Insertional Mutants of *Chlamydomonas reinhardtii* That Require Elevated CO_2 for Survival[1]

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Aquatic photosynthetic organisms live in quite variable conditions of CO_2 availability. To survive in limiting CO_2 conditions, *Chlamydomonas reinhardtii* and other microalgae show adaptive changes, such as induction of a CO_2-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 CO_2 in *C. reinhardtii* is unidentified, and it is not known how they sense a change of CO_2 level. The limiting CO_2 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 CO_2 acclimation. Eight independently isolated mutants of *C. reinhardtii* that require high CO_2 for photoautotrophic growth were tested by complementation group analysis. 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 (*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 CO_2 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 CO_2 can vary substantially in aquatic habitats and represents the major substrate for photosynthetic CO_2 fixation via the enzyme Rubisco, CO_2 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 CO_2. Because the CO_2 diffusion rate in water is much slower than that in air (Badger and Spalding, 2000), the CO_2 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; Kaplan and Reinhold, 1999).

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 periplasmic carbonic anhydrase (CA) (pCA1, encoded by the *Cah1* gene; Fujiwara et al., 1990; Fukuzawa 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 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. Whatever the limiting-CO_2 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 CO_2 acclimation is through the analysis and characterization of mutants specifically defective in growth in limiting CO_2, 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,

[1] This work was supported by the U.S. Department of Agriculture National Research Initiative (grant nos. 97–35100–4210 and 99–35100–7569 to M.H.S.). This is journal paper no. J–19297 of project no. 3578 of the Iowa Agriculture and Home Economics Experiment Station (Ames) and was supported by the Hatch Act and State of Iowa funds.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010333.

1990), a collection of insertionally generated high CO_2-requiring (HCR) mutants unable to grow in limiting CO_2 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 CO_2-minimal medium. Cells from each of more than 7,000 transformant colonies were suspended in air-minimal medium and grown on plates in high CO_2 (5% [v/v] CO_2 in air), normal air, and low CO_2 (50–100 μL L^{-1} CO_2). HCR mutants, defined as those showing little or no growth either in normal air or in low CO_2, should include mutants, like *cia-5*, that are defective in acclimation to limiting CO_2, 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 CO_2 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 CO_2 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 CO_2. The second group, including HCR86 and HCR105, showed a stringent HCR phenotype both in air and in low CO_2. HCR89 and HCR95, comprising the third group, had a leaky HCR phenotype both in air and in low CO_2. HCR3510 lacked a significant growth phenotype in air but had a stringent phenotype in low CO_2.

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 Arg^+ and HCR phenotypes (Table II). Five of the eight mutants showed cosegregation 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 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

Table I. *Strains of C. reinhardtii used in the study*

Strain	Genotype	Description	Reference
ars301	*cw15 sr-u-2-60 mt*$^+$	Generated by transformation of CC425 with *Arg7*; used as wild type in liquid growth experiments	Provided by John P. Davies (Exelixis, Inc., South San Francisco)
CC124	mt$^-$	Wild type (137C)	Harris (1989)
CC425	*arg2 cw15 sr-u-2-60* mt$^+$	Cell wall-less, Arg-requiring, and streptomycin-resistant mutant	Harris (1989)
CC849	*cw10* mt$^-$	Cell wall-less mutant used as wild type in RNA analyses	Harris (1989)
CC1068	*arg2 nr-u-2-1* mt$^-$	Arg-requiring and kanamycin-resistant mutant	Harris (1989)
CC2702	*cia-5* mt$^+$	No acclimation to limiting CO_2	Moroney et al. (1989); Spalding et al. (1991)
	cia-5 mt$^-$	Generated by CC2702 × CC124	This report
CC1219	*ca1-1* mt$^+$	Defective in *Cah3*, thylakoid lumen CA	Spalding et al. (1983a); Funke et al. (1997); Karlsson et al. (1998)
	ca1-1 mt$^-$	Generated by CC1219 × CC801	Provided by Kensaku Suzuki (Tohoku National Agri Research, Morioka, Japan)
CC1860	*pmp1-1* mt$^+$	Deficient in C_i transport	Spalding et al. (1983b)
	pmp1-1 mt$^-$	Generated by CC1860 × CC124	This report
CC2648	*pgp1-1* mt$^+$	Deficient in phosphoglycolate phosphatase	Suzuki et al. (1990)
	pgp1-1 mt$^-$	Generated by CC2648 × CC124	This report
	HCRP34 mt$^-$	Generated by HCRP34 × CC1068	This report
	HCR209 mt$^-$	Generated by HCR209 × CC1068	This report
	HCR3510 mt$^-$	Generated by HCR3510 × CC1068	This report
	HCR86 mt$^-$	Generated by HCR86 × CC1068	This report
	HCR89 mt$^-$	Generated by HCR89 × CC1068	This report
	HCR90 mt$^-$	Generated by HCR90 × CC1068	This report

Table II. *Characteristics of HCR mutants*

Mutants	High CO_2 Requirement[a]		*Arg7* Insert[b]	Vector Sequence Present	Cosegregation with Arg^+ Phenotype and *Arg7* Insert	Diploid Analysis
	Air	Low CO_2				
cia-5	Leaky[c]	Stringent[c]	NA[d]	NA	NA	Recessive
HCRP34	Leaky	Stringent	1	+[e]	Yes	Recessive
HCR209	Leaky	Stringent	2	+	ND[f]	Recessive
HCR3510	Wild type	Stringent	1	+	Yes	Recessive
HCR86	Stringent	Stringent	1	+	Yes	Recessive
HCR89	Leaky	Leaky	1	−	Yes	Recessive
HCR90	Leaky	Stringent	1	+	Yes	Recessive
HCR95	Leaky	Leaky	1	+	No	Recessive
HCR105	Stringent	Stringent	1	ND	No	Recessive

[a] Growth phenotype in low CO_2 was determined by spot test on agar in high CO_2 (5% [v/v] CO_2 in air) versus either air (300–500 μL L^{-1} CO_2) or low CO_2 (50–100 μL L^{-1} CO_2). [b] *Arg7* insert detected by Southern analysis using 1.3-kb *Sal*I fragment of *Arg7* as probe. [c] Leaky, Cells grow very slowly; stringent, cells did not grow at all. [d] NA, Not applicable. [e] +/− Indicate the presence (+) or absence (−) of vector sequence. [f] ND, Not determined.

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

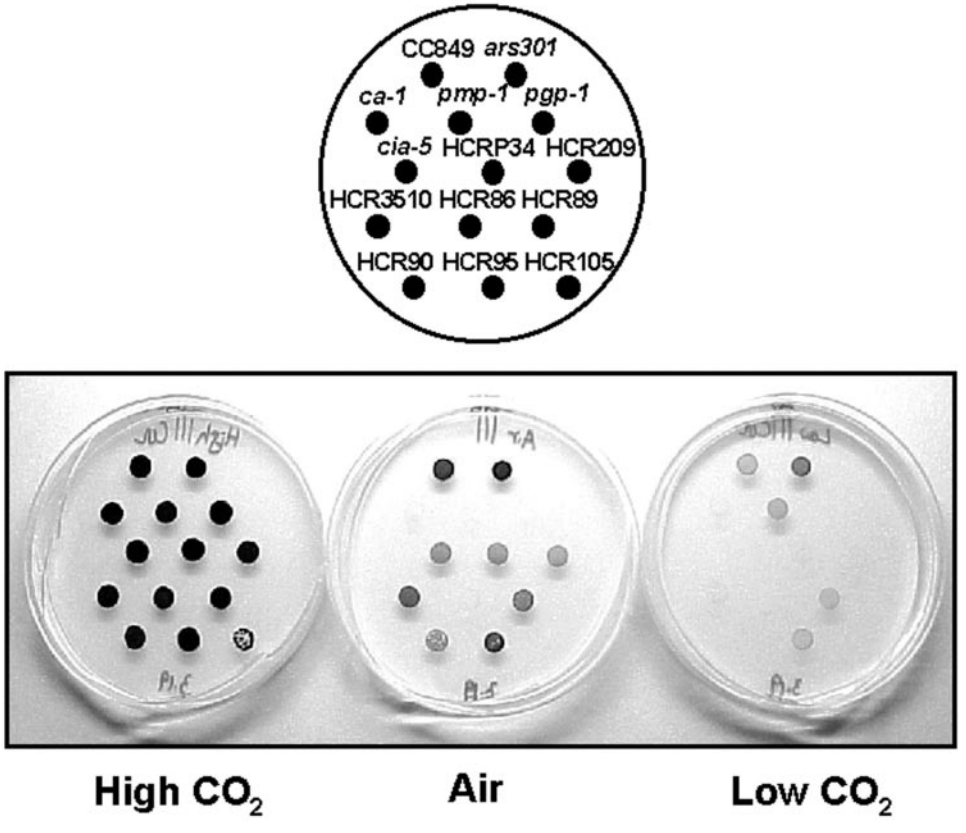

Figure 1. Spot tests for growth response to different CO_2 concentrations for wild-type strains (CC849 and ars301), four previously described HCR mutants (*cia-5*, *ca-1*, *pmp-1*, and *pgp-1*), and eight new HCR mutants. Plates were kept either at high CO_2 (5% [v/v] CO_2), at air level of CO_2, or at low CO_2 (50–100 μL L^{-1}) for 10 d.

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 (5×10^4 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).

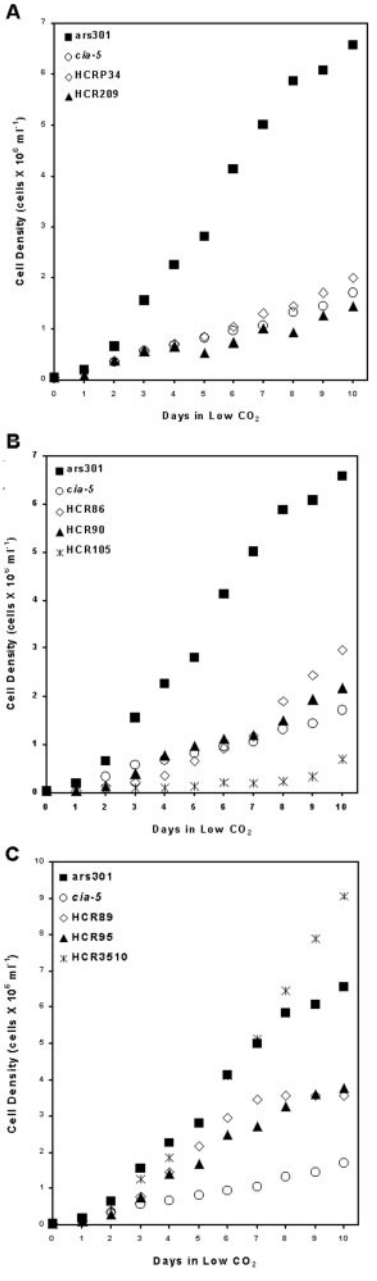

Figure 2. Liquid cell growth curves for wild type (ars301), *cia-5*, and HCR mutants grown at pH 7 on an orbital shaker without aeration. A, HCRP34 and HCR209. B, HCR86, HCR90, and HCR105. C, HCR89, HCR95, and HCR3510. The growth curves shown are averages of three independent growth experiments.

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

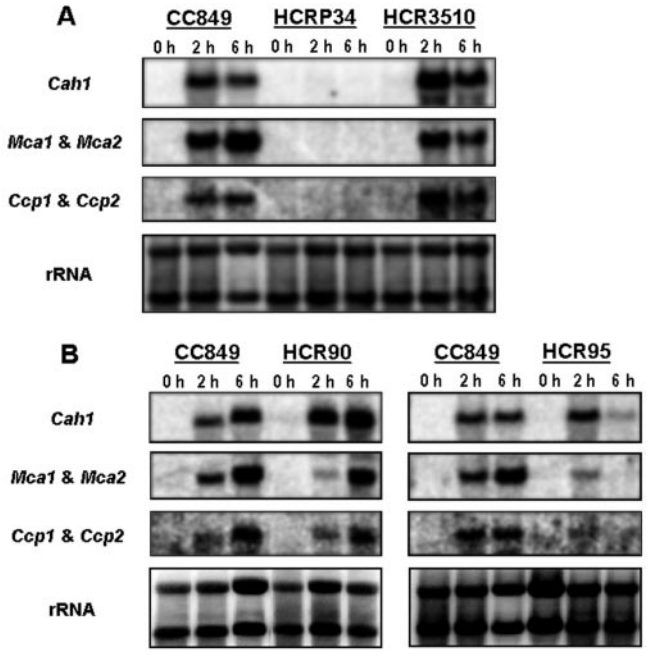

Figure 3. Northern-blot analyses for wild type (CC849) and HCR mutants. A, HCRP34 and HCR3510. B, HCR90 and HCR95. Total RNA (10 μg per lane) was isolated 2 h to 6 h after transfer of cells to air levels of CO_2 from high CO_2. *Cah1* mRNA was probed with the 1.4-kb *Bgl*II and *Nco*I fragment of *Cah1* cDNA (Van and Spalding, 1999). *Mca1* and *Mca2* mRNA was probed with the full-length *Mca2* cDNA (Eriksson et al., 1996, 1998). *Ccp1* and *Ccp2* mRNA was probed with the 1.2-kb *Eco*RI and *Hin*dIII fragment of *Ccp1* G1 (Chen et al., 1997). The rRNA was probed with 25S and 5.8S rDNA (Marco and Rochaix, 1980).

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, and HCR105) did not show reproducibly different patterns of expression for the three low CO_2-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 C_3 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 CO_2 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 CO_2 (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 C_i (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-CO_2 acclimation responses, such as induction of CCM, up-regulation of low CO_2-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 CO_2. The gene responsible for this mutation (*Cia5*) 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 CO_2.

Because many changes involved in acclimation to limiting CO_2 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 CO_2 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 CO_2 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 CO_2-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 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 transcripts 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 level 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^+ 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 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 *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 insertional 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 air or 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 (5×10^4 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., Piscat-

away, NJ) was used for blotting. After phoporimager 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, 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 CO_2 (50–100 μL L^{-1}). Because 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.

Received April 9, 2001; returned for revision June 4, 2001; accepted July 2, 2001.

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