCR89</td>
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<tr>
<td>HCR90</td>
<td></td>
<td>Generated by CC1060 × CC124</td>
<td>This report</td>
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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 were tested in crosses with another arg2 mutant (CC1068, Table I) for linkage of the 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 cosegregate 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, cosegregation 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

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 CO$_2$ conditions (50–100 μL L$^{-1}$ CO$_2$) after mating with HCR mutants, this indicates they are not allelic to each other, because these known mutants also show HCR phenotypes.

Rapid allelism tests were used to place the various HCR mutants into different complementation groups. Complementation analysis was tested with the eight HCR mutants (Table II) along with cia-5, ca-1, pmp-1, and pgp-1 (Table I). Only crosses between cia-5 × HCRP34, cia-5 × HCR209, and HCRP34 × HCR209 failed to generate wild type colonies. Thus, HCR3510, HCR86, HCR89, HCR90, HCR95, and HCR105 each define a new HCR locus. HCRP34 and HCR209 have been confirmed as defective in the same locus as cia-5 by comparison of the sequence of the DNA flanking the inserts with a cloned cia-5 gene (Xiang et al., 2001) and by complementation with a cloned cia-5 gene (data not shown).

**Liquid Growth Experiments**

Growth experiments showed patterns of high CO$_2$ requirement for photoautotrophic growth consistent with those seen in spot tests (Fig. 1). Active, 1-d-old air-adapted cells were inoculated into liquid minimal medium with similar starting cell densities ($5 \times 10^4$ cells ml$^{-1}$), 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 cia-5, grew very similar to cia-5 in air (Fig. 2A). The growth rates of HCR86 and HCR90 also were only slightly better than that of cia-5 in air (Fig. 2B), but the growth rates of HCR89 and HCR95 were intermediate between wild type (ars301; see Table I) and 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 CO$_2$-Inducible Transcripts**

Because the expression of low CO$_2$-inducible polypeptides (pCA1, mtCA1, mtCA2, Ccp1, and Ccp2) has been reported to change differentially during acclimation to limiting CO$_2$ (Villarejo et al., 1996, 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 CO$_2$, had reduced expression of only 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 mRNA abundance (data not shown). HCR3510, which showed a wild-type phenotype in air but a stringent HCR phenotype in low CO$_2$, 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 was confirmed up to 24 h (data not shown). The other HCR mutants (HCR86, HCR89, 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 (Somerville and Ogren, 1982) and barley (Hordeum vulgare) (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 an HCR phenotype in C. reinhardtii (Spalding et al., 1983a, 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, 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; Burrow et al., 1996). This mutant is thought to be defective in the signal transduction pathway for acclimation to limiting CO₂. The gene responsible for this mutation (Caia5) has been cloned recently (Fukuzawa et al., 2001; Xiang et al., 2001), and its characterization suggests it may encode a transcription factor. The identification of this important gene opens the way for more rapid progress in delineation of the signal transduction pathway for acclimation to limiting CO₂.

Because 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. As new alleles of cia-5, HCRP34 and
and HCR209 may 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 transcript accumulations that were not reproducibly different from those of wild type (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 levels of low CO_2-inducible transcripts (Fig. 3B).

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 cosegregates with the mutant phenotype, i.e. if the insert is responsible for the mutation. As judged by the Arg^7 phenotype, the Arg7 inserts in mutants HCRP34, HCR3510, HCR86, HCR89, and HCR90 cosegregate 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 cosegregation of the Arg^+ and HCR phenotypes has not been demonstrated for HCR209.

It is unfortunate that the inserts in mutants HCR95 and HCR105 do not cosegregate 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 HCR90) remain as viable candidates for identification of novel genes essential for acclimation of *Chlamydomonas 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 *Chlamydomonas reinhardtii* strains (Table I) were grown as previously described (Geraghty et al., 1990). Cells were cultured on an orbital shaker under aeration with 5% (v/v) 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% (v/v) CO_2 to aeration with normal air for 2 h to 6 h. For growth on solid media, cells were maintained under 5% (v/v) CO_2 in air (high CO_2), normal air, or 50 to 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 insertion mutants on CO_2-minimal medium, CC425 (Table I) was transformed with linearized p-Arg7.8 (Debuchy et al., 1989) containing the structural gene (Arg7) for argininosuccinate lyase to complement the arg2 mutation. Each of more than 7,000 colonies was screened by spot tests to identify HCR mutants. After replica plates with transformants were made, each plate was placed in high CO_2 and low CO_2. Mutants identified in this primary screen as having HCR phenotypes 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 CO_2 for 10 d (Harris, 1989).

For liquid growth experiments, active, 1-d-old air-adapted cells were inoculated into liquid minimal medium at similar cell densities (5 x 10^4 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). Total RNA was purified with TRIzol reagent (Life Technologies, Gaithersburg, MD) from air-induced cells exposed to limiting CO_2 (aeration with normal air) and Hybond N^- nylon transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ).
away, NJ) was used for blotting. After phororimager 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 (ar-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 kana-

mycin resistance (sr-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, Ames).

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 CO2 (50–100 μL L⁻¹). Because all HCR mutants required elevated CO2 for survival, wild-type colonies were observed in low CO2 only if the cross generated wild-type recombinant progeny.

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**LITERATURE CITED**


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