CO_2 concentrating mechanism and signal transduction pathway associated with limiting CO_2 acclimation in *Chlamydomonas reinhardtii*

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TABLE OF CONTENTS

Chapter 1. General introduction and literature review

General Introduction

Although present in small quantities in the air, carbon dioxide (CO_2) is one of the most important gases on earth. Photosynthetic organisms absorb CO_2 as the major substrate to support photosynthesis, the beginning of energy flow into living things and one of the primary processes which compose the global carbon cycle. Therefore, changes in CO_2 concentrations can have profound influences on photosynthetic reactions and many related carbon metabolic processes, which subsequently influence the living environments on earth. The overall global atmospheric CO_2 concentration remains nearly constant in the short term. However, the CO_2 concentrations in localized environments for photosynthetic organisms can change frequently and often do so rapidly. Photosynthetic organisms have evolved a series of adaptive mechanisms through which they constantly monitor their surroundings and adjust their physiology accordingly. Many aquatic photosynthetic microorganisms possess CO_2 concentration mechanisms (CCMs) that typically are induced by limiting CO_2 environments, allowing them to optimize carbon acquisition. In particular, an array of regulatory devices appears present to facilitate the sensing of CO_2 availability and the regulation of metabolic pathways. Although significant advances have been made over the past several years in understanding the CCM and its regulation, many components involved in limiting CO_2 acclimation are still unknown or uncharacterized.

Recent developments in genomics and molecular tools have enabled new insights into the nature of the CCM and its regulation. We are particularly interested in low CO_2 acclimation in *Chlamydomonas reinhardtii*, a unicellular green alga, since the CCM and its regulation in this model photosynthetic eukaryote provide an excellent system to study cell acclimation to the environment. We have applied insertional mutagenesis, together with physiological and genetic analyses, to identify and characterize non-acclimating mutants, which have proved to be valuable assets for deciphering the CCM and limiting CO_2 acclimation at the molecular level.

Literature Review

As the primary machinery for carbon fixation on the earth, a photosynthetic organism must constantly respond to a changing environment. The shortage of carbon supply is a major stress frequently confronted by plants, not only due to low ambient CO_2 concentrations, but also due to the low CO_2 affinity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), a key enzyme in carbon fixation. Several strategies have evolved in plants to maintain a high photosynthetic rate by raising the CO_2 concentration at the site of Rubisco, including different C4 carbon cycles operating in C4 and CAM plants. In many aquatic photosynthetic microorganisms, however, a different way of concentrating inorganic carbon (Ci) has been adopted, frequently called the CO_2 concentration mechanism (CCM).

The CCM represents an adaptation to environments with limiting CO_2 availability. Induction of the CCM results in a large intracellular pool of Ci, therefore improving photosynthetic efficiency under low ambient CO_2 conditions. This process involves active Ci transport, inter-conversion between CO_2 and bicarbonate (HCO_3^-), and dehydration of accumulated HCO_3^- in the chloroplasts at sites of Rubisco localization. The CCM represents an inducible response specifically to limiting Ci environments. When there is sufficient supply of CO_2, or under heterotrophic growth conditions, the operation of the CCM is repressed. The CCM is activated only when the CO_2 supply is limited. During acclimation, rapid changes in gene expression and biochemical events occur that are believed to be regulated by a signal transduction pathway as yet not fully identified.

During the past several decades, the CCM has been extensively studied in *C. reinhardtii*, a unicellular green alga with a photosynthetic apparatus similar to that of higher plants. Its short life cycle and well characterized genetic background, in combination with the increasingly available genetic and molecular tools and growing knowledge about its genome, make *C. reinhardtii* an attractive model system for studying the interaction of photosynthetic eukaryotic cells with their environment. The techniques for nuclear, mitochondrial and chloroplast genome transformation have been well developed in this organism, and the majority of its genome has been sequenced. With physiological and biochemical studies having already provided many details on the CCM and its regulation, the previously mentioned advances in genetic studies and molecular tool development are promising a more integrated picture at the molecular level.

Acclimation of *C. reinhardtii* to limiting CO_2

CO_2 concentrating mechanism

Like many other photosynthetic microorganisms, *C. reinhardtii* displays a distinguishable physiological state when grown under limiting CO_2 conditions. Following a change from enriched CO_2 concentrations (1-5% CO_2 in air) to limiting CO_2 concentrations (air, ~0.03% CO_2), one significant acclimation appears as a much higher efficiency in photosynthetic carbon assimilation. It has been reported that *C. reinhardtii* cells grown in limiting CO_2 have an apparent photosynthetic $K_{½}$ for dissolved Ci 10-100 fold lower than that in high CO_2 grown cells. Such high efficiency can be attributed to its capacity for accumulating a large intracellular Ci pool. Indeed, *C. reinhardtii* has been demonstrated to accumulate intracellular Ci at least 20-fold over ambient CO_2 levels. The high Ci level maintains a saturated or near saturated CO_2 concentration at the site of Rubisco, favoring its carboxylation activity over its oxygenation activity and thus efficiently promoting photosynthesis and suppressing photorespiration. Despite a lack of characterization of major components involved in the CCM, it is believed that at least two essential components comprise the integrated parts of the CCM: (i) an active Ci transport system; and (ii) an enzymatic system catalyzing rapid interconversion between CO_2 and HCO_3^-.

i. Ci transport system

It has been demonstrated that the induction of an active Ci transport system occurs at both the plasma membrane and the chloroplast envelope (Sultemeyer et al., 1989; Palmqvist et al., 1994). Vanadate, an inhibitor of the plasma membrane bound ATPase, was shown to inhibit Ci uptake, suggesting that Ci transport at the plasma membrane is energy dependent (Palmqvist et al., 1988). It is generally believed that CO_2 is the species predominantly transported across the plasma membrane (Spalding, 1998), but it is not clear which Ci species is transported into the plastid.

Active Ci uptake across the chloroplast envelope was shown to be induced in cells grown photoautotrophically in air, but not in cells grown photoheterotrophically or in CO_2-enriched air (Moroney et al., 1987; Sültemeyer et al., 1988). Although there is substantial evidence demonstrating the existence of an active transport system at the chloroplast envelope, none of its components have been physically characterized. Disruption of the plastid *ycf10* open reading frame was found to decrease the uptake of Ci in chloroplasts of *C. reinhardtii*

(Rolland et al., 1997). The product of the chloroplast *ycf10* gene is localized in the inner chloroplast envelope and displays sequence homology with the cyanobacterial *CotA* product, which is altered in mutants defective in CO_2 transport and proton extrusion (Katoh et al., 1996a, b). It is not clear how this protein functions in plastid Ci transport. Other proteins which might be involved in the chloroplast Ci transport include two limiting CO_2 induced polypeptides, encoded by *Ccp1* and *Ccp2* genes, and located in the chloroplast envelope (Chen et al., 1996). Ccp1 and Ccp2 show sequence homology with the mitochondrial carrier protein family. Im and Grossman (2001) also have identified a limiting CO_2-induced gene (*Mrp1*) encoding a putative ABC-transporter. Although suggested by these authors to be a platid protein, the deduced Mrp1 sequence indicates that the product of this gene probably enters the endomembrane system, and thus may be a plasma membrane protein. Recently many CO_2 responsive genes have been identified in large scale analyses of gene expression profiles in response to different CO_2 conditions (Miura et al., 2004), among which several CO_2 responsive genes have been suggested as genes encoding putative Ci transporters, including *Mrp1*, *LciB*, *LciC* and *LciA*. The expression of these putative transporter genes is up-regulated in response to limiting CO_2, and the up-regulation disappears in the *pmp1* mutant. These authors therefore suggested that Pmp1 was involved in regulating the expression of Ci transporters (Miura et al., 2004).

Compromised Ci transport can lead to cell death under limiting CO_2 conditions, as has been demonstrated in a high CO_2 requiring mutant, *pmp-1*, which lacks the ability to accumulate Ci and dies when grown in air levels of CO_2 (Spalding et al., 1983). Recent studies showed that this mutant is actually able to grow under very low CO_2 conditions (50-100 ppm CO_2), indicating that differentially regulated Ci transport systems may exist under different limiting CO_2 concentrations (Van et al., 2001; Spalding et al., 2002).

ii. Carbonic anhydrases

The interconversion between CO_2/HCO_3^- is catalyzed by carbonic anhydrases (CAs). Three independent evolutionary lines of carbonic anhydrase, α–, β–, and γ–type, with a variety of functions have been identified from living organisms. Despite different CA isoforms present in *C. reinhardtii*, so far only one plastid CA (α–type, encoded by *Cah3*) has been confirmed as essential for limiting CO_2 acclimation. Mutants defective in *Cah3*, *ca1-1* and *cia3*, cannot

survive under limiting CO_2 conditions (Funke et al., 1997; Karlsson et al., 1995; Karlsson et al., 1998). Cah3 is constitutively expressed and associated with the thylakoid membranes. Association of this CA with the PSII fraction during chloroplast fractionation, and the presence of a putative thylakoid targeting sequence suggest a lumenal location for Cah3 (Park et al., 1999). A hypothesis has been offered regarding the function of Cah3, in which it plays an essential role for CO_2 concentrating in chloroplasts by dehydrating stromal HCO_3^- in the acidic lumen after transport or diffusion into the thylakoid lumen. The CO_2 thus released upon lumen acidification in the light then diffuses back into stroma and enters the Calvin cycle. Since CO_2, rather than HCO_3^-, is the substrate used by Rubisco, this hypothesis requires a low CA activity in the stroma, at least at the site of Rubisco, to minimize CO_2 hydration in the HCO_3^--favoring alkaline environment of the stroma. Another proposed role for Cah3 is its involvement in PSII function (Villarejo et al., 2002), although a recent study demonstrated that PSII electron transport was not affected by the *cia3* mutation (Hanson et al., 2003).

Under limiting CO_2 conditions, the majority of the CA activity in *C. reinhardtii* is found in the periplasmic space. The products of two genes, *Cah1* and *Cah2*, were identified as α-type CA isoforms associated with periplasmic CA activity (Fukuzawa et al., 1990, Fujiwara et al., 1990). The regulation of *Cah1* and *Cah2* expression by Ci availability shows opposite trends. While the less abundant *Cah2* gene product (pCA2) is expressed at high Ci concentrations and repressed at low Ci concentrations, the *Cah1* gene product (pCA1), which accounts for the majority of the extracellular CA activity, is expressed only at limiting Ci concentrations. The regulation of *Cah1* gene expression during limiting CO_2 acclimation has been extensively studied. However, its role in the CCM has been controversial, at least until a null-mutant with a disrupted *Cah1* gene was identified (Van and Spalding, 1999). The *cah1* mutant cells did not show any distinguishable phenotype differing from the wild type cells, questioning the essentiality of pCA1 in the CCM. It is possible that pCA1 does not play any important physiological role for an individual cell. It may, however, play an ecological role for a population. Under natural conditions, the localized depletion of dissolved Ci in water can occur when the cell density is high and photosynthesis is active. Therefore, a periplasmic CA may be required to accelerate the CO_2 exchange at the air-water interface.

Other identified intracellular CA isoforms include two mitochondrial CAs encoded by very

similar genes, *βCa1* and *βCa2* (Eriksson et al., 1996), also called *Mca1* and *Mca2* (Spalding, 1998). Unlike Cah3, pCA1 and pCA2, mtCA1 and mtCA2 belong to the β-type CA family, which has a different evolutionary origin from α-type CA. The expression of *Mca1* and *Mca2* is regulated by CO_2 availability at the transcriptional level, with the abundant *Mca1* and *Mca2* transcripts induced by limiting Ci. However, the absence of any mutants deficient in the mitochondria CAs has kept their function unknown. It has been suggested that they might be needed for buffering pH in mitochondria to neutralize ammonia released during photorespiration (Eriksson et al., 1996). Recently evidence has indicated that β-type CAs have an ancient evolutionary origin and are ubiquitously distributed in almost all living organisms (Smith et al., 1999). A study showed that a β-type CA is essential for the growth of *Corynebacterium glutamicum* under atmospheric conditions (Mitsuhashi et al., 2004), in that a deficiency in a specific β-type CA in this organism causes a high CO_2 requiring phenotype. Although we do not know if the mitochondrial βCAs in *C. reinhardtii* play roles similar to their bacterial counterpart, it seems likely that the importance of these CA isoforms may include a more general role in carbon utilization. For example, Giordano et al. (2003) proposed that products of *Mca1* and *Mca2* might be involved in anaplerotic carbon recycling in *C. reinhardtii* to balance carbon and nitrogen assimilation.

Metabolic acclimation under limiting Ci conditions and its interaction with the CCM

In photosynthetic eukaryotes, different metabolic processes interact dynamically. Photosynthesis, photorespiration, respiration and other metabolic processes are not only connected by common key metabolites and redox equivalents, but also are mutually regulated energetically. Under conditions with changing Ci availability, these processes must be regulated coordinately. The CCM is not an isolated process during limiting CO_2 acclimation. On the contrary, it is highly dependent on other metabolic events during limiting CO_2 acclimation and reciprocally exerts its own influence on them.

i. Photosynthesis

Once induced, the CCM could be considered an integrated functional component in photosynthesis. Meanwhile, photosynthesis plays an essential role for the CCM, by providing an energy supply and by playing a regulatory role. Induction of the CCM disappeared either

in the presence of DCMU, an inhibitor of photosynthetic electron transport, or in a mutant deficient in Rubisco activity (Spalding and Ogren, 1982; Spencer et al., 1983). In both cases photosynthetic electron transport would be inhibited, therefore diminishing the energy supply from the chloroplasts. It is believed that photosynthesis provides energy for the operation of CCM. As in the model mentioned above, acidification of the thylakoid lumen may be involved in dehydration of HCO_3^- to supply CO_2, and, if so, then this process should directly dissipate part of the proton gradient built up by photosynthetic electron transport. Furthermore, it is possible that photosynthetic electron transport may participate in Ci transport in ways other than generating proton gradients. In cyanobacteria, an NADPH dehydrogenase (NDH) mediated cyclic electron transport has been demonstrated to contribute to CO_2 uptake (Ogawa and Kaplan, 2003). Although such an NDH complex has not been demonstrated in *C. reinhardtii* chloroplasts, there is evidence for a plastoquinone-mediated alternative electron transport pathway (chlororespiration pathway). In addition to its role in energy supply, photosynthesis also is required for the expression of low CO_2 inducible genes and regulates the induction of the CCM (see later discussion).

ii. Photorespiration

Oxygen and CO_2 are competing substrates for Rubisco. Oxygenase activity of Rubisco generates phosphoglycolate as the first product in the photorespiratory pathway, and phosphoglycolate is metabolized rapidly to glycolate by phosphoglycolate phosphatase (PGPase) and continues along the photorespiration pathway. In unicellular green algae, glycolate oxidase and peroxisomes are absent. Instead, a mitochondrial glycolate dehydrogenase (GDH) catalyzes the oxidation of glycolate to form glyoxylate. It also has been reported that a glycolate-quinone oxidoreductase system might exist in chloroplasts to oxidize glycolate (Goyal and Tolbert, 1996). This alternative glycolate oxidizing pathway in chloroplasts, together with the low level of GDH detected in *C. reinhardtii* once questioned the significance of GDH in handling glycolate flux under limiting Ci conditions. However, a mutant (*gdh1*) deficient in GDH was recently reported that requires high CO_2 to grow, supporting the contention that GDH does play an essential role in the photorespiratory pathway during limiting Ci acclimation (Nakamura et al., 2005).

When *C. reinhardtii* cells grown in elevated CO_2 are exposed to limiting CO_2, a large amount of glycolate is excreted into the medium. In contrast, limiting CO_2-acclimated cells

secrete almost no glycolate. It was therefore assumed that operation of the CCM could effectively suppress photorespiration. However, using inhibitors for photorespiration, Moroney et al. (1986) found that the CCM could not totally diminish photorespiration in limiting CO_2 even in fully acclimated cells. It seems likely that limiting CO_2 acclimated cells have a higher capacity for metabolizing the glycolate. Indeed, several enzymes in the photorespiration pathway were found to be up-regulated by limiting Ci, including an alanine:α-ketoglutarate aminotransferase (ATT), GDH and PGPase (Chen et al., 1996; Marek and Spalding, 1991; Nakamura et al., 2005). The importance of photorespiration also is demonstrated by a PGPase deficient mutant and a GDH deficient mutant, both of which require elevated CO_2 concentrations to survive (Suzuki et al., 1990; Nakamura et al., 2005). Furthermore, like photosynthesis, photorespiration also has been suggested to play a role in regulating gene expression associated with limiting CO_2 acclimation (see later discussion).

iii. Respiration

C. reinhardtii cells can be grown either autotrophically, with CO_2 as the sole carbon source, or heterotrophically, by consuming acetate. It is known that respiratory substrates, such as acetate, inhibit induction of the CCM under mixotrophic growth conditions (Spalding and Ogren, 1982; Coleman et al., 1992), presumably by suppressing the expression of genes involved in the CCM. However, it is not clear what role respiration plays under autotrophic growth conditions. Relocation of mitochondria in *C. reinhardtii* during limiting Ci acclimation has been reported (Geraghty and Spalding, 1996), but the precise cause and the significance of such a change in cell organization is still unknown. Although mitochondrial respiration-driven Ci transport seems to occur in some marine microalgae (Huertas et al., 2002), it is generally accepted that the energy supply required for the CCM is provided by photosynthesis in most green algae and cyanobacteria. Mitochondria may play other critical roles in limiting Ci acclimation, such as scavenging carbon flux generated in photorespiration, or anaplerotically recycling the released CO_2 generated in mitochondria. Giordano et al. (2003) have reported that conditions favoring the expression of the mitochondrial carbonic anhydrases (mtCAs) usually also stimulate anaplerosis. For example, expression of the mtCA at limiting CO_2 concentrations was diminished when NH_4^+ supply restricted the rate of photoautotrophic growth, and the expression of mtCA was induced at a

supra-atmospheric partial pressure of CO_2 if the NH_4^+ concentration was increased in the growth medium (Giordano et al., 2003).

Furthermore, respiration could influence limiting CO_2 acclimation by regulating energy distribution. Studies have demonstrated that respiration influences linear photosynthetic electron transport and state transitions in *C. reinhardtii* (Cardol et al., 2003). *C. reinhardtii* mutants defective in mitochondrial electron transport had lower linear photosynthetic electron transport activity and stabilized state 2, which favors cyclic over linear electron transport in the chloroplast. Therefore mitochondria might play an indirect role in CCM by influencing the energy distribution. In addition, since limiting Ci will lead to stress situations similar to high light conditions in which dissipation of excess energy is needed, respiration may be necessary at least for early events in limiting Ci acclimation to avoid irreparable damage by photoinhibition before the CCM is fully induced.

Gene expression and signal transduction associated with limiting CO_2 acclimation

Like many acclimation responses to changing environments, new polypeptides are induced in *C. reinhardtii* upon transition from high CO_2 to limiting CO_2. In addition, recent studies have revealed that many genes are either up- or down-regulated quantitatively during the acclimation (Im and Grossman 2001; Miura et al., 2004).Some key signals commonly involved in cellular differentiation and development, such as blue light and cAMP, have been implicated as being involved in regulating limiting CO_2 acclimation. Furthermore, various metabolic events, cell cycle, and circadian rhythm all have been reported to contribute to the regulation of Ci responsive genes (see below). However, little information is available about the signal transduction pathway specifically regulating the functional CCM, and the identity of the signal triggering the adaptive changes remains unknown.

i. Changes in gene expression during limiting CO_2 acclimation

Carbonic anhydrases are among the most abundant proteins induced by limiting CO_2. So far four genes encoding different CAs have been identified as CO_2 responsive genes. Their gene products include periplasmic carbonic anhydrases pCA1 and pCA2, and mitochondrial carbonic anhydrases mtCA1 and mtCA2. Other identified limiting CO_2 induced polypeptides include an alanine-α-ketoglutarate aminotransferase, and two chloroplast proteins of unknown function encoded by *Ccp1* and *Ccp2* (Spalding, 1998).

The limiting CO_2 induced genes show no or a very low expression in high CO_2, but with a great increase in expression upon limiting CO_2 induction. Many other genes, however, maintain their transcripts in both high CO_2 and low CO_2, but exhibit up- or down-regulation upon changing CO_2 concentration. For example, the transcript level of thylakoid lumen CA (*Cah3*) in limiting CO_2 is 2 fold higher than that in high CO_2 (Karlsson et al., 1998). Other genes showing quantitative changes with changing CO_2 levels include *Pgp1*, *Gdh1* and genes encoding several chloroplast ribosome subunits (Suzuki et al., 1990; Nakamura et al., 2005; Wang and Spalding, see Chapter 5). Recent large-scale studies of global gene expression identified many genes that change their expression in quantitatively under different CO_2 levels (Im and Grossman, 2001; Miura et al., 2004). In addition to regulation at the transcriptional level, transient up- or down-regulation at the translational level also has been reported, e.g., transient translational down-regulation of the large and small subunits of Rubisco by limiting CO_2 (Winder et al., 1992).

Differential display and microarray techniques are proving useful to identify limiting CO_2 inducible genes (Im and Grossman, 2001; Miura et al., 2004). Identifying more CO_2 responsive genes will undoubtedly shed new light on our understanding of limiting CO_2 acclimation, but, it also is important to note that not all apparently CO_2-responsive genes are directly involved in the CCM. Since any limitation on the Ci supply affects many metabolic pathways and triggers stress responses, some changes will reflect general responses to changing environments rather than a specific response to Ci supply. Therefore, other approaches are necessary to elucidate the functions of the apparent CO_2 responsive genes in the CCM. Recently RNAi techniques have been shown to be accessible for down-regulation of gene expression in *C. reinhardtii* (Soupene et al., 2004), making it a promising tool for clarifying the functions of some CO_2 responsive genes.

ii. CO2 responsive genes are differentially regulated

There is evidence indicating that different CO_2 responsive genes might be regulated differently during limiting Ci acclimation. Villarejo et al. (1996) reported that several limiting CO_2 inducible polypeptides did not follow the same expression pattern even under the same induction condition. While pCA1 could be induced in darkness, mtCA1/mtCA2 and Ccp1/Ccp2 apparently were induced only in the presence of light. Differential gene expression also was reported in response to the effect of a glycolate pathway inhibitor. While

inhibition of the glycolate pathway depressed the expression of pCA1 and mitochondrial CAs, it had no effect on the synthesis of Ccp1/Ccp2 proteins (Villarejo et al., 1997). Even being highly homologous, the *Ccp1* and *Ccp2* genes exhibited different timing in their expression upon limiting CO_2 induction (Chen et al., 1997). It seems likely that, although all the CO_2 responsive genes respond primarily to changing CO_2, they are regulated by different elements or by multiple regulatory pathways.

iii. Signal(s) regulating CO_2 responsive genes

Carbohydrate metabolism has been implicated as a major influence in regulating gene expression associated with limiting CO_2 acclimation. Photosynthesis and photorespiration both have been suggested to contribute significantly to the regulation of gene expression in response to changing Ci. Several experiments indicated that functional photosynthesis is necessary for induction of CO_2 responsive genes. In the dark or in the presence of photosynthetic inhibitors, no *Cah1* transcripts are induced by limiting CO_2 (Fukuzawa et al., 1990). Based on results using inhibitors of the photorespiratory pathway, Ramazanov and Cardenas (1992) argued that photorespiration and the glycolate pathway are involved in the induction of CA activity and the CCM. It has been suggested that the signal for induction of the CCM depends on the intermediate metabolites from photosynthesis or photorespiration (Spalding et al., 1983; Ramazanov and Cardenas, 1992). However, considering the complexity of interaction among different metabolic processes, generation of the signal may depend on interaction of multiple metabolic pathways.

Although limiting CO_2 concentration seems to be the primary trigger for the changes in gene expression associated with the CCM, limiting CO_2 may not be the signal directly regulating gene expression. Several studies have reported that induction of CO_2 responsive genes, such as *Cah1*, is triggered not only by a low CO_2 concentration, but apparently also by an increased O_2/CO_2 ratio. With increased O_2, transcripts of *Cah1* were reportedly induced in high CO_2 concentrations (Ramazanov and Semenenko, 1984; Villarejo et al., 1996). However, recent work varying the CO_2/O_2 ratio under carefully controlled conditions has called into question any role of O_2 or of photorespiration in regulating at least *Cah1* and *Gdh1* expression (Vance and Spalding, 2005). O_2 might exert its influence on carbon metabolism in many ways, e.g., the O_2 concentration can directly affect the redox state in chloroplasts and the photosynthetic electron transport chain and can change the composition

of metabolites by draining carbon into photorespiration. Many photosynthetic genes have been demonstrated to be regulated by key photosynthetic metabolites or by the redox state of electron carriers (Pfannschmidt et al., 2001; Oswald et al., 2001). Therefore, O_2 may influence the induction of the CCM in an indirect way by affecting photosynthesis and carbon metabolism.

Limiting CO_2 can be viewed as an inductive signal for limiting CO_2 induced gene expression. However, it also is possible that high CO_2 is a suppressive signal. The repression of limiting CO_2 inducible responses by alternative carbon sources (acetate) as well as high CO_2 suggests a possible analogy with other metabolic regulation systems, such as the carbon catabolite repression in yeast. This repression system allows the organism to avoid devoting its resources to unnecessary synthetic activities when a preferred carbon source is present. If this is the case in CO_2 acclimation, then high CO_2 or other available carbon sources may produce a signal to repress the expression of genes necessary for surviving in a limiting CO_2 condition, and in limiting CO_2 such a repression signal for gene expression would be de-repressed.

The known metabolic regulation of gene expression in eukaryotes is complicated. In yeast, the glucose repression signals are transmitted through a series of proteins to the corresponding genes, involving activators, repressors and intermediary elements. Such a regulatory complex may be also involved in controlling CO_2 responses in *C. reinhardtii*, and if so, its elucidation will mostly rely on the identification of components in the signal transduction pathways associated with limiting CO_2 acclimation.

iv. Signal transduction pathway regulating the limiting Ci responses

The major impediment to understanding the signal transduction pathway(s) associated with limiting CO_2 acclimation is inadequate information about the components in the pathway(s). Only one regulatory gene, *Cia5* (*Ccm1*), has been identified (Moroney et al., 1989; Marek and Spalding, 1991, Xiang et al., 2001; Fukuzawa et al., 2001). Cia5 must be among the most important regulators triggering low CO_2 responses, because *cia5* mutants lack all known limiting CO_2 inducible characteristics, including Ci transport activity, induction of CO_2 responsive genes and cell structural changes, observed in wild type cells during limiting CO_2 acclimation.

Identification of Cia5 had been regarded as the most important impetus in studying regulation of the CCM in *C. reinhardtii* Recently two labs were able to clone the *Cia5* (*Ccm1*) gene through different approaches (Xiang et al., 2001; Fukuzawa et al., 2001). The Cia5 protein is a 699-aa hydrophilic protein. A putative zinc-finger motif in its N-terminal region and a glycine repeat characteristic of transcriptional activators suggest that it may be a transcription factor. Near its C-terminus exist several putative phosphorylation sites, and it was speculated that the C-terminus of Cia5 was post-translationally modified in response to a limiting CO_2 signal. Complementation of the *cia5* mutant by a truncated Cia5 protein lacking its C-terminus was reported to confer a constitutive expression of low CO_2 inducible genes (Xiang et al., 2001). However, recent results suggest that the constitutive expression might not be due to the truncation of Cia5 (Weeks, personal communication). It is still not clear how Cia5 regulates CO_2 responsive genes or how this protein associates with other signal transduction components. It seems likely that a limiting CO_2 signal causes modification of the Cia5 protein, and Cia5 then either directly regulates CO_2 responsive genes or regulates downstream signal transduction components. Elucidation of the Cia5 signaling pathway will give us a better insight into regulatory acclimation in changing CO_2 environments.

v. Pathways associated with low CO_2 and very low CO_2 acclimation

In most published studies, the normal atmospheric level of CO_2 (350-400 ppm) is called low CO_2 and used to induce the CCM, while air supplemented with 5% CO_2 is called high CO_2. The relationship of different limiting CO_2 concentrations and induction of the CCM was studied in *Chlorella ellipsoidea*, demonstrating that the expression of dissolved inorganic carbon (DIC) transport was inversely correlated with external DIC concentrations and occurred over a relatively narrow range of DIC concentrations (Matsuda et al., 1998). CO_2 concentrations lower than those in the normal atmosphere have occasionally been used to study the CCM or to screen high CO_2 requiring mutants. Karlsson et al. (1994) compared *C. reinhardtii* cells acclimated in air levels of CO_2 (400ppm) and very low CO_2 (70ppm), and found that cells acclimated in 70 ppm CO_2 showed a higher photosynthetic rate than those acclimated in 400 ppm CO_2. These authors concluded that the maximal capacity to accumulate DIC is inversely related to the CO_2 concentration in the medium. While extracellular and intracellular CA activities were similar in cells grown under both conditions, more ATP was hydrolyzed in the cells grown at 70 ppm CO_2. These data

suggested that a higher capacity of the CCM in very low CO_2 resulted from an active (energy requiring) pumping system.

It has been generally assumed that concentration differences of different limiting CO_2 levels only quantitatively affect CCM induction, an assumption that may be not true, however. Recent studies with the *pmp1* mutant revealed that at least two Ci uptake systems exist, and that they are controlled by different limiting CO_2 levels. The most conspicuous characteristic observed in this mutant is that, in addition to growing in high CO_2 (5% CO_2 in air), it can grow in very low CO_2 (<200ppm), but it dies in air levels of CO_2 (350-400 ppm). These observations suggest that in this mutant, while the CCM is still functional under very low CO_2 conditions, a specific component controlling the active accumulation of Ci is missing at the atmospheric level of CO_2. The *pmp1* mutant could be deficient in a Ci transport system, or a regulator controlling the expression of this transport system. Villarejo et al. (2001) reported that this mutant had reduced activity of chloroplast CA in limiting Ci compared with wild type cells, but it is not clear if the apparent decrease in chloroplast CA is due to a regulatory deficiency. Identification of the *Pmp1* gene would help clarify the function of its gene product and the Ci transport essential for air level CO_2 acclimation. More physiological and biochemical investigations of the very low CO_2 acclimation state also will be necessary.

vi. Blue light, cell cycle and other factors influencing limiting CO_2 acclimation

Evidence suggests that the signal transduction pathway associated with limiting CO_2 acclimation must be integrated with other signal pathways for controlling gene expression. Pathways associated with the blue light signaling, cell cycle and circadian rhythm either are required or interfere with limiting CO_2 acclimation. Dionisio et al (1989a) reported two light requiring steps, a photosynthesis-dependent step and a photosynthesis-independent (blue light) step, controlling *Cah1* induction during limiting CO_2 acclimation. The induction of the *Cah1* gene product, pCA1, also was inhibited by potassium iodide, a flavin quencher, suggesting that flavin may be the blue light sensor in this process (Dionisio et al., 1989b). It is not clear how the blue light signal interferes with the regulation of CO_2 responsive genes, or how this pathway interacts with the Cia5 signal transduction pathway. Regulation of CO_2 responsive genes also is complicated by the cell cycle, which itself is influenced by circadian rhythm (Spalding, 1998). Rawat and Moroney (1995) reported a circadian rhythm in the expression of *Cah1* when using synchronously grown cells, where the expression of *Cah1* in

limiting CO_2 cells was reported to increase in the light period and decrease in darkness. This circadian pattern of gene expression may reflect the complex regulation of CO_2 responsive gene expression by both Ci and the cell cycle (Spalding, 1998).

Perspective

Many components of the CCM have yet to be revealed in *C. reinhardtii*. It is expected that more future work will focus on identification of the functional components in the CCM. This task can be achieved by identification and characterization of non-acclimating mutants, analysis of global gene expression profiles and analysis of the whole genome sequence. The recent availability of the nearly complete *C. reinhardtii* genome sequence (http://genome.jgi-psf.org/chlre2/chlre2.home.html) provides an immense advantage in this area. Taking advantage of this data, a reverse genetic approach can be applied to reveal the functions of many putative CCM components once they are identified via analysis of expression profiles of CO_2 responsive genes and/or analysis of genome sequences. In addition, proteins interacting with known regulatory proteins, such as Cia5, will help us to elucidate the connections and interactions among signal transduction components. Advances in the understanding of carbon metabolism and its regulatory pathways in *C. reinhardtii* will give us better insight into the integrated network associated with carbon acquisition and utilization in photosynthetic organisms.

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Chapter 2. An inorganic carbon transport system responsible for acclimation specific to air levels of CO_2 in *Chlamydomonas reinhardtii*

Abstract

Many photosynthetic microorganisms are capable of acclimating to CO_2 limited environments by induction and operation of CO_2 concentrating mechanisms (CCMs). The CCM is driven by different energy-coupled Ci transport systems which allow these organisms to accumulate a large amount of dissolved inorganic carbon (Ci) within the bulk intracellular spaces. In the green alga *Chlamydomonas reinhardtii*, a *pmp1* mutant has been described with deficiencies in Ci transport, and a Pmp1 protein associated Ci uptake system has been suggested to be responsible for the Ci uptake in low CO_2 (air level) acclimated cells. However, little is known about the identity of Pmp1 and the Pmp1 associated Ci transport system. In the current study, we identified and characterized a mutant, *ad1,* which can not grow in low CO_2 (350ppm) but can grow either in high CO_2 (5% CO_2 in air) or in very low CO_2 (below 200ppm). Genetic and molecular analyses revealed that the *ad1* mutant is allelic to *pmp1*, and the Ad1/Pmp1 protein was discovered to be encoded by *LciB*, a gene previously identified as a CO_2 responsive gene. The *LciB* gene and three related genes in *C. reinhardtii* compose a unique gene family. As demonstrated in yeast two-hybrid analyses, interactions between different members in this family may be important to their function in Ci uptake. Physiological analyses indicated that *C. reinhardtii* possesses multiple Ci transport systems which are responsible for acclimation to different levels of limiting CO_2, and an Ad1/Pmp1 associated transport system is specifically required for low (air level) CO_2 acclimation.

Introduction

In nature, the ambient CO_2 concentrations for photosynthetic organisms can vary across orders of magnitude, and often become the limiting factor for carbon acquisition. To maximize photosynthesis under limited CO_2 conditions, many photosynthetic organisms have evolved different CO_2 concentrating strategies to accommodate the CO_2 variability by increasing CO_2 concentrations at the site of the primary carbon fixation enzyme, ribulose

bisphosphate carboxylase/oxygenase (Rubisco). Some well-known examples of such strategies are C4 photosynthetic pathways adopted by C4 and CAM plants in which CO_2 enrichment is achieved by spatial or temporal separation of ambient CO_2 fixation into C4 acids, and re-release of CO_2 into the C3 pathway at the site of Rubisco. For aquatic photosynthetic organisms, CO_2 limitation often becomes more severe because of the slow diffusion rate of dissolved inorganic carbon (Ci) in aquatic environments. Many aquatic photosynthetic microorganisms have adopted a strategy known as a CO_2 concentrating mechanism (CCM) which allows them to raise the CO_2 concentration to high levels in close proximity to Rubisco.

As demonstrated by many physiological and biochemical studies, the CCM in many aquatic photosynthetic microorganisms operates in an inducible manner, and therefore allows cells to cope with environments with changing CO_2 in a very efficient and flexible way (Spalding 1998; Kaplan and Reinhold 1999). The photosynthesis of cells grown in enriched CO_2 often displays a characteristic similar to that of C3 plants. Once cells confront environments with limited CO_2 availability, their photosynthesis switches to a different status similar to that in C4 photosynthesis, with much higher apparent affinity for CO_2. However, unlike the CO_2 enrichment in C4 or CAM cells, CCMs in aquatic photosynthetic microorganisms is achieved by accumulating a large amount Ci within the bulk intracellular spaces, mainly in the form of HCO_3^-, which is driven by different energy-coupled C_i (CO_2 and/or bicarbonate) transport systems and requires enzymes catalyzing the interconversion between CO_2 and HCO_3^-.

As key components of CCM operation, C_i transporter systems have been extensively studied in the prokaryotic model organisms, cyanobacteria. With the aid of mutant studies and the recent availability of several genomes, at least 4 transport modes involved in C_i uptake have been identified and characterized in different species of cyanobacteria, including two HCO_3^- transporters and two CO_2 uptake systems associated with the operation of specialized NDH-1 complexes (Badger and Price, 2003). These C_i transport systems display different affinities for Ci, and have been demonstrated to express differentially with varying CO_2 availability. In eukaryotic photosynthetic microorganisms, however, Ci transport has been the least understood step in the CCM. Although the unicellular green alga *Chmydomonas reinhardtii* has served as the model system to study CCM for many years, and many genes involved in

limiting CO_2 acclimation have been identified, to date, no Ci transport system has been unambiguously identified and fully characterized in this organism. Recently, several genes have been proposed as candidates for Ci transporter by comparing gene expression profiles of cells grown under different CO_2 conditions, but their roles in the CCM have not been functionally confirmed (Im and Grossman, 2002; Im et al., 2003; Miura et al., 2004). Spalding et al (1983) identified a *C. reinhardtii* mutant, *pmp1*, in which Ci transport apparently was impaired. A recent study has demonstrated that the expression profiles of several CO_2 responsive genes in *pmp1* are different from that in wild type, and suggested that the *Pmp1* gene product might play a role in regulating the expression of genes involved in Ci transport (Miura et al., 2004). Another recent observation in *pmp1* is its air-dieing phenotype (Van et al., 2001; Spalding et al., 2002); it grows well in either high (5%) or very low (<200 ppm) CO_2, but dies in air level of CO_2 (low CO_2, 350-450 ppm). This conspicuous phenotype distinguishes the *pmp1* mutant from many other high-CO_2-requiring (HCR) mutants, and indicates the existence of multiple Ci transport systems in *C. reinhardtii* that correspond to different acclimation states for multiple CO_2 levels. Indeed, a physiological study has demonstrated that at least 3 distinct CO_2 acclimation states exist in *C. reinhardtii*, corresponding to: High CO_2 (H-CO_2), $\geq$0.5% CO_2; Low CO_2 (L-CO_2), 0.4% - 0.03% CO_2; and Very-Low CO_2 (VL-CO_2), $\leq$0.01% CO_2 (Vance and Spalding, 2005). Therefore, the Pmp1 protein must either be directly involved, or play a regulatory role, in a Ci transport system specific for the L-CO_2 (air level of CO_2) acclimation state.

To understand the mechanism of L-CO_2 acclimation in eukaryotic photosynthetic organisms, we have taken an insertional mutagenesis approach in an effort to identify functional components involved in low CO_2 acclimation in *C. reinhardtii*. Here we describe the identification and characterization of a mutant, *ad1,* that displays an air-dieing phenotype. Our results have shown that *ad1* is allelic to *pmp1*, and that the defective gene in each, *Pmp1/Ad1* belongs to a small family of genes encoding an apparently unique group of proteins in *C. reinhardtii.*

Results

Identification of the *air dier 1* (*ad1*) mutant

To isolate and identify *C. reinhardtii* mutants unable to acclimate to low (air level) CO_2, we performed insertional mutagenesis. A plasmid carrying the Ble^R gene (pSP24s; Lumbreras et al., 1998) was used to transform a wall-less, wild type strain, *cw10*. The air dieing phenotype of putative mutants was evaluated in spot tests, based on their ability to grow in high, low or very low CO_2 concentrations. From approximately 2500 transformants, two mutants displayed the air-dieing phenotype, and therefore were named as "*air dier*" (*ad*) mutants. One mutant, *ad1*, was selected for the further investigation. Figure 1 shows the growth of *ad1*. As with *pmp1*, *ad1* can grow as well as wild type in either high CO_2 or very low CO_2, but dies in low (air level) CO_2. In contrast, another mutant, *cia5*, which is defective in acclimation responses to any level of limiting CO_2, can grow similar to wild type in high CO_2, somewhat more slowly in low CO_2, but dies in very low CO_2.

In addition to the *air dier* phenotype, *pmp1* has been shown previously to have reduced Ci transport in comparison to wild type (Spalding et al., 1983). We tested the Ci transport activity in the *ad1* mutant, which exhibited a much reduced accumulation of Ci compared to wild type (Figure 2A).

Photosynthetic O_2 evolution response to C_i concentrations for low CO_2 acclimated and very low CO_2 acclimated wild type and *ad1* cells was compared (Figure 2B). The *ad*1 mutant cells acclimated in low CO_2 showed dramatically decreased photosynthetic activity compared to wild type cells grown under the same condition. When grown in very low CO_2, the photosynthetic O_2 evolution of *ad1* and wild type showed similar responses to external C_i concentrations. These results demonstrate that the *air dier* phenotype in *ad1* is due to an impaired photosynthetic ability in low (air level) CO_2, presumably caused by a deficiency in C_i transport in this CO_2 concentration.

Miura et al. (2004) have reported that the induction of several CO_2-responsive genes by limiting CO_2 was abolished in *pmp1*. These include two putative Ci transporter genes *LciA* and *Mrp1*, and *LciB*, a gene encoding a protein with unknown function. We compared the low CO_2 induced expression of *LciA* and *Mrp1* in the *ad1* mutant with that in wild type (Figure 3A). In wild type cells, expression of both *LciA* and *Mrp1* genes can be induced upon exposure of high CO_2 grown cells to low (air levels) CO_2. In *ad1*, although the expression of

both *LciA* and *Mrp1* could be induced by low CO_2, the expression level was dramatically reduced compared to that in wild type cells. In *pmp1*, the expression of both genes in low CO_2 also was observed, similar to that seen in *ad1* (Figure 3B). The failure in observing the induction of *LicA* and *Mrp1* gene expression in the *pmp1* mutant by Miura et al (2004) may result from their short induction time and different growth conditions.

Identification of the *Ad1* gene

The *ad1* strain was crossed with wild type strain CC620 to determine whether the *air dier* phenotype in the *ad1* mutant co-segregated with the inserted Ble^R gene. More than one hundred random progeny were tested for their growth in different levels of CO_2 and their resistance to zeocin. Fifty random progeny with the air-dieing phenotype all exhibited zeocin resistance, while all zeocin-sensitive progeny showed wild type growth in low CO_2, indicating co-segregation of the *ad* phenotype in *ad1* with the Ble^R insert. DNA gel blot analysis with probes specific for the Ble^R gene and pBluescript sequences indicated a single insertion present in the *ad1* mutant (data not shown).

As detailed in Methods and Materials, DNA flanking the Ble^R gene in the *ad1* mutant was isolated from the *ad1* genomic DNA by inverse PCR. This sequence was used to BLAST against the *C. reinhardtii* genome draft (http://genome-.jgi-psf.org/chlre1/chlre1.home.html), and the site of insertion was shown to be located on scaffold 42 of the genome draft (Figure 4A). Further PCR and DNA gel blot analyses revealed a deletion of a segment of the genomic DNA sequence at the site of insertion in *ad1*, presumably caused by the integration of the Ble^R insert. The deletion was shown to span a region of approximately 36kb that contains several predicted open reading frames. Based on the available sequence of the *C. reinhardtii* genome and fine PCR analysis of the deleted region in *ad1*, we were able to recover the sequence flanking the opposite end of the Ble^R insert by PCR using primers complementing the inserted plasmid and the genomic DNA flanking the insertion.

A sequence flanking the Ble^R insert was amplified by PCR and used as probe to screen a *C. reinhardtii* bacterial artificial chromosome (BAC) library to identify clones containing wild type genomic DNA overlapping the site of the insertion. DNA from two identified clones was used to transform the *ad1* mutant cells and successfully complemented the *ad* phenotype. Complemented cell lines exhibited wild type growth in low (air level) CO_2. The expression of a putative Ci transporter gene, *Mrp1*, in the complemented lines also was

shown to recover to the wild type level from the reduced level seen in *ad1* (Figure 5A). However, attempts to complement the *ad*1 mutant phenotype by using sub-cloned fragments corresponding to several predicted open reading frames in the deleted region were not successful. In addition, Southern and PCR analysis failed to show any of the predicted genes in the deleted region to be present in all complemented lines.

Since several gaps are present on the available genomic sequence at the site of the deletion, we assumed that the *Ad1* gene might be present in one of these gaps. BAC sequencing revealed that one gap contained a missing sequence that is located on another scaffold, scaffold 874 (Fig. 4A), which includes a gene identical in sequence to a previously reported CO_2 responsive gene, *LciB* (Miura et al., 2004). *LciB* was found to be present in all BAC complemented *ad1* lines.

To confirm whether the *LciB* gene is the *Ad1* gene, we amplified a genomic DNA fragment containing the *LciB* gene by PCR from the wild type BACs. The DNA fragment could successfully complement the *ad1* mutant. Complemented *ad1* lines grew as well as wild type in both low and very low CO_2 (Figure 1). Southern analysis indicated that all the complemented lines carried the genomic DNA of the *LciB* gene, which is absent from the *ad1* mutant (Figure 5B). In addition, RNA gel blot analysis showed that all complemented lines recovered the expression of *LciB* (Figure 5C). Complementation of the *ad1* mutant also was achieved by expressing the *LciB* cDNA under the control of the constitutive *PsaD* promoter and terminator (data not shown).

_ad1_ is _pmp1_ allele

The *ad1* mutant appears very similar to the *pmp1* mutant in its *air dier* phenotype, impaired C_i transport and the decreased expression of several putative Ci transporter genes. Crosses between the *ad1* and the *pmp1* mutants failed to produce recombinants or diploids with wild type growth phenotype, suggesting that *ad1* was likely to be a *pmp1* allele. Both the genomic DNA and cDNA of the *LciB* gene that complemented the *ad1* mutant also successfully complemented the *pmp1* mutant (Fig 1, Figure 5B). Comparison of DNA sequences of the *LciB* gene from the *pmp1* mutant and those from the wild type revealed a single nucleotide mutation (C > A) at nucleotide position 105. This mutation results in a stop codon in the place of tyrosine at amino acid 35 of the wild type gene product (Figure 6).

LciB* gene family in *Chlamydomonas

BLAST searches and domain searches of several databases with *LciB* revealed no significant recognizable domains nor significant homologies, except for three additional genes in the *C. reinhardtii* genome (Figure 6): a similar CO_2 responsive gene, *LciC* (Miura et al., 2004), on scaffold 9, and two previously unreported genes, *LciD* and *LciE*, both on scaffold 4 (Figure 4B.).

The *LciB* and *LciC* gene products are quite similar in their amino acid sequence (57% identity; 73% similarity), as are *LciD* and *LciE* (71% identity; 78% similarity), with these two protein pairs also sharing substantial similarity with each other (40-44% identity; 62-65% similarity), thus constituting an LciB protein family. *LciD* and *LciE* are aligned head to head along the genome with another pair of CO_2 responsive genes, *Ccp1* (*LIP36 G1*) and *Ccp2* (*LIP36 G2*) in an apparent inverted repeat (Figure 4B). The inverted regions flank another set of CO_2 responsive genes, *Cah1* and *Cah2*, forming a cluster of six CO_2 responsive genes within a 75 kB region on scaffold 4. The significance of this arrangement is not clear. As noted by Miura et al. (2004), *LciB* and *LciC* gene products are predicted to be soluble proteins, probably targeted into the plastid. This also is the case for *LciD* and *LciE*.

Genes of LciB family as CO_2 responsive genes

Miura et al. (2004) demonstrated that *LciB* and *LciC* were up-regulated by limiting CO_2. Since the two genes share high similarity in their coding sequence, we used gene-specific probes for *LciB* and *LciC* by amplifying unique 3' UTR regions from each cDNA. Northern analysis showed that the *LciB* and *LciC* genes have very similar patterns in terms of the low CO_2 induced expression (Figure 7). In wild type, both genes showed constitutive expression with low abundance mRNA levels under high CO_2 conditions, while both mRNA levels increased dramatically when cells were transferred into either low CO_2 (400 ppm) or very low CO_2 (100 ppm). A level of CO_2 (1500 ppm) intermediate between low and high CO_2 also induced the expression of *LciB* and *LciC* (data not shown), indicating that the up-regulation of the *LciB* and *LciC* gene expression by low CO_2 does not require a CO_2 level as low as that of air. This observation is consistent with the response observed for *Cah1* and *Gdh1* expression (Vance & Spalding, 2005).

There were no ESTs available for *LciD* and *LciE*, indicating that they either are genes with low expression or are genes expressed under conditions different from those used for EST

identification and from those inducing *LciB/LciC* expression. We amplified putative 3'UTR sequences of *LciD* and *LciE* from a cDNA library based on their predicted genomic sequences, and used these specific, amplified probes to analyze the expression of *LciD* and *LciE*. The *LciD* gene showed low CO_2 inducible expression patterns similar to those of *LciB* and *LciC*, but with relative low mRNA abundance (Figure 7). We noticed that there were two bands on RNA gel blots hybridized to the *LciD* probe. We could not detect *LciE* on RNA gel blots by using a predicted *LciE* specific probe, although we did successfully verify the existence of this 3'UTR and the expression of *LciE* by amplifying a partial *LciE* cDNA from a cDNA library and subsequent sequencing of the PCR product. Attempts to identify the full-length *LciD* and *LciE* cDNA by screening a cDNA library with predicted *LciD* and *LciE* probes (coding region) yielded 5 cDNA clones. Sequencing all 5 cDNA clones revealed that they were all from the *LciD* gene, but with two different 3'UTR sequences. Comparison of 3'UTR sequences of *LciD* cDNA clones revealed two 3'UTR sequences with different lengths, indicating that alternative splicing occurred and explaining the two *LciD*-hybridizing bands on gel blots. Both versions of *LciD* mRNA showed similar responses to low CO_2. Attempts to identify a full-length *LciE* cDNA by screening the cDNA library were not successful. It appears that the expression of *LciE* is relatively low compared to other genes in this family.

Proteins interacting with LciB

Physiological and biochemical studies have demonstrated that the LciB protein is involved directly or indirectly in C_i transport. However, being predicted to be soluble, LciB itself can not perform the function as a transporter which requires some transmembrane domains. To be functional in C_i transport, LciB must interact with other proteins either to be directly involved in C_i transport or to regulate C_i transport in some way. To better understand the role played by LciB in C_i transport, we used yeast two-hybrid screens to identify proteins interacting with the LciB protein. In yeast two-hybrid screens with LciB as the bait, 2 classes of LciB interacting proteins (from 28 clones showing strong interaction) were identified from a *C. reinhardtii* cDNA expression library (Figure 8). Sequencing and PCR analysis revealed that one class of the putative interacting proteins (23 of 28 clones) was LciC, either the full length, or near full length amino acid sequence, while another class of was LciB itself (5 of 28 clones). Therefore, it is most likely that LciB and LciC form a protein complex, e.g., a

hetero-dimer, hetero-tetramer or higher-order hetero-complex containing at least LciB and LciC.

Discussion

Ad1/Pmp1, transporter or regulator?

Despite being the major components in the CCM, the C_i transport systems in eukaryotic photosynthetic organisms remain largely unknown. It has been demonstrated that the induction of active C_i transport systems occurs at both the plasma membrane and the chloroplast envelope in *C. reinhardti* (Sultemeyer et al., 1989, Palmqvist et al., 1994). However, to date, no components of Ci transport systems have been unambiguously identified or physically characterized in *C. reinhardti* or other eukaryotic photosynthetic organisms. Mutants defective in C_i transport provide an excellent opportunity to unravel the C_i transport mechanism. Since being identified, the *pmp1* mutant has been touted as demonstrating the C_i transport requirement in the CCM (Spalding et al., 1983). In addition, recent studies suggested that Pmp1 could be involved in regulating the expression of C_i transporters (Miura et al., 2004). In this work, we generated a new mutant allele of *pmp1*, *ad1*, by insertional mutagenesis, and identified the *Ad1/Pmp1* gene. We demonstrated that a lesion in *LciB* (*Ad1/Pmp1*) caused the *ad* phenotype and greatly decreased C_i transport in *ad1* and *pmp1*, presumably only in low CO_2. Neither *ad1* nor *pmp1* mutants can make a functional LciB protein, either because it would be a truncated form *(pmp1)* or because the gene is deleted (*ad1*).

It has been postulated that Pmp1 is a functional component involved in Ci transport. In low CO_2 grown *ad1/pmp1*, both Ci transport and photosynthetic activity were shown to be dramatically reduced. However, based on the amino acid sequence of LciB protein, it seems unlikely that Pmp1/Ad1 (LciB) itself can perform as an intact active Ci transporter. In contrast with most transporters, which always contain hydrophobic transmembrane domains, LciB was predicted to be a soluble protein with no obvious transmembrane regions.

Miura et al. (2004) reported that in *pmp1*, induction or up-regulation of several putative transporter genes, including *LciB* itself, disappeared. They therefore proposed that *Pmp1* is involved in regulation of multiple Ci-uptake systems. In fact, the suggestion by Miura et al. that LciB might be involved in Ci transport was based on its apparent regulation by Pmp1. In

this work we have shown that transcripts of these putative transporters were still present in *pmp1* and *ad1*, although the messenger level in low CO_2 was dramatically reduced. Nevertheless, it appears Pmp1 is involved in some processes that regulate transcript level of putative Ci transporter genes, either by indirectly affecting synthesis of new transcripts or the stability of these transcripts. Unlike Cia5, which is a transcriptional regulator and localized in the nucleus (Fukuzawa et al., 2001; Xiang et al., 2001), LciB is predicted to localize in plastids and is not likely to be directly involved in regulating transcription.

Lacking sequence similarity to any known protein domains or motifs makes it difficult to predict the role played by LciB in Ci transport. Using FingerPRINTScan program (http://www.ebi.ac.uk/printsscan/), we could identify two small motifs in the LciB sequence showing weak similarity to 2 out of 8 elements from a fingerprint providing a signature for the HCO_3^- transporter superfamily. Similar motif sequences also are present in other members in the LciB family. This fingerprint was derived from an initial alignment of 18 sequences from two classes of HCO_3^- transporters in animal cells: Anion exchanger proteins exchange HCO_3^- for Cl^- in a reversible, electroneutral manner, and Na^+/HCO_3^- co-transport proteins mediate the coupled movement of Na^+ and HCO_3^- across plasma membranes, often in an electrogenic manner. These fingerprint elements have not been functionally characterized. As noted previously, LciB also has a weak similarity to a putative GTP-binding motif (DLPGL) conserved in dynamin families (Miura et al. 2004).

Since only limited information on LciB is available, we can not draw any definite conclusion regarding its function. Despite the fact that LciB must somehow be involved in regulating the expression of putative Ci transporter genes, the possibility of direct involvement of LciB in Ci uptake can not be excluded. Physiological evidence from *pmp1/ad1* for LciB being associated with Ci transport, and the presence of putative Ci transporter elements in LciB suggests that LciB may be associated with a Ci transporter, or may be part of a transporter complex. The presence of putative fingerprint motifs belonging to a HCO_3^- transporter superfamily and a putative GTP-binding motif suggests that its function could be involved in an energy dependent transporting process, or a regulatory process. It is possible that LciB regulates the expression of Ci transporter genes by transporting/sensing CO_2 simultaneously, in which case CO_2 sensing and subsequent signaling could be tightly coupled on a Ci transport system associated with LciB.

LciB gene family and LciB/LciC complex

The similarity among the proteins of the LciB family indicates that they are functionally related. However, in *ad1* and *pmp1* a defect in only one gene from this gene family, *LciB*, causes the *ad* phenotype, indicating that genes in the LciB family are not functionally redundant. Although having high sequence similarity, as well as limiting CO_2 inducible expression similar to *LciB*, the *LciC* gene can not compensate for the defects caused by the lesion of *LciB* in *ad1* and *pmp1*, implying that LciC either carries out a different function from LciB (or carries out the similar functions in a different location), or that LciC is required to join LciB to form a complex. LciB/LciC and LciB/LciB interactions demonstrated in our yeast two-hybrid screens indicate that the latter case appears to be true and suggest that these interactions may be required for functioning of an LciB-related Ci transport system. In addition, an LciB/LciC complex may require interaction with other proteins, either to form a functional transporter, or to play a regulatory role in C_i uptake. If so, it will be important to identify other LciB (or LciC) interacting partners of this complex to gain better understanding of its function. Meanwhile, investigation on another pair of proteins in this family, LciD/LciE, may also help us to clarify the roles in Ci uptake played by this gene family.

LciB and Ci transporters in low CO_2 acclimation

The distinct *ad* phenotype of *pmp1/ad1* indicates the essentiality of *Pmp1/Ad1* for low (air level) CO_2 acclimation. This system, although expressed in very low CO_2, appears to be vital only for low CO_2 acclimation, and thus to be dispensable for acclimation at very low concentrations of CO_2, where the *pmp1/ad1* mutants grow as well as wild type. Although we cannot exclude the possibility that an LciB-related Ci transport system contributes to very low CO_2 acclimation, Ci transport systems other than that associated with LciB must be sufficient to provide normal Ci accumulation under very low CO_2 conditions. Similar to other nutrient transport or scavenging systems, an LciB-associated C_i transport system may be a system with relatively low affinity for C_i, while transport systems functioning in very low CO_2 acclimation should be systems with high affinities for C_i. In cyanobacteria, two classes of HCO_3^- transporters have been identified: BCT1, encoded by the *cmp*ABCD operon, is a high affinity Ci transporter belonging to the bacterial ATP binding cassette (ABC) transporter family, and is expressed under severe Ci limitation (Omata et al. 1999);

while the gene product of *sbtA* is a sodium-dependent HCO_3^- transporter with relatively low affinity for Ci (Shibata et al., 2002a, b). In *C. reinhardtii*, two limiting CO_2 inducible genes have been proposed to encode putative Ci transporters: *Mrp1* encodes a protein belonging to an ABC transporter superfamily; *LciA* encodes a protein similar to the chloroplast nitrite transporter, NAR1. Although neither Mrp1 nor LciA has been functionally characterized, it appears likely that they are involved in Ci uptake based on the fact that they are limiting CO_2 responsive transport proteins. In *ad1/pmp1*, expression of both *LciA* and *Mrp1* genes are impaired. If both LciA and Mrp1 are involved in C_i uptake in low CO_2, then down-regulation of both in *ad1/pmp1* might possibly be responsible for the impaired C_i uptake in these mutants. Another possible explanation is that the down-regulation of *Mrp1* and *LciA* expression are the consequence of impaired Ci transport in *ad1/pmp1*, and an as yet unidentified transporter associated with LciB is responsible for the Ci uptake in low CO_2 acclimation. It would be interesting to know how LciB regulates the expression of *Mrp1* and *LciA*, and if there is any physical interaction between LciB and Mrp1 or LciA.

Acclimation to multiple levels of CO_2

The photosynthesis of *ad1* was impaired only in low CO_2 grown cells, which apparently is caused by defective Ci transport. In very low CO_2 grown cells, photosynthesis of *ad1* recovered to a level similar to that in wild type cells. These results revealed the existence of a distinct state for very low CO_2 acclimation, which is different from that in low CO_2 (air level) acclimation in *C. reinhardtii*. Therefore, limiting CO_2 acclimation in *C. reinhardtii* requires at least two suites or combinations of proteins that are differentially expressed or activated in different levels of limiting CO_2. LciB obviously is associated with those specific for the low CO_2 (air level) acclimation. The transporter(s) responsible for very low CO_2 acclimation must be either inactive or not induced in low CO_2.

Our photosynthetic measurements also showed that very low CO_2 grown cells have a relatively high affinity for Ci, but lower photosynthesis at near saturated Ci concentrations. This is consistent with the recent report on different physiological states for limiting CO_2 acclimation in *C. reinhardtii* (Vance and Spalding, 2005). The difference in $K_{½}$ and V_{max} between low CO_2 grown cells and very low CO_2 grown cells implies that the transport system specific for low (air level) CO_2 has a relatively low affinity for Ci, but higher transport capacity, while the system(s) specific for very low CO_2 has a high affinity for Ci,

but a lower capacity. This represents an excellent survival strategy in *C. reinhardtii* for acclimation to different levels of limiting CO_2: in very low CO_2, a Ci uptake system with a high affinity and low capacity would allow *C. reinhardtii* cells to grow at a reasonable rate without depleting all available Ci; while in low CO_2 (air), a high capacity for Ci uptake could maintain optimal growth, and a transporter with relatively low affinity would be sufficient to accommodate the Ci uptake in low CO_2.

The majority of past research on limiting or low CO_2 acclimation in *C. reinhardtii* and other microalgae has focused mainly on air level CO_2 acclimation, while targeted research on very low CO_2 acclimation has been limited. Future investigation of this distinct state should definitely help fill the gap in our understanding of the multiple levels of CO_2 acclimation in *C. reinhardtii*.

Materials and Methods

C. reinhardtii strains, culture, and gas conditions

C. reinhardtii strains CC849, CC620 and CC125 were obtained from *Chlamydomonas* Genetics Center, Duke University. The *pmp1* mutant and the *cia5* mutant have been described previously (Spalding et al., 1983, Moroney et al., 1989). Wild type cells and high CO_2 requiring mutants were maintained on agar plates with CO_2 minimal medium (Geraghty et al., 1990) and kept in Plexiglas chambers at room temperature. Liquid cultures were grown on an orbital shaker at 125 rpm. In both plate and liquid culture, continuous gas flow was maintained through either the growth cambers or the culture flasks. Three gas conditions used in this study were: high CO_2 (5% CO_2 in air v/v) which was obtained by mixing compressed CO_2 gas with normal air; low CO_2 (normal air, 350-400 ppm); and very low CO_2 (50-150 ppm) which was obtained by mixing normal air with either compressed CO_2 free air or CO_2 depleted air (air passed through a saturated sodium hydroxide solution).

Isolation of *air dier* mutants, growth spot tests and genetic analysis

C. reinhardtii wall-less strain CC849 (*cw10*, *mt*$^-$) was transformed with linearized pSP124s plasmid (a gift from Saul Purton, University of London; Lumbreras et al., 1998) by the glass bead method (Kindle, 1990). Transformed cells were kept in high CO_2 and selected on plates with minimal medium supplemented with 10 μg/ml zeocin. Once visible, zeocin resistant transformants were transferred to duplicate plates and subjected to screening by growth spot

tests in high CO_2, low CO_2 and very low CO_2. Mutants exhibiting an air-dier phenotype were then maintained in the high CO_2 chamber.

Spot growth tests were performed by suspending actively growing cells of different strains in minimal medium to similar, low cell densities ($<10^6$/ml), then spotting 3 μL of each cell suspension onto agar plates. Test plates were kept in high CO_2, low CO_2 or very low CO_2 chambers for 8 days.

Genetic crosses and tetrad analysis were performed as previously described (Harris 1989).

Photosynthetic O_2 evolution and Ci uptake

Photosynthetic O_2 evolution was measured at 25 °C with a Clark-type O_2 oxygen electrode (Rank Brothers, Cambridge, England). Cells from liquid cultures were collected by centrifugation and suspended in N_2 saturated MOPS-Tris (25mM, pH 7.3) to a final chlorophyll concentration of 20 μg/ml. Internal and external Ci were first depleted under illumination (500 μmol photons m^{-2} s^{-1}) as judged by cessation of O_2 evolution. The measurements were then initiated by the addition of different concentrations of $NaHCO_3$. The Ci uptake by *C. reinhardtii* cells was measured by the silicone oil filtration technique (Badger et al. 1980; Moroney et al. 1985).

DNA- and RNA-Blot Analysis

Genomic DNA was isolated from cells grown as patches on plates. Cells were resuspended in 250μL of H_2O, followed by addition of 250 μL lysis buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 80 mM Tris-HCl pH 8.0). After extraction twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v), RNase was added and DNA samples were incubated in 37 °C for 30 minutes. The NaCl concentration was then adjusted to 0.7M by adding concentrated NaCl solution, followed by addition of 50 μL of 10% CTAB in 0.7M NaCl and incubation of the samples at 50 °C for 10 minutes. DNA was precipitated with ethanol following a final chloroform extraction.

Total RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (1987).

Southern and Northern analyses were performed by standard procedures (Sambrook et al.1989), and membranes were scanned using a PhosphorImager (Storm)

Isolation of sequences flanking the *Ble*^{R} insert from *ad1* by inverse PCR

*Bam*HI was used to digest the genomic DNA isolated from *ad1* because Southern analysis indicated that this enzyme produced a fragment with a size around 1.5kb, including the partial inserted pSP124s vector and its flanking genomic DNA. *Bam*HI digested *ad1* genomic DNA (0.2 μg) was circularized with 1 unit of T4 DNA ligase (Invitrogen) and then precipitated. The circularized product was used as template for inverse polymerase chair reaction (PCR) using standard PCR procedures. Three pairs of primers were designed, with each pair of primers complementing the sequence of pSP124s in opposite orientations. All three pairs of primers produced PCR products with the correct sizes based on Southern analysis. PCR amplified DNA from one pair of primer (5'-CTGGACCGCGCTGATGAACA-3' and 5'-GGAGGTCGTGTCCACGAACT-3') was sequenced to determine the sequence flanking the insert.

Identification of BAC clones containing the wild type *Ad1* gene and complementation of *ad1* and *pmp1*

DNA flanking the site of insertion in *ad1* was amplified by PCR based on the sequence of the DNA from inverse PCR and the *C. reinhardtii* genome (http://genome-.jgi-psf.org/chlre1/chlre1.home.html). Using the amplified DNA as probe six wild type bacterial artificial chromosome (BAC) clones containing wild type DNA from the inserted region were identified from a BAC library (Clemson University, http://www.genome.clemson.edu/groups/bac/)..

All complementation was performed by the glass bead transformation procedure (Kindle 1990). After transformation, cells were kept in low (air level) CO_2 to observe wild type growth of complemented mutants. Cells transformed with the empty vector or mock DNA were used as controls. For BAC complementation of *ad1*, DNA isolated from 26B2 and 14L14, two BAC clones identified from the BAC library, were used to transform *ad1*.

In complementing *ad1* and *pmp1* with *LciB* genomic DNA, a 4.6 kb fragment of genomic DNA containing the *LciB* coding region and putative promoter region was amplified from a BAC clone (26B2) by PCR using a pair of primers: upper primer, 5'-GAGTAGGCGTCGCGTCGTAA-3'; lower primer 5'-CGACACTGACGGCGCAATTA-3'. The amplified DNA was used to transform *ad1* and *pmp1*.

In complementing *adl* and *pmpl* with *LciB* cDNA, *LciB* cDNA was amplified from a cDNA library (the expression cDNA library for yeast two hybrid screens as detailed next) by PCR with specific primers that introduced a *Nde*I site overhanging the start codon ATG at the 5'end, and an *Eco*RI site following the stop codon at the 3' end. The amplified cDNA was digested by *Eco*RI and *Nde*I and ligated into *Eco*RI/*Nde*I digested pGenD plasmid (Fischer and Rochaix, 2001), which placed the *LciB* cDNA between the *PsaD* promoter and terminator. This plasmid was linearized and used to transform *adl* and *pmpl*.

Identification of proteins interacting with LciB by yeast two-hybrid screens

HybriZAP 2.1 two-hybrid system (Stratagene, La Jolla, CA) was used for yeast two-hybrid analysis of components that interact with LciB. A pooled mRNA (gift from Dr. John Davies) was used to construct the *C. reinhardtii* expression library. This pooled mRNA was the same as the one used for constructing the core libraries described by Shrager et al. (2003), which was isolated from cells grown to mid-log phase in TAP (acetate-containing) medium in the light, TAP medium in the dark, HS (minimal) medium in ambient levels of CO_2 and HS medium bubbled with 5% CO_2 respectively. The cDNA was synthesized using a cDNA synthesis kit (Stratagene) according to the manufacturer's protocol. To reduce secondary structure in the mRNA template, the reverse transcription reaction was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 50°C as suggested by Shrager et al (2003). The cDNA inserts were ligated into the HybriZAP2.1 vector, and all following steps were performed according to the manufacturer's protocol, including packaging, amplification of the HybriZaP2.1 library, *in vivo* excision of the pAD-GAL4-2.1 phagemid vector and final amplification of the excised phagemid library.

To construct the *LciB* bait vector, a cDNA region encoding LciB (amino acid 79-448) was amplified by PCR with specific primers that introduced an *Eco*RI site and a *Sal*I site at 5'end and 3'end, respectively: upper primer, 5'-CTGAATTCCACTCCGAGCTGATCAAG-3'; lower primer, 5'-GATGGTCCGTCGACATGTTGTTAGC-3'. The amplified PCR product was digested with *Eco*RI and *Sal*I, and ligated into the pBD-GAL4 Cam phagemid vector to obtain the *LciB* bait plasmid.

The yeast strain YRG-2 was co-transformed with the bait plasmid expressing *LciB* and the target plasmids containing the cDNA library by sequential transformation according to the manufacturer's protocol. Protein-protein interaction was evaluated by the expression of both

the *HIS3* reporter gene (the growth of transformed cells on selective media without histidine) and the *LacZ* reporter gene (the detection of β-galactosidase activity by the filter lift assay). Plasmid DNA containing candidate cDNAs was isolated from yeast colonies showing a strong interaction (His^+-$LacZ^+$), and then was used to transform the *E. coli* (DH5α) competent cells. The amplified target plasmids and the *LciB* bait plasmid were then reintroduced into the yeast strains YRG-2 by co-transformation to verify the interaction. The specificity of the interaction between the bait and target proteins was verified by co-transformation of the bait plasmid or the target plasmid, with the control plasmids into yeast cells. All control plasmids were described in the manual of HybriZAP 2.1 two-hybrid system. The identity of the cDNA from candidate plasmids was determined by sequencing or PCR with specific primers complementing known genes.

Screening of the cDNA library

For identification of the *LciD* cDNA, a pair of primers was designed based on the sequence flanking the 3'end of the predicted *LciD* coding region: upper primer, 5'-AAGAAAGGCCTCGCTTAACG-3' and lower primer, 5'-GGTACTGGGTGCAAGCTAAT-3', and was used to amplify the putative 3'UTR of *LciD* from the HybriZaP2.1 library by PCR. The amplified PCR product was used as a probe to screen the HybriZaP2.1 library. Five cDNA clones were identified and sequenced.

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Figure Legends

Figure 1. Spot test for growth of *C. reinhardtii* strains in high CO_2 (5% [v/v]), low CO_2 (400 ppm) and very low CO_2 (100 ppm). The strains include wild type (*cw10*, CC125), mutants *cia5*, *pmp1* and *ad1*, and *LciB* complemented *ad1* and *pmp1* cell lines (ad-lb-p1, ad-lp-p3, pmp-lb-p2, pmp-lb-p4). CO_2 conditions are as described in Methods and Materials.

Figure 2. Inorganic carbon (Ci)-dependent photosynthetic O_2 evolution and internal Ci accumulation in wild type *C. reinhardtii* (*cw10*) and the *ad1* mutant. **2A,** (a) Total ^{14}C uptake, and (b) acid- labile intracellular Ci accumulation by low CO_2-acclimated wild type (*cw10*) and *ad1* cells. Cells were acclimated in low CO_2 for 24 hours to induce active Ci uptake. The Chl concentrations were 20 μg/ml, and the initial external Ci concentration was 50 μM ($NaH^{14}CO_3$). The ^{14}C-uptake was measured at pH 7.3 in the light after depletion of external and internal Ci. **2B,** Response of Ci-dependent photosynthetic O_2 evolution to $NaHCO_3$ concentrations in low CO_2-acclimated wild type, *ad1* and complemented *ad1* (ad-lb-p3) cells (left), and very-low-CO_2 acclimated cells (right). The rates for O_2 evolution were measured at pH 7.3, and the Chl concentrations were 20 μg/ml.

Figure 3. *Mrp1* and *LciA* transcript accumulation in wild type, *ad1* and *pmp1* cells. **3A**, RNA gel blot analysis of *Mrp1* and *LciA* expression in wild type (*cw10*) and *ad1* cells. Total RNA (10 μg per lane) was isolated from high CO_2-grown (H) cells and from cells acclimated to low CO_2 (L) for time durations as indicated. RNA loading levels are indicated by ethidium bromide staining of rRNA. *Mrp1* and *LciA* mRNAs were probed with fragments corresponding to *Mrp1* and *LciA* coding region respectively. **3B,** RNA gel blot analysis of *Mrp1* expression in *pmp1*. Total RNA (5 μg per lane) was isolated from high CO_2-grown (H) and low CO_2-acclimated (L) *pmp1* cells. Other experiment conditions were same as in Figure 3A.

Figure 4. Cloning of *Ad1* and genomic organization of the *LciB* gene family. **4A,** Integration of pSP124s and a deletion of ~36 kb of genomic DNA on scaffold 32 in the draft *C. reinhardtii* genome (http://genome.jgi-psf.org/chlre2/chlre2.home.html). Gene models and genomic structure are shown. One gap within the deleted region on scaffold 32 contains a missing genomic sequence located on another scaffold, 874, which includes the *LciB* genomic DNA. A schematic presentation of the exon–intron structure of *LciB* is shown with

exons of the coding region (solid boxes) and introns (black lines) indicated. **4B,** Genomic organization of *LciD* and *LciE* on scaffold 4, illustrating a cluster of six CO_2 responsive genes, including *LciD* and *LciE*, present within a 75 kB region. *LciD, LciE , Cah1, Cah2, Ccp1* and *Ccp2* are indicated by arrows.

Figure 5. Complementation of *ad1* and *pmp1* by *LciB*. **5A,** *Mrp1* transcript accumulation in wild type (*cw10*), the *ad1* mutant, and the complemented *ad1* strains. Total RNA (10 μg per lane) was isolated after high CO_2 grown cells (0 hour) were transferred into low CO_2 for the indicated time durations. RNA loading levels are indicated by ethidium bromide staining of rRNA. Ad-26B5 and Ad-14-1 are two complemented *ad1* strains with DNA isolated from BAC clones containing the *LciB* gene. **5B,** Southern blot analysis indicating the presence of the *LciB* gene in complemented *ad1* and *pmp1* strains (ad-lb-p1 to ad-lb-p5, complemented *ad1* strains; pmp-lb-p1 to pmp-lb-p5, complemented *pmp1* strains). Mutants *ad1* and *pmp1* were complemented with a PCR amplified genomic DNA fragment containing the *LciB* gene. Genomic DNA from wild-type (*cw10*) and complemented *ad1* or *pmp1* strains was digested with *Sal*I, and separated by agarose gel electrophoresis. After transfer of DNA to a membrane, hybridization was performed with a probe specific for the *LciB* gene. **5C,** Northern blot analyses indicating the expression of *LciB* recovered in complemented *ad1* strains (ad-lb-p1, ad-lb-p2 and ad-lb-p3). Total RNA (10 μg per lane) was isolated after high CO_2 grown cells (0 hour) were transferred into low CO_2 for 2 and 4 hours. The hybridization was performed with PCR fragments corresponding to the *LciB* 3'UTR which is specific for *LciB*.

Figure 6. Sequence similarity among the deduced proteins of the LciB family. Amino acids in a blackened or grey-shaded background represent identical or conserved residues, respectively, for the four sequences. The arrow indicates the site of mutation in *LciB* of *pmp1* converting a tyrosine codon to a stop codon.

Figure 7. Expression of genes of the *LciB* family under different CO_2 conditions. RNA gel blot analysis of *LciB, LciC and LciD* expression performed in wild type (*cw10*), *ad1* and *cia5* mutants. Total RNA (10 μg per lane) was isolated from high CO_2-grown and low CO_2 (air) or very low CO_2 (50 ppm) acclimated cells. The time durations for low CO_2 or very low CO_2 acclimation was as indicated. RNA loading levels are indicated by ethidium bromide staining

of rRNA. *LciB* , *LciC* and *LciD* mRNA was probed with PCR fragments specifically corresponding to *LciB, LciC* and *LciD* 3'UTR regions respectively.

Figure 8. Strong interaction between LciB and LciC, and between LciB and LciB as demonstrated in yeast two hybrid analysis. A fragment containing the *LciB* coding region was cloned in-frame into the pBD-GAL4cam plasmid (BD-LB). AD-LC and AD-LB are two plasmids from positive clones identified from the yeast two hybrid screens for LciB interacting proteins. AD-LC is a pAD-GAL4 plasmid containing partial *LciC* coding region without first 39 amino acids. AD-LB is a pAD-GAL4 plasmid containing full length of *LciB* coding region. Pairs of BD and AD plasmids were co-transformed and inoculated on SD plates lacking leucine and tryptophan. Positive control consisted of yeast co-transformed with pBD-WT and pAD-WT, a pair of control plasmids. Interaction between LciB and LciC, or LciB and LciB was scored by streaking co-transformed colonies on SD plates lacking leucine, tryptophan and histidine and observing growth after 3 days. No interaction was observed when plasmids containing LciB or LciC were co-transformed with corresponding control plasmids as recommended in the manual for HyvriZAP-2.1 two-hybrid system.

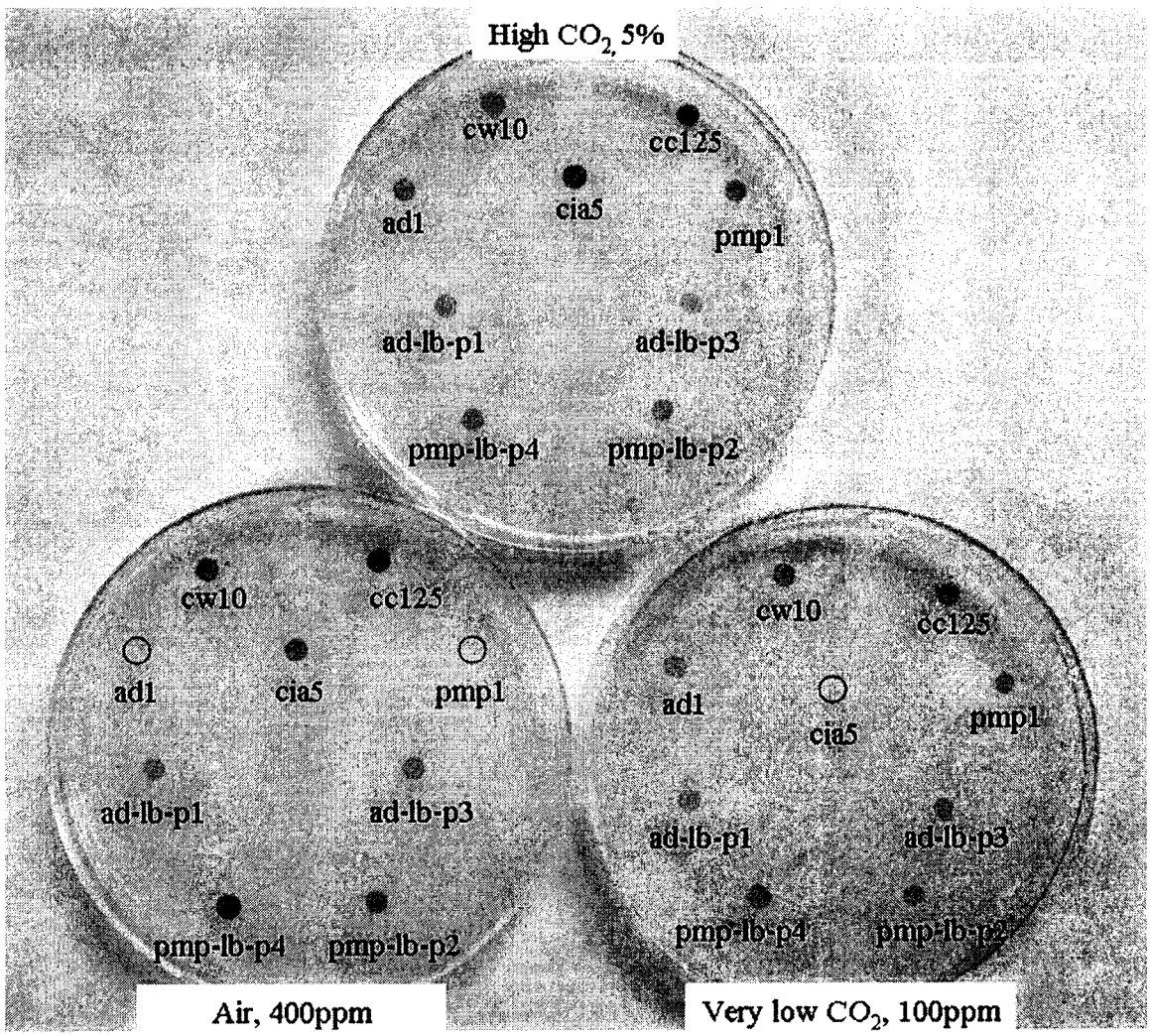

High CO_2, 5%
cw10
cc125
ad1
cia5
pmp1
ad-lb-p1
ad-lb-p3
pmp-lb-p4
pmp-lb-p2
cw10
cc125
ad1
cia5
pmp1
ad-lb-p1
ad-lb-p3
pmp-lb-p4
pmp-lb-p2
Air, 400ppm
cw10
cc125
ad1
cia5
pmp1
ad-lb-p1
ad-lb-p3
pmp-lb-p4
pmp-lb-p2
Very low CO_2, 100ppm

Figure 1.

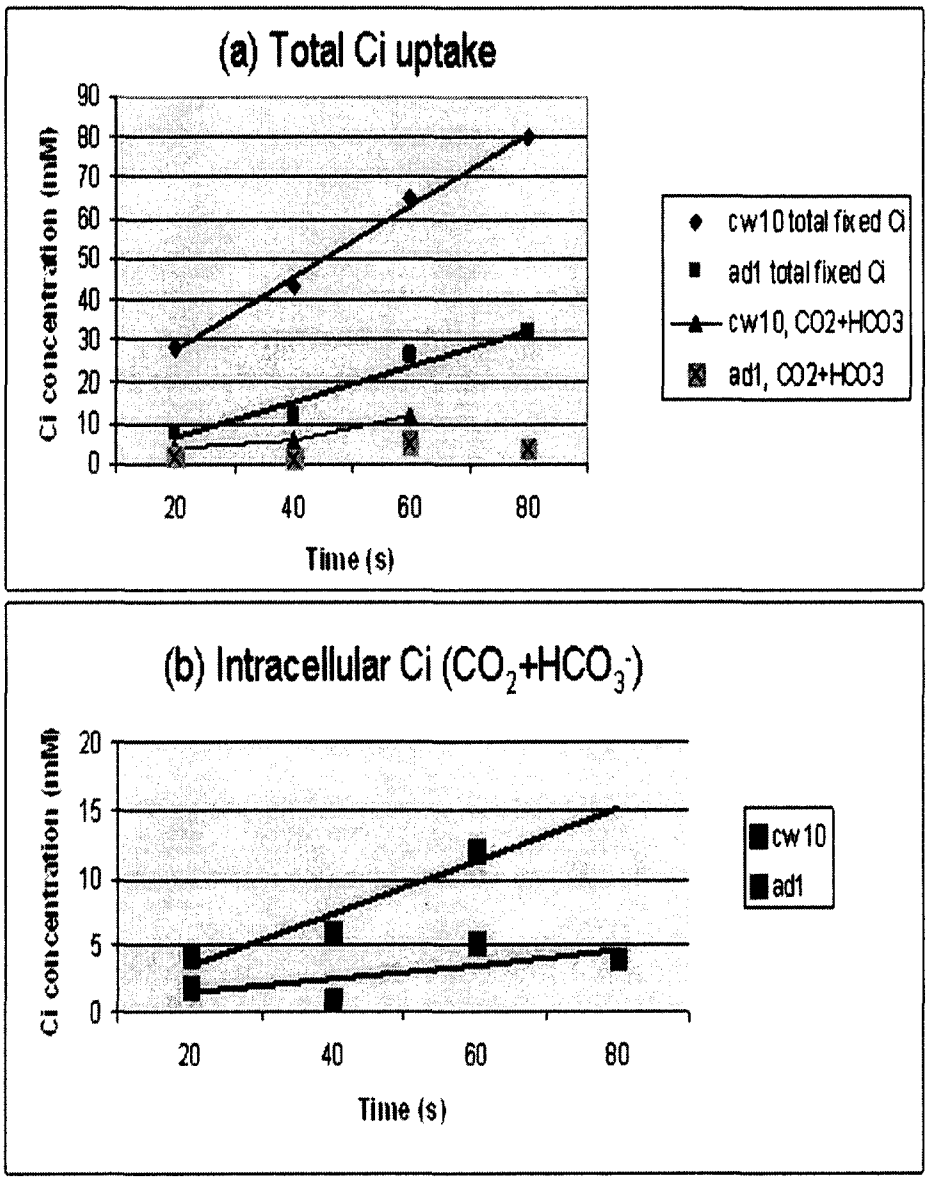
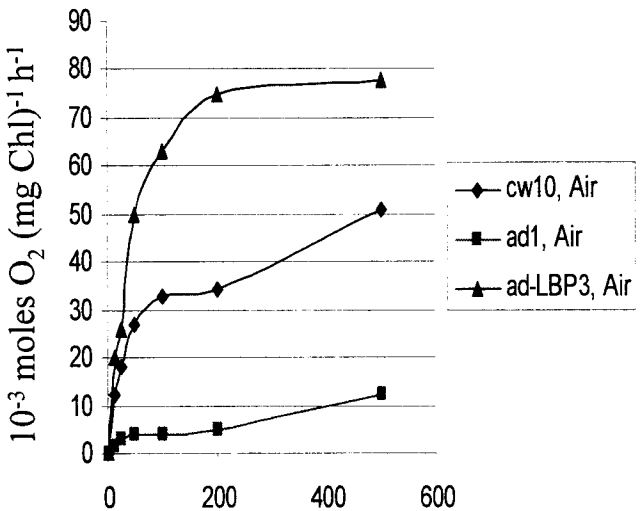

(a) Total Ci uptake
Ci concentration (mM)
90
80
70
60
50
40
30
20
10
0
20
40
60
80
Time (s)
cw10 total fixed Ci
ad1 total fixed Ci
cw10, CO2+HCO3
ad1, CO2+HCO3
(b) Intracellular Ci (CO_2+HCO_3^-)
Ci concentration (mM)
20
15
10
5
0
20
40
60
80
Time (s)
cw10
ad1

Figure 2A.


90
80
70
60
50
40
30
20
10
0
0
200
400
600
10^{-3} moles O_2 (mg Chl)$^{-1}$ h^{-1}
cw10, Air
ad1, Air
ad-LBP3, Air
10^{-3}M $NaHCO_3$

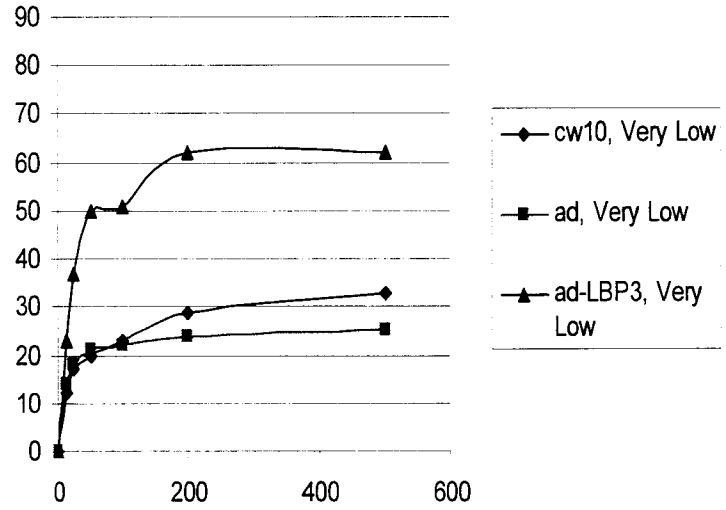

90
80
70
60
50
40
30
20
10
0
0
200
400
600
cw10, Very Low
ad, Very Low
ad-LBP3, Very Low
10^{-3}M $NaHCO_3$

Figure 2B.

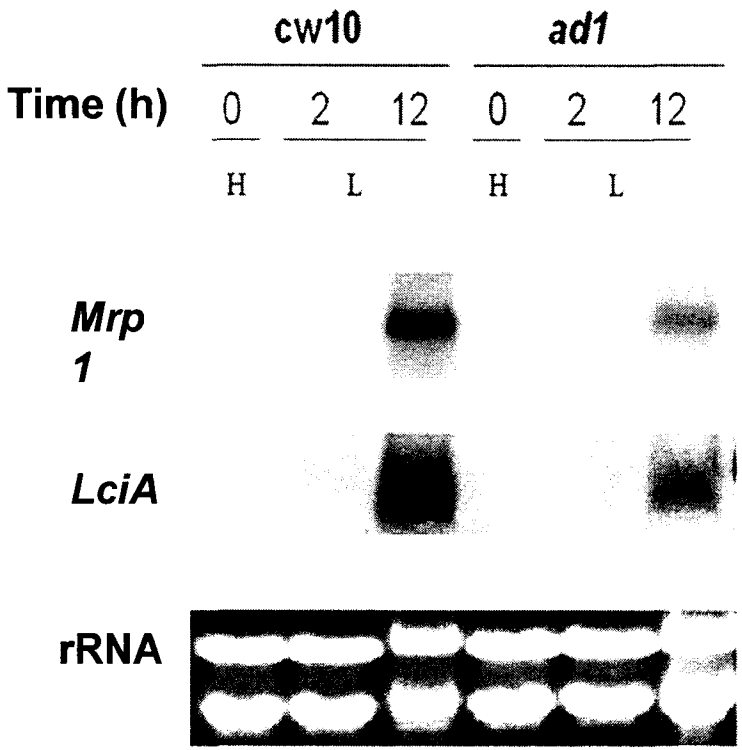


Figure 3A.

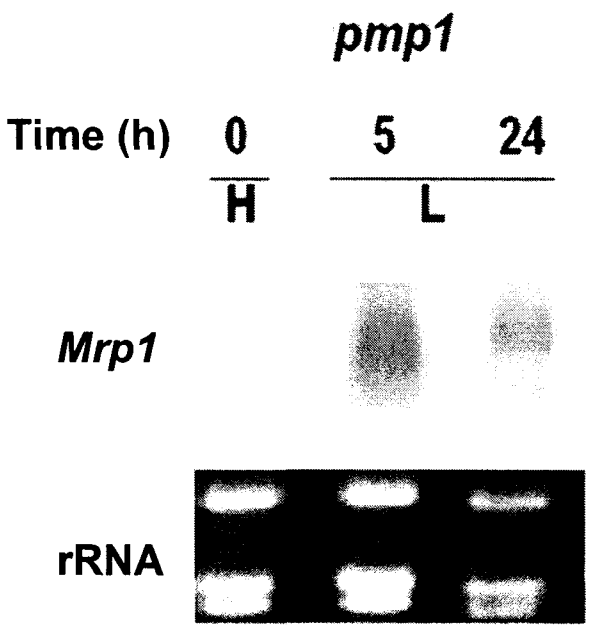


Figure 3B.

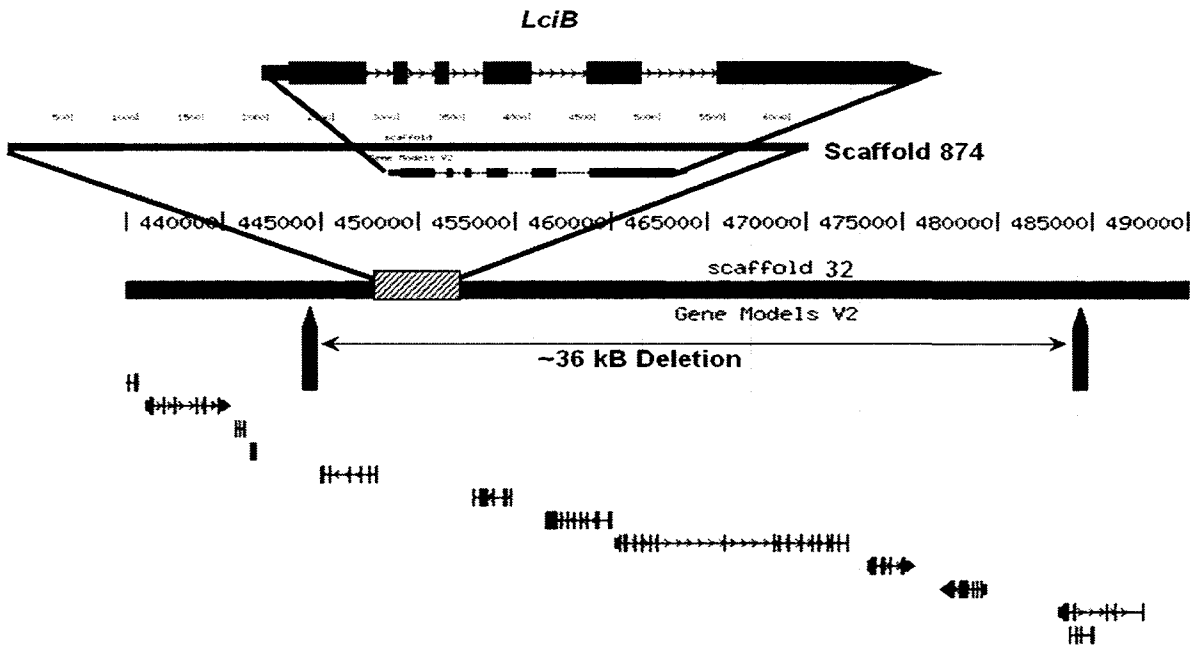


Figure 4A

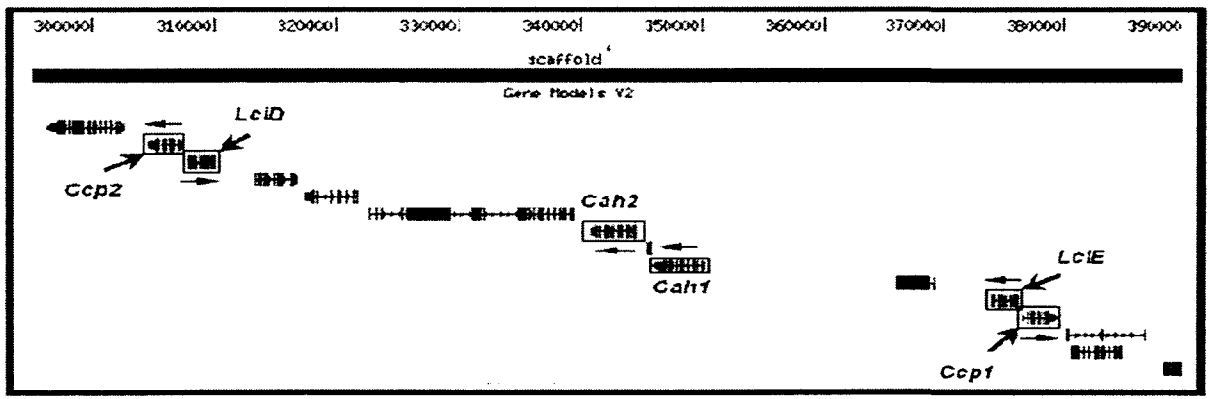


Figure 4B

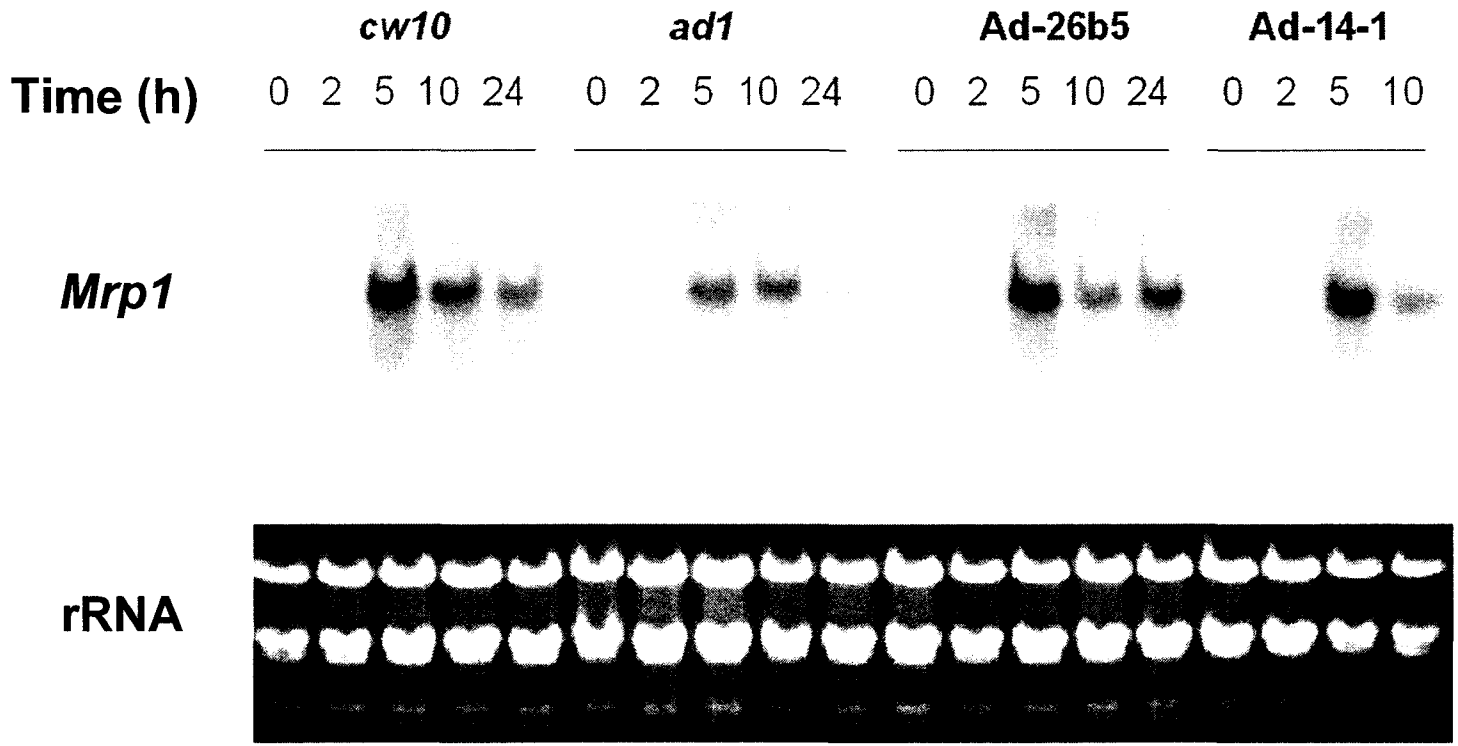

cw10
ad1
Ad-26b5
Ad-14-1
Time (h)
0 2 5 10 24
0 2 5 10 24
0 2 5 10 24
0 2 5 10
Mrp1
rRNA

Figure 5A.

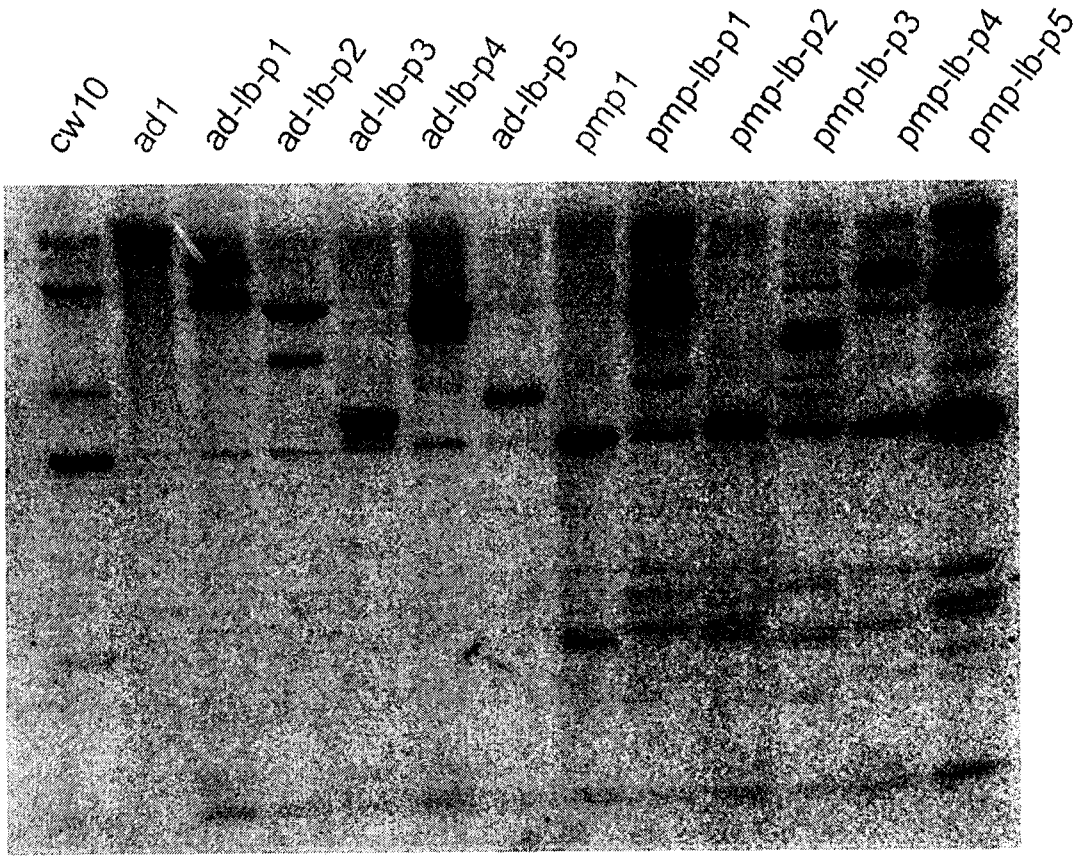

cw10
ad1
ad-lb-p1
ad-lb-p2
ad-lb-p3
ad-lb-p4
ad-lb-p5
pmp1
pmp-lb-p1
pmp-lb-p2
pmp-lb-p3
pmp-lb-p4
pmp-lb-p5

Figure 5B.

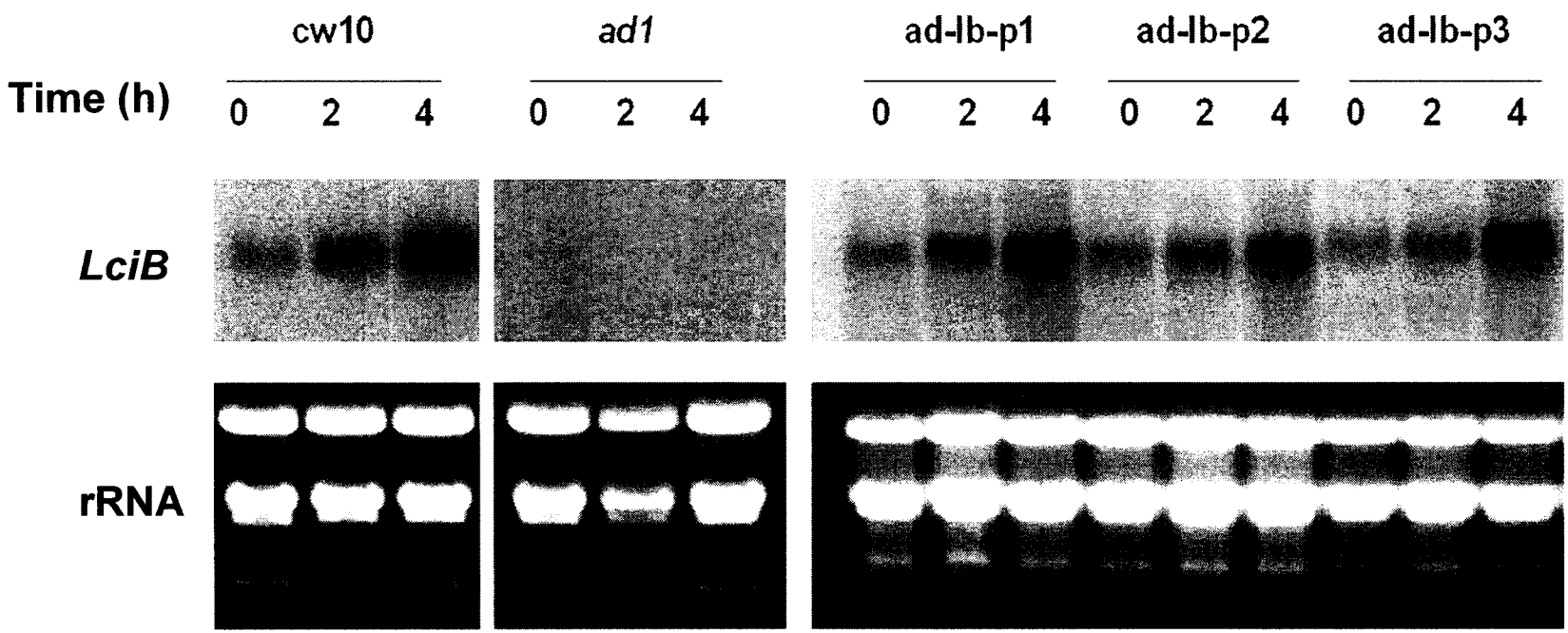

cw10
ad1
ad-lb-p1
ad-lb-p2
ad-lb-p3
Time (h)
0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
LciB
rRNA

Figure 5C

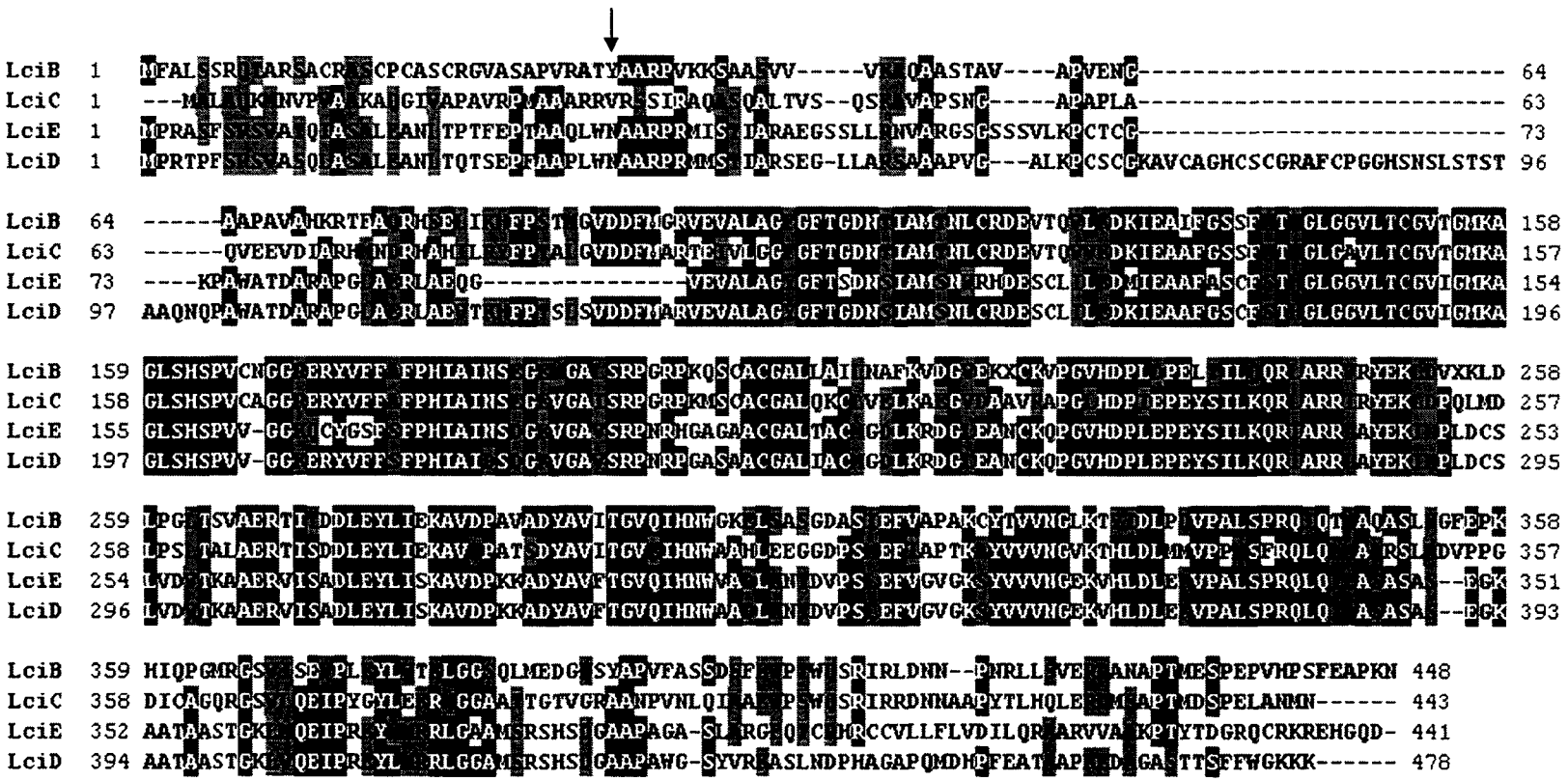


Figure 6

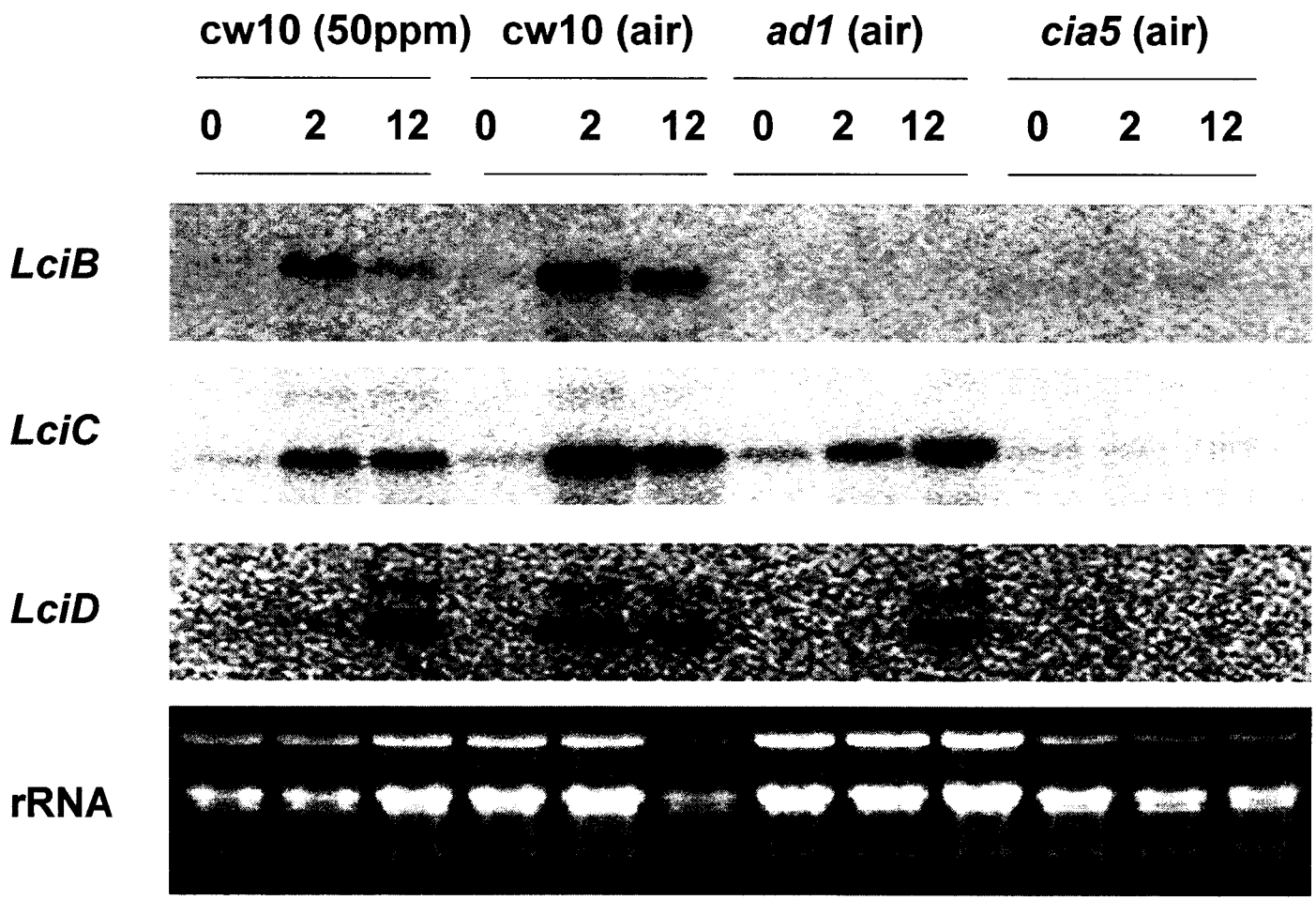

cw10 (50ppm)
cw10 (air)
ad1 (air)
cia5 (air)
0 2 12 0 2 12 0 2 12 0 2 12
LciB
LciC
LciD
rRNA

Figure 7.

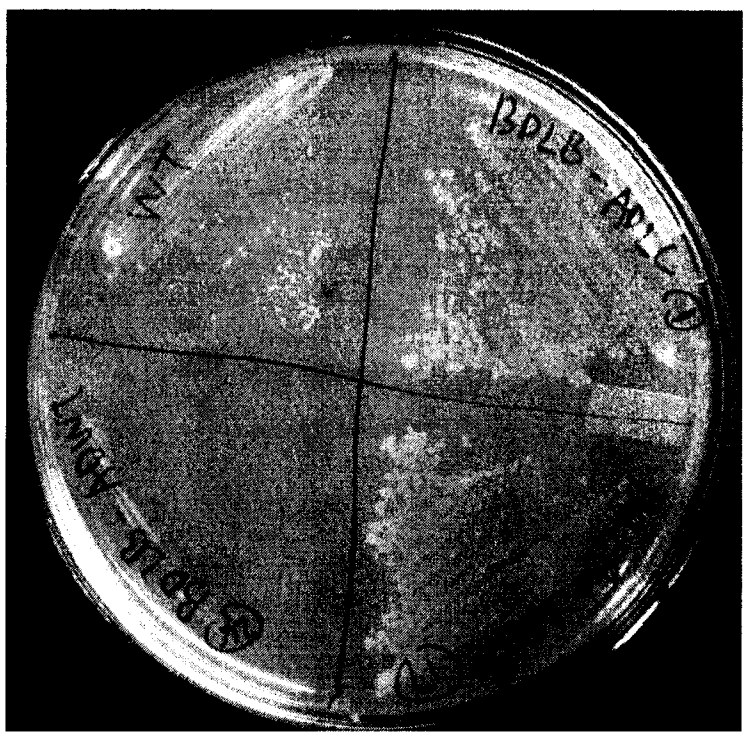


Figure 8.

Chapter 3. Defective 2-oxoglutarate dehydrogenase causes an acetate sensitive phenotype in *Chlamydomonas reinhardtii*

Abstract

A *Chlamydomonas reinhardtii* mutant, *HCR 96*, with a high CO_2 requiring (HCR) phenotype and an acetate sensitive (Acs^-) phenotype was generated by insertional mutagenesis and characterized in this study. *HCR96* exhibited poor photoautotrophic growth in both low (air level) and very low CO_2, and its growth was inhibited by acetate under mixotrophic growth conditions. The mutant, grown either in high CO_2 or low CO_2, displayed a low rate of respiration. Genetic and molecular analyses revealed that the disruption in a gene, designated as *Ogd2*, in *HCR96* was responsible for the Acs^- phenotype. The *Ogd2* gene encodes a mitochondrion targeted protein with similarity to the 2-oxoglutarate dehydrogenase complex (OGDC) E2 subunit from other organisms. The wild type *Ogd2* gene was cloned, and was able to complement the Acs^- phenotype in *HCR96*. Possible explanations for the acetate toxicity in acetate-non-utilizing mutants, as well as the relationship between acetate metabolism and other metabolic pathways with respect to gene regulation are discussed.

Introduction

Species of *Chlamydomonas* are among the acetate flagellates, the term originally used to describe both green and colorless cells which grow well on acetate or other small organic substrates as sole carbon source but cannot use glucose (Harris, 1989). As the name indicates, *Chlamydomonas* species can grow heterotrophically by consuming acetate, although in their native growing environments, they most likely either grow photoautotrophically with CO_2 as sole carbon source, or grow mixotrophically by using both CO_2 and acetate. The ability to dispense with photosynthesis and to use acetate as the sole carbon source makes *Chlamydomonas reinhardtii* a remarkable model organism to study many aspects of photosynthesis and chloroplast biogenesis, as mutants impaired in photosynthesis can be easily isolated and maintained on acetate in the dark.

Like in many other acetate flagellates and organisms that can grow on acetate as the sole carbon source, in *C. reinhardtii*, acetate is believed to be assimilated through the glyoxylate

bypass, an epicycle of the tricarboxylic acid (TCA) cycle (Harris, 1989). In this pathway, acetate is first converted to acetyl coenzyme A (acetyl-CoA), a nearly universal metabolite, by the enzyme acetyl-CoA synthetase. Acetyl-CoA in turn condenses with oxaloacetate to form citrate which is then converted to isocitrate as in the conventional TCA cycle. From there, two key enzymes unique for the glyoxylate cycle, isocitrate lyase and malate synthase, diverge the carbon flux to the bypass that skips two CO_2 releasing oxidative reactions catalyzed by isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase respectively. Therefore, instead of degrading acetyl-CoA into two CO_2 as in the TCA cycle, the net effect of the glyoxylate bypass is to incorporate two molecules of acetyl-CoA to generate a 4-carbon carboxylic acid, which replenishes the intermediate metabolites for the TCA cycle in order to compensate for the loss of carbon skeletons that are continuously withdrawn from the TCA cycle for biosynthetic reactions. Other destinations of acetyl CoA include being oxidized into CO_2 in the conventional TCA cycle, or being used for fatty acid synthesis and many other biosynthetic pathways.

Because acetyl-CoA is a pivotal metabolite centered in anaplerotic, catabolic and biosynthetic pathways, the metabolism of acetyl-CoA and its precursor, acetate, must be dynamically regulated in response to different needs in an organism, as has been demonstrated in bacteria and many other microorganisms. In bacteria, for example, expression of many TCA cycle and glyoxylate bypass enzymes affecting the destination of acetate is regulated in response to the carbon source (Saier et al., 1996; Oh et al. 2002; for review: see Cozzone, 1998). In photosynthetic organisms, however, metabolism of acetate or acetyl-CoA and its regulation are less well understood. Nevertheless, the metabolism and regulation of acetyl-CoA in photosynthetic organisms arguably must be more complex because of the intertwining of photosynthetic metabolism with other metabolic pathways and the need for acetyl-CoA metabolism and generation in multiple compartments (Rawsthorne 2002; Nikolau et al., 2000).

While acetate metabolism is highly regulated, it can also exert its influence on many other metabolic pathways in, e.g., *C. reinhardtii*, where evidence has demonstrated that acetate metabolism under mixotrophic growth conditions can influence many aspects of photosynthesis. For example, Endo and Asada (1996) reported that acetate transiently inhibits photosynthesis, and a recent study using stable carbon isotope ratio mass

spectrometry demonstrated that the fraction of carbon biomass resulting from photosynthesis declined dramatically after acetate was provided, even with saturating light and CO_2 (Heifetz et al., 2000). Photosynthetic carbon gain in *C. reinhardtii* can even be nearly abolished under limiting light or limiting CO_2 conditions, as shown by acetate suppression of photosynthesis via repression of the expression of photosynthetic genes (Goldschmidt-Clermont, 1986; Kindle 1987, Kovacs et al., 2000). In maize, transcription of various genes involved in photosynthesis also was repressed by acetate (Sheen 1990). In addition to photosynthesis, acetate also appeared to repress limiting CO_2 induction of the CO_2 concentrating mechanism in *C. reinhardtii*. Expression of limiting CO_2 inducible genes, such as periplasmic carbonic anhydrase (CA), was decreased when acetate was supplemented (Spalding and Ogren, 1982; Coleman et al., 1991; Fett and Coleman, 1994). In contrast, respiration was stimulated by acetate in *C. reinhardtii* (Fett and Coleman, 1994; Endo and Asada, 1996). Acetate was shown to induce or up-regulate isocitrate lyase and malate synthase in *C. reinhardtii* and higher plants (Martinez-Rivas and Vega, 1993; Petridou et al., 1997; Nogales et al., 2004; Graham et al., 1992). The acetate regulation of metabolism and gene expression appears to reflect the dynamic interaction between photosynthesis, respiration and other metabolic processes in response to changing environments and nutrient conditions.

Because the utilization of acetate provides a sophisticated and flexible model system to study metabolic sensing mechanisms, mutants with defects in acetate metabolism have been extensively investigated in yeast and other eukaryotic organisms (McCammon, 1996; Apirion 1965; Armitt et al., 1976; Owen et al., 1992; Todd et al., 1977). In yeast, many mutants with defects in acetate metabolism (acetate non-utilizing mutants, Acn^-) have been isolated and characterized (McCammon 1996). The defective genes in most such Acn^- mutants appeared to be related to TCA cycle and glyoxylate bypass enzymes, although some mutants appeared to be regulatory mutants defective in acetate metabolism. In photosynthetic organisms, acetate metabolism is intertwined with many metabolic pathways and present in several cellular compartments, making it an intriguing system to study complex metabolic interactions, especially regulation and communication among photosynthesis, mitochondrial respiration and chlororespiration. Recently, several Arabidopsis Acn^- mutants deficient in their ability to utilize or sense acetate were isolated and characterized (Hooks et al., 2004,

Turner et al., 2005). However, the metabolic and signaling network associated with acetate metabolism in photosynthetic cells still remains largely unexplored.

In *Chlamydomonas* species, mutants unable to grow under heterotrophic conditions (dark + acetate), so called *dark dier* (*dk*) mutants, have been investigated with a long history (Lewin 1954; Neilson et al., 1972; Wiseman et al. 1977; Matagne et al., 1989; Colin et al., 1995). Except the *C. dysosmos* mutant described by Lewin (1954) as defective in isocitrate lyase synthase (Neilson et al., 1972), most *dk* mutants identified so far have been characterized as dark$^-$ uniparental minus inheritance (dum) mutants in which defects apparently are associated with the mitochondrial genome (Wiseman et al., 1997, Colin et al., 1995). The normal functions of the defective genes have been demonstrated as mostly being involved in respiratory electron transport. Few *Chlamydomonas* mutants with defects in acetate metabolism have been identified or characterized. In the current research, we describe a *C. reinhardtii* mutant that is unable to grow in the presence of acetate, regardless of whether in light or dark, and thus shows an acetate sensitive (Acs$^-$) phenotype. Molecular characterization revealed that a defect in 2-oxoglutarate dehydrogenase (OGDC) caused the acetate-sensitive phenotype in this mutant.

Results

Identification of *HCR96*, a mutant with high CO_2 requiring (HCR) and acetate sensitive (Acs$^-$) phenotype

HCR96 was identified as a high CO_2 requiring (HCR) mutant in a screen to identify HCR mutants with putative defects in limiting CO_2 acclimation responses. In the screen, insertional mutagenesis was used to isolate mutants with defects in limiting CO_2 acclimation as described in the Methods and Materials section. Basically, strain CC425 (an mt$^+$ wall-less *arg7* mutant) was transformed with linearized plasmids carrying the *Arg7* structural gene as the selectable marker to complement the arginine requiring phenotype, allowing transformants to grow without added arginine. Because transformation in *C. reinhardtii* proceeds by non-homologous recombination, the linearized plasmid integrates into the genome at random locations and may disrupt any gene. Transformants were subsequently screened for growth in high CO_2 *vs* limiting CO_2. Any mutant unable to grow in limiting CO_2

was designated a high CO_2 requiring (HCR) mutant and was selected for further characterization.

As shown in Figure 1, *HCR96* grew very poorly under limiting CO_2 conditions (air level or very low CO_2), while in high CO_2 (5% CO_2 in air) it grew, although apparently somewhat more slowly than wild type. When acetate was provided, most other HCR mutants could grow as well as wild type in low CO_2 or in the dark, but no growth in *HCR96* was observed under those conditions. Therefore, in addition to its HCR phenotype, *HCR96* appeared to be an acetate non-utilizing (Acn^-) mutant. Further tests revealed that *HCR96* could not grow even in high CO_2 in the presence of acetate, even though it otherwise grew well in high CO_2 in the absence of acetate. Thus acetate appeared to inhibit the growth of *HCR96* under all CO_2 conditions. This acetate sensitive (Acs^-) phenotype in *HCR96* distinguished this mutant from other HCR mutants and Acn^- mutants.

To determine genetically whether the insertion of the *Arg7* plasmid caused the HCR and/or Acs^- phenotype, we crossed *HCR96* with CC1068 (mt^- *arg7*) to test for co-segregation of the plasmid with the two phenotypes. If the insertion is linked with the HCR and/or Acs^- phenotype, then all progeny able to grow without arginine should also show the HCR and/or Acs^- phenotype. Otherwise we should be able to observe arginine prototrophic progeny with wild type growth or with the HCR and Acs^- phenotypes separated. Through several attempts, we were unable to obtain any viable arginine prototrophic progeny with either *HCR96* phenotype from tetrad analysis. When random progeny analysis was performed, we obtained arginine prototrophic colonies lacking the HCR or Acs^- phenotype. However, later analysis of these colonies indicated they were not real progeny, but instead were diploids generated during the mating of *HCR96* and CC1068. Thus it appears that a mutation in *HCR96* either prevented the germination of zygotes, or prevented the survival of progeny with the *HCR96* phenotype.

Cloning the defective gene in *HCR96*

Although genetic analysis failed to indicate if the mutant phenotype in *HCR96* co-segregated with the insert, we were able to clone the sequence flanking one end of the insert. Southern analysis revealed a single insert in *HCR96* (data not shown), and the genomic sequence flanking the vector end of the insert was cloned by plasmid rescue. Sequencing of this flanking DNA indicated that the insertion could be localized on scaffold 10 based on the

available *C. reinhardtii* genome (http://genome.jgi-psf.org/chlre2/chlre2.home.html). The disrupted genome region contains an expressed gene, named as *Ogd2* in this research, encoding a protein similar to the 2-oxoglutarate dehydrogenase E2 subunit from other organisms. As indicated by the genome sequence, EST sequences and gene model prediction, the *Ogd2* gene is around 5.3 kb and consists of 14 introns (15 exons). The insert disrupted the *Ogd2* gene inside its 13th exon according to the flanking sequence from the 5'end (Figure 2A). This was confirmed by Southern analysis (data not shown). Although we were unable to clone the flanking sequence from the other end of the insert, PCR analysis revealed that sequence outside the 3'end of the disrupted *Ogd2* gene was still present in the genome, indicating that no large deletion occurred in that region.

Complementation of the Acs$^-$ phenotype

Two BAC clones overlapping the disrupted genome in *HCR96* were identified from a *C. reinhardtii* BAC library using a fragment from the flanking DNA as probeand were used to transform *HCR96* cells. Since the Acs$^-$ phenotype was more stringent in *HCR96* than the HCR phenotype (although very slow, some mutant cells can grow in low or very low CO_2), transformed *HCR96* cells were kept on acetate plates for direct selection of complementation. DNA from both BAC clones was able to complement the Acs$^-$ phenotype of *HCR96* using acetate as the selection, and BAC complemented *HCR96* could grow as well as wild type in the presence of acetate.

To determine if disruption of the *Ogd2* gene was responsible for the Acs$^-$ phenotype, an 8 kb DNA fragment was digested from the BAC DNA with *Nde*I and purified from an agarose gel after separation by electrophoresis, then used to transform *HCR96* cells. This genomic DNA fragment, which includes only the *Ogd2* gene based on the genome sequence, could also successfully complement the Acs$^-$ phenotype in *HCR96*. Southern analysis revealed the presence of the wild type *Ogd2* genomic DNA in all complemented cell lines (Figure 2B) in addition to the defective *Ogd2* gene. Accumulation of *Ogd2* transcripts was detected in wild type cells and complemented *HCR96* lines, while no *Ogd2* transcripts were detected in *HCR96* (Figure 2C). Therefore, we concluded that the defective *Ogd2* gene caused the Acs$^-$ phenotype in *HCR96*.

In the colonies showing wild type growth from tetrad analysis, the presence of the wild type *Ogd2* gene, as well as the disrupted *Ogd2* gene, was also detected by Southern, indicating

these colonies were diploids containing both wild type and mutant genomes (last two lanes in Figure 2B). Because the wild-type *Ogd2* gene complemented the defective copy, these diploid lines demonstrate that the Acs$^-$ defect in *HCR96* is recessive.

Although complementation of the Acs$^-$ phenotype in *HCR96* could be demonstrated, complementation of the HCR phenotype in these cell lines was not conclusive because of the leaky HCR phenotype in *HCR96*. In contrast to the stringent Acs$^-$ phenotype, spot tests have shown that *HCR96* exhibited a leaky HCR phenotype in air or in very low CO_2. Although complemented *HCR96* lines appeared to grow better in low CO_2, they did not grow as well as wild type cells (Figure 1). Therefore, it is not clear whether the HCR phenotype is linked with the Acs$^-$ phenotype and the *Arg7* insert in *HCR96*.

Sequence analysis of 2-oxoglutarate dehydrogenase E2 subunit gene

The nucleotide sequence of the *Ogd2* gene and corresponding ESTs were obtained from the *C reinhardtii* genome. The ESTs sequences and the gene model prediction revealed a 5.3 kb open reading frame, with a predicted transcript length of 2237 bp, a coding sequence of 1353 bp and a deduced protein consisting of 450 amino acids. A BLAST search indicated that *Ogd2* was very similar to genes encoding the 2-oxoglutarate dehydrogenase complex (OGDC) E2 subunit from other organisms: 73% identity with an OGDC gene from *Arabidopsis* (gi:4210332; Machuy et al., unpublished), and 68% identity with a gene from rat (Nakano et al., 2002). Domain analysis revealed that Ogd2 contains a lipoyl binding domain at its N-terminal and a 2-oxoacid dehydrogenases acyltransferase (catalytic) domain at its C-terminal. A mitochondrial targeting peptide at the N-terminal of Ogd2 was predicted (TargetP 1.1; http://www.cbs.dtu.dk/services/TargetP/).

Like other 2-oxoacid dehydrogenase complexes, OGDC includes multiple copies of three components: a 2-oxoacid dehydrogenase/decarboxylase (E1 subunit), a dihydrolipoyl acyltransferase (E2 subunit) and dihydrolipoyl dehydrogenase (E3 subunit). In OGDC, E2 subunits carry the function of the dihydrolipoamide succinyltransferase, which transfers a succinyl group to coenzyme A to generate succinyl-CoA. Since E2 subunits from different 2-oxoacid dehydrogenase share high similarities, we compared the Ogd2 amino acid sequence with the E2 sequence from other 2-oxoacid dehydrogenase complexes (Figure 3A, B). Sequence comparison and phylogenetic analysis indicated that Ogd2 is more similar to the

E2 subunit from 2-oxoglutarate dehydrogenase than that from other pyruvate dehydrogenase complexes.

Decreased respiration in HCR96

The mitochondrial 2-oxoglutarate dehydrogenase complex (OGDC) catalyzes the oxidative decarboxylation of 2-oxoglutarate, and occupies a central position in the TCA cycle. Disruption of *Ogd2* therefore will break the TCA cycle and affect respiration. Indeed, *HCR96* exhibited decreased respiration. The mutant, either grown in high CO_2 or low CO_2, displayed a relatively lower rate of respiration compared to that of wild type (Table 1). Photosynthesis in *HCR96*, however, was not detectably different from that in wild type (data not shown).

Expression of *Ogd2* and other genes in *HCR96*

We determined the expression of *Ogd2* in *HCR96* and wild type cells grown in high CO_2 and cells transferred from high-CO_2 to limiting-CO_2. *Ogd2* exhibited a constitutive expression in wild type cells, and the expression in high CO_2 and limiting CO_2 grown cells did not exhibit an obvious difference (Figure 4A). *HCR96* did not show any *Ogd2* transcript accumulation under any conditions, while the expression of *Ogd2* was recovered in *HCR96* lines complemented by the wild type *Ogd2* gene (Figure 2C).

We also tested the expression of several genes in wild type cells and in *HCR96*. They include the genes involved in respiration and genes responsive to CO_2 (Figure 4A, B). Isocitrate lyase (ICL) and malate synthase (MS) are two enzymes essential for operation of the glyoxylate cycle. RNA gel blot analyses indicated both genes exhibited a nearly steady constitutive expression in wild type under different CO_2 conditions (Figure 4A, B). In addition, except that *ICL* exhibited a relatively lower expression in *HCR96* than in wild type, no obvious differences between *HCR96* and wild type were observed for the expression of *MS* gene and several other genes, including alanine amino transferase gene (*AAT*, Figure 4A), three glutamine synthetase genes (*GS1*, *GS2* and *GS3*, Figure 4B) and a periplasmic anhydrase gene (*Cah1*, not shown).

Discussion

Acetate is a near-universal nutrient for most so-called lower organisms. Many bacteria and eukaryotic organisms, including *C. reinhardtii* and yeast, can use acetate as the sole carbon source to survive. The metabolism of acetate depends on the concomitant operation of the TCA cycle and the glyoxylate bypass. In bacteria and yeast, assimilation of acetate into biosynthesis pathways, i.e., anaplerotic reactions, depends on the glyoxylate bypass, while the degradation of acetate for energy generation, i.e., the catabolic reactions, more depends on the operation of the TCA cycle. In yeast, many acetate non-utilizing (Acn^-) mutants were identified as mutants with defective enzymes in the TCA cycle or the glyoxylate bypass, which demonstrates that both pathways are essential for acetate metabolism (McCammon 1996).

In photosynthetic organisms, the energy generation by mitochondrial respiration appears dispensable since energy can be generated from photosynthesis. Indeed, many *C. reinhardtii dark dier* mutants with defective respiratory electron transport chain can grow photoautrophically in the light (Wiseman et al., 1977; Colin et al., 1995). Although *HCR96* appeared similar to other *dark dier* mutants in terms of acetate non-utilizing (Acn^-) phenotype, its growth was inhibited by acetate in light, which makes *HCR96* different from many other *dark dier* mutants. The acetate sensitive phenotype (Acs^-) displayed in *HCR96* also has been observed in yeast and higher plant respiratory mutants. McCammon (1996) isolated many yeast Acn^- mutants with various defects in enzymes of TCA cycle, glyoxylate bypass, gluconeogenesis, and regulatory factors associated with respiratory carbon metabolism. He observed that acetate inhibited the growth of almost all of the yeast Acn^- mutants when other carbon sources were supplied. Similarly, the Arabidopsis *acn1* mutant and its alleles, with defects in an acyl-CoA synthetase that catalyzes acetyl-CoA formation, also exhibited sensitivity to exogenous acetate (Hooks et al., 2004; Turner et al., 2005). McCammon (1996) proposed two possible mechanisms to explain this surprising toxicity of acetate for yeast growth: (1) defects blocking acetate metabolism cause accumulation or depletion of intermediates that inhibit oxidative metabolism; (2) acetate is preferentially used over other carbon sources for oxidative metabolism even when the cells are unable to metabolize acetate. However, the Acs^- phenotype observed in *HCR96* and Arabidopsis Acn^- mutants suggests that these may not be the case, at least not in photosynthetic organisms.

First, *C. reinhardtii dark dier* mutants, although defective in mitochondrial respiration, exhibited normal growth under photoautotrophic conditions, and a nearly wild type mixotrophic growth in the presence of acetate (Colin et al., 1995). These observations indicate that photosynthesis can compensate for the defective oxidative respiration with regard to energy generation. In addition, the defect in acetyl-CoA synthesis, the first step in acetate metabolism, causes the same Acn$^-$ phenotype in the *Arabidopsis acn1* mutant, suggesting that acetate toxicity must not result from altered levels of intermediate metabolites in acetate metabolism. It seems more likely that acetate itself, when not activated, as in the form of acetyl-CoA, is toxic to cells. High concentrations of acetate have been shown to inhibit *C. reinhardtii* growth (Chen and Hohns, 1997). Expression of a yeast acetyl CoA hydrolase in the mitochondrion of tobacco plants, which should increase the ratio of acetate to acetyl-CoA, was shown to inhibit growth and to restrict photosynthesis (Bender-Machado et al., 2004). In Acn$^-$ mutants, blocking acetate metabolism elevates the intracellular acetate concentrations, and acetate or its acid form, acetic acid, may be detrimental to normal cellular function at such high concentrations.

Acetate has been demonstrated to repress photosynthesis and the CO_2 concentrating mechanism by regulating gene expression (Kovacs et al., 2000; Fett and Coleman, 1994). However, it is not clear by what means acetate is involved in this gene regulation. It was suggested that the effect of acetate might result from the intermediate metabolites in acetate metabolism or acetate involved metabolic pathways (Fett and Coleman, 1994). In addition, as demonstrated in a study by Kovacs et al. (2000), consumption of ATP by acetate assimilation in the glyoxylate cycle resulted in decreased ATP content and subsequent retarded carbon assimilation by the Calvin cycle, and that the increased NADPH/ATP ratio brought about an over-reduction of the inter-photosystem electron transport components. These authors suggested that regulation of photosynthesis gene expression by acetate metabolism was controlled by the redox state. We tested the expression of several genes in different CO_2 conditions, which includes genes involved in the glyoxylate cycle, photorespiration and possibly limiting CO_2 acclimation, and found no obvious difference between *HCR96* and wild type. It will be interesting to investigate whether acetate controlled gene expression is altered in *HCR96*, which may help in gaining a better understanding of acetate regulated gene expression.

Molecular analyses indicated that *HCR96* was a respiratory mutant defective in the *Ogd2* gene, which encodes the 2-oxoglutarate dehydrogenase E2 subunit. Although *HCR96* exhibited an HCR phenotype, we were unable to confirm whether the HCR phenotype was caused by the defective *Ogd2* gene. It is very likely that the impaired TCA cycle activity resulting from the defective OGDC would affect other metabolic pathways. In photosynthetic eukaryotic organisms, photosynthesis, photorespiration, respiration and other metabolic processes are not only connected by common key metabolites and redox equivalents, but also are mutually regulated energetically. The mitochondrial OGDC occupies a central position in cellular metabolism within the TCA cycle, and its substrate, 2-oxoglutarate, is located at a pivotal interface between carbon and nitrogen metabolism. Being one of the essential carbon metabolites in carbon metabolism and the major carbon skeleton for nitrogen assimilation, 2-oxoglutarate connects the TCA cycle, nitrogen assimilation, photorespiration and amino acid biosynthesis. The level of 2-oxoglutarate therefore reflects the carbon/nitrogen status. 2-oxoglutarate has been suggested as a signal molecule involved in the regulation of carbon/nitrogen metabolism. Several studies suggest that this organic acid participates in the PII signaling system that regulates carbon/nitrogen distribution in bacteria and cyanobacteria (Hodges 2002). The binding of 2-oxoglutarate with the PII protein has been demonstrated. In *Arabidopsis*, a putative 2-oxoglutarate sensing protein, GLB1 was discovered and appeared to be involved in carbon/nitrogen sensing (Hsieh et al, 1998). Furthermore, 2-oxoglutarate also is a direct regulator of enzymes such as cytosolic pyruvate kinase and PEP carboxylase, mitochondrial citrate synthase and alternative oxidase (Hodges, 2002). Therefore, in *HCR96*, the possible increased level of 2-oxoglutarate caused by the defective OGDC could have a profound influence on carbon/nitrogen metabolism. Since not many mutants with defects in respiratory carbon metabolism have been identified in photosynthetic organisms, *HCR96* could serve as a valuable system to study the role of 2-oxoglutarate in regulation of carbon and nitrogen metabolism.

Methods and Materials

Cell strains and culture conditions

C. reinhardtii strains, CC125 (wild type), CC849 (*cw10 mt⁻*), CC425 (*arg2 cw15 mt⁻*) and CC1068 (*arg2, mt⁻*) were obtained from the *Chlamydomonas* Genetics Center (Duke

University). All strains were maintained on plates with CO_2 minimal medium as previously described (Geraghty et al., 1990), except that arginine was supplemented for strains CC425 and CC1068. Liquid cultures were grown on an orbital shaker under aeration with 5% CO_2 in air (high CO_2-grown cells) or with different limiting CO_2 concentrations. Cell cultures were switched from elevated CO_2 to one or more limiting CO_2 concentrations for different durations of induction as indicated in the experiments.

Both on plates and in liquid cultures, continuous gas flow was maintained through either the growth cambers or the culture flasks. Three gas conditions used in this study were: high CO_2 (5% CO_2 in air v/v) which was obtained by mixing compressed CO_2 gas with normal air; low CO_2 (normal air, 350-400 ppm); and very low CO_2 (50-150 ppm) which was obtained by mixing normal air with either compressed CO_2 free air or CO_2 depleted air (air passed through a saturated sodium hydroxide solution).

Generation of mutants, growth spot tests and genetic analysis

Glass bead transformations were performed as described previously (Kindle, 1989). A linearized pArg7.8 plasmid was used to transform CC425 cells. Transformed colonies were screened by spot tests in different CO_2 concentrations to identify limiting CO_2 non-acclimating mutants. After transformant colonies were transferred to replica plates, replicas of each plate were placed in high CO_2, low CO_2 and very low CO_2 chambers.

Mutants identified in this primary screen as having a high CO_2-requiring (HCR) phenotype were screened again using more careful spot tests. For spot growth tests, active growing cells were suspended to similar cell densities with minimal medium, spotted (3 µl) onto agar plates and incubated with different concentrations of CO_2 for 10 d (Harris, 1989). To identify acetate non-utilizing mutants or acetate sensitive mutants, suspended cells were spotted onto plates with CO_2 minimal medium supplemented with 0.01M sodium acetate. Genetic analyses were performed by crossing *HCR96* with strain CC1068 (*arg*$^-$, *mt*$^-$) according to the protocol of Harris (1989).

DNA and RNA blot analysis

Genomic DNA was isolated from cells grown as patches on plates. Cells were resuspended in 250µL of H_2O, followed by addition of 250 µL lysis buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 80 mM Tris-HCl pH 8.0). After extraction twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v), RNase was added and DNA samples were incubated in 37 ^{o}C for 30

minutes. The NaCl concentration was then adjusted to 0.7M by adding concentrated NaCl solution, followed by addition of 50 μL of 10% CTAB in 0.7M NaCl and incubation of the samples at 50 °C for 10 minutes. DNA was precipitated with ethanol following a final chloroform extraction.

Total RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (1987).

Southern and Northern analyses were performed as standard procedures (Sambrook et al.1989). Hybond-N^+ nylon transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used for blotting, and membranes were scanned using a PhosphorImager (Storm)

Cloning of the genomic sequence flanking the insertion by plasmid rescue

Southern analysis was performed to evaluate restriction enzymes to be used in plasmid rescue, identifying *Pvu*II as releasing a fragment with a proper size for plasmid rescue and containing the vector and DNA flanking the insert. Genomic DNA (0.5 μg) isolated from the *HCR96* mutant was digested with *Pvu*II, and then circularized using 1 unit of T4 DNA ligase (Invitrogen) after phenol extraction. The circularized DNA was used to transform *E. coli* strain DH5α. Purified plasmids were then verified by restriction enzyme digestion and sequenced.

Identification of BAC clones and complementation of mutants

A 1.4kb fragment was purified from an agarose gel after electrophoresis of *Sst*I digested plasmid containing *HCR96* genomic flanking DNA. This DNA was used as a probe to identify BAC clones (Clemson University, http://www.genome.clemson.edu/groups/bac/) containing the wild type *Ogd2* DNA.

All complementation was performed by the glass bead transformation procedure (Kindle, 1990). After transformation, cells were kept in selective conditions (plates with CO_2 minimal medium and 0.01M sodium acetate) to observe wild type growth of complemented mutants. Cells transformed with either the empty vector or mock DNA were used as controls.

Respiratory oxygen consumption

Respiratory oxygen consumption was measured at 25 °C with a Clark-type O_2 oxygen electrode (Rank Brothers, Cambridge, England). Cells from liquid cultures were collected by centrifugation and suspended in MOPS-Tris (25mM, pH 7.3) to a final chlorophyll

concentration of 20μg/ml. The respiratory oxygen consumption was measured after the cells were transferred into the reaction vessel and kept in the dark.

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Figure legends

Figure 1. Spot test for growth of *C. reinhardtii* wild type (*cw10*), mutants *cia5*, *pmp1* and *HCR96*, and *Ogd2* complemented *HCR96* cell lines (N2B1, N2B2, 3c1, 3c2, 3c2, 2b1 and 2b2) in high (5% [v/v]), low (400 ppm) and very low (100 ppm) CO_2. CO_2 conditions are as described in Methods and Materials.

Figure 2. Cloning of the *Ogd2* gene and complementation of *HCR96* by *Ogd2*. (A) Restriction map and gene organization of *HCR96*. The *Arg* insertion site is indicated by an inverted triangle. The sequence flanking the *Arg* insert was cloned by plasmid rescue through the indicated *Pvu*II sites. The *Ogd2* gene is shown with boxes as exons and black lines as introns. The probe for Southern blot analysis also is shown. (B) Southern blot analysis of *Pvu*II/*Dra*I digested genomic DNA from wild type (CC425), *HCR96* and complemented *HCR96* strains using the *Ogd2* gene probe. The last two lanes contain DNA isolated from the diploid colonies with wild type growth (see Results). The *HCR96* mutant was complemented with either the DNA from BAC clones containing *Ogd2* (3c1, 3c2, 3c2 and 2b2) or with 8kb *Nde*I digested genomic fragments (N2B1, N2B2) as indicated in 2A. All complemented cells grew in the presence of acetate. (C) Northern blots analysis of *Ogd2* expression in wild type, *HCR96*, and complemented *HCR96*. Total RNA was isolated from high CO_2-grown cells (H) or air adapted cells (L) for 10 hours. Hybridization was performed with the *Ogd2* gene probe.

Figure 3. Comparison of Ogd2, 2-oxoglutarate dehydrogenase E2 subunit (OGD2) and pyruvate dehydrogenase E2 subunit (PDH2) from other organisms. **3A,** Alignment of Ogd2 and other OGD2s and PDH2s. *C. reinhardtii* Ogd2 (C_100022) and PDH2 (PDE2) sequences were obtained from *C. reinhardtii* genome site (http://genome.jgi-psf.org/chlre2/chlre2.home.html). Accession numbers for the OGD2 sequences are as follow: (*Saccharomyces cerevisiae*, P19262; *Arabidopsis*, XP_472312 CAA11553; *E. coli*, NP_415255; Human, BAA06836; Rat, Q01205); and for the PHD2: (*S. cerevisiae*, P12695;*Arabidopsis*, CAA86300; *E. coli*., NP_414657; Rat, NP_112287). **3B**, Unrooted phylogenetic tree of Ogd2, OGD2 and PDH2 protein sequences. Sequences were analyzed and the phylogenetic tree generated using ClustalX.

Figure 4. Gene expression time courses in wild type and *HCR96* cells. **4A**, RNA gel blot of *Ogd2, ILC, ATT* expression in wild type (*cw10*) and *HCR96* cells. Total RNA (10 μg per lane) was isolated from high CO_2-grown (0h) and air-acclimated cells at the indicated times

after transfer. RNA loading levels are indicated by ethidium bromide staining of rRNA. The constitutive expression of a gene coding a G-protein β-subunit-like polypeptide is shown as a control. **4B,** RNA gel blot of *MS, GS1, GS2, GS3* expression in wild type and *HCR96.* Experiment conditions were the same as in Figure 4A.

Table 1.

Respiration of *HCR96* and wild type cells
($nmolO_2/mgCHL/min$)

	High CO_2		Low CO_2	
		SD (n=3)		SD (n=3)
cw10	430.9448	12.33749	480.4805	34.40372
HCR96	176.1655	8.717932	222.9532	49.44353

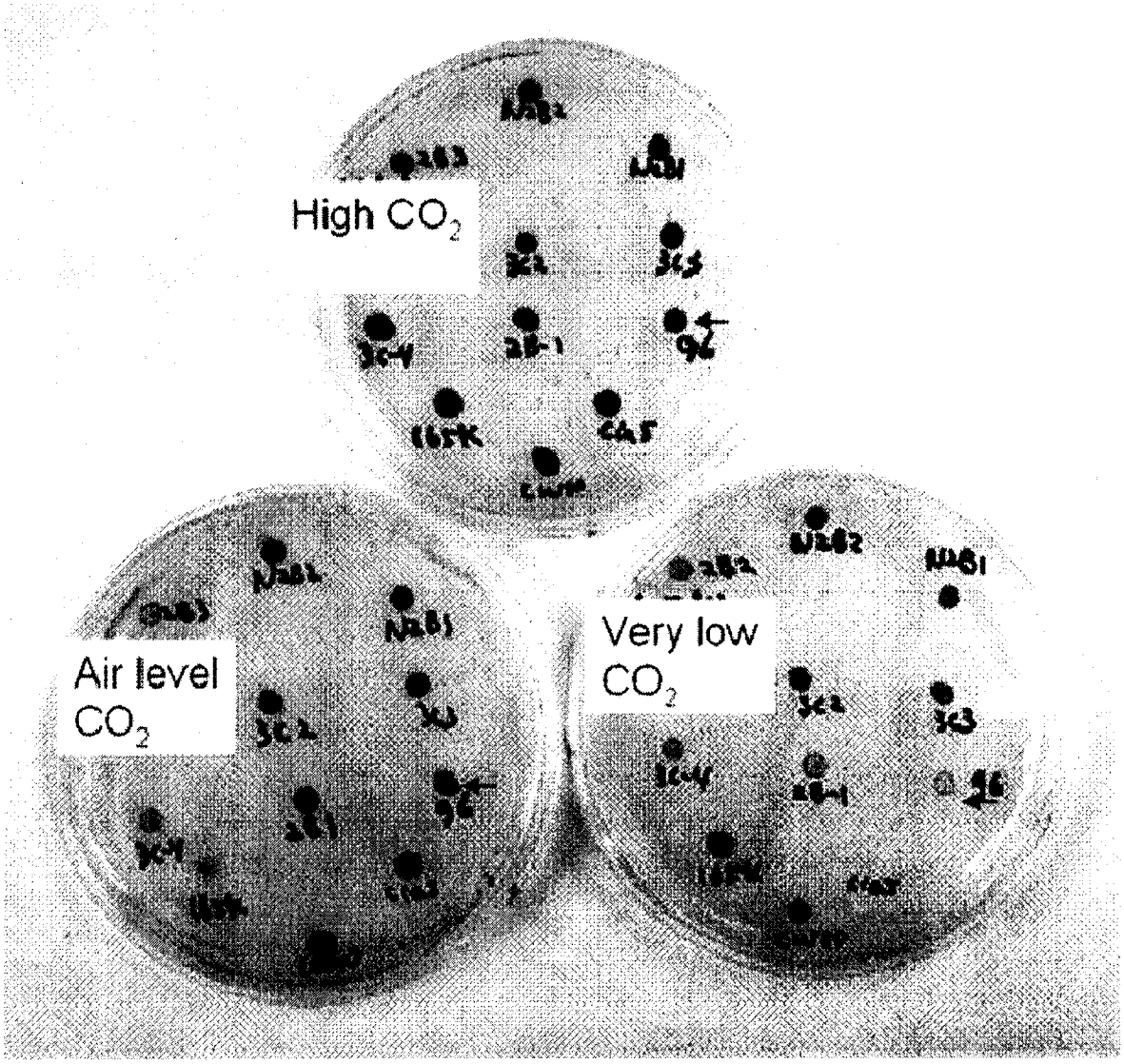

Figure 1.

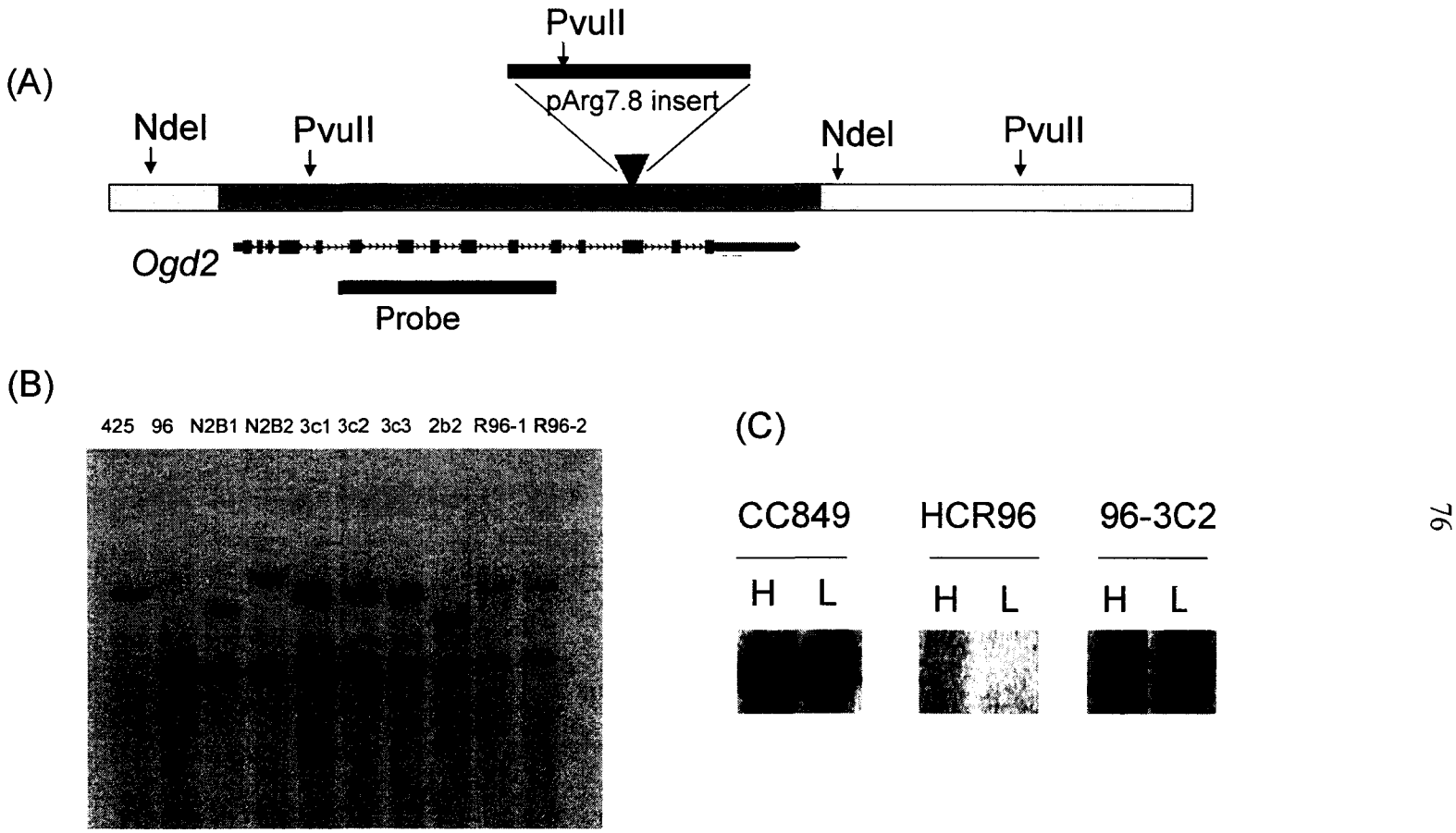
(A)
PvuII
pArg7.8 insert
NdeI
PvuII
NdeI
PvuII
Ogd2
Probe
(B)
425 96 N2B1 N2B2 3c1 3c2 3c3 2b2 R96-1 R96-2
(C)
CC849
HCR96
96-3C2
H L
H L
H L

Figure 2.

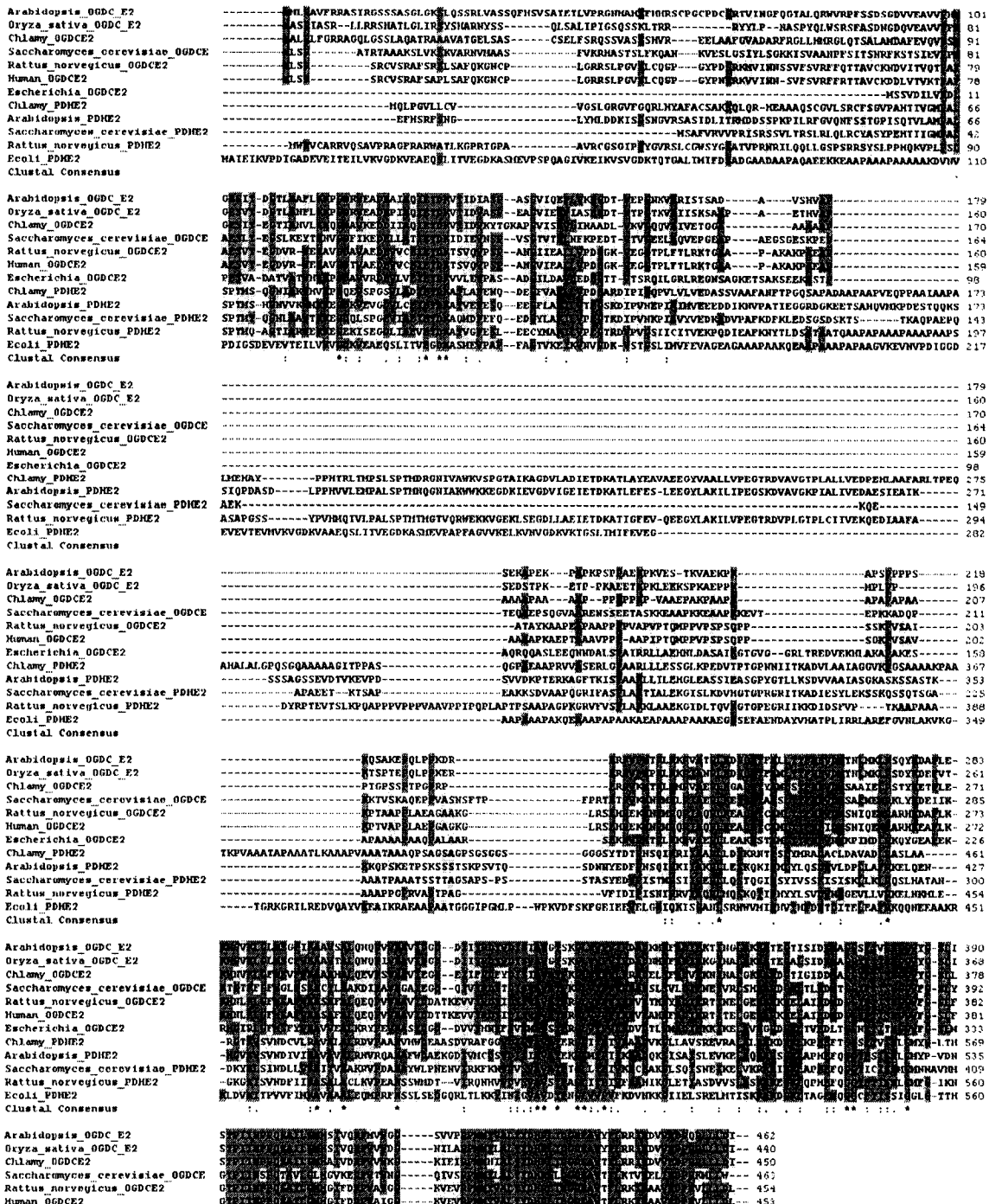

Figure 3A.

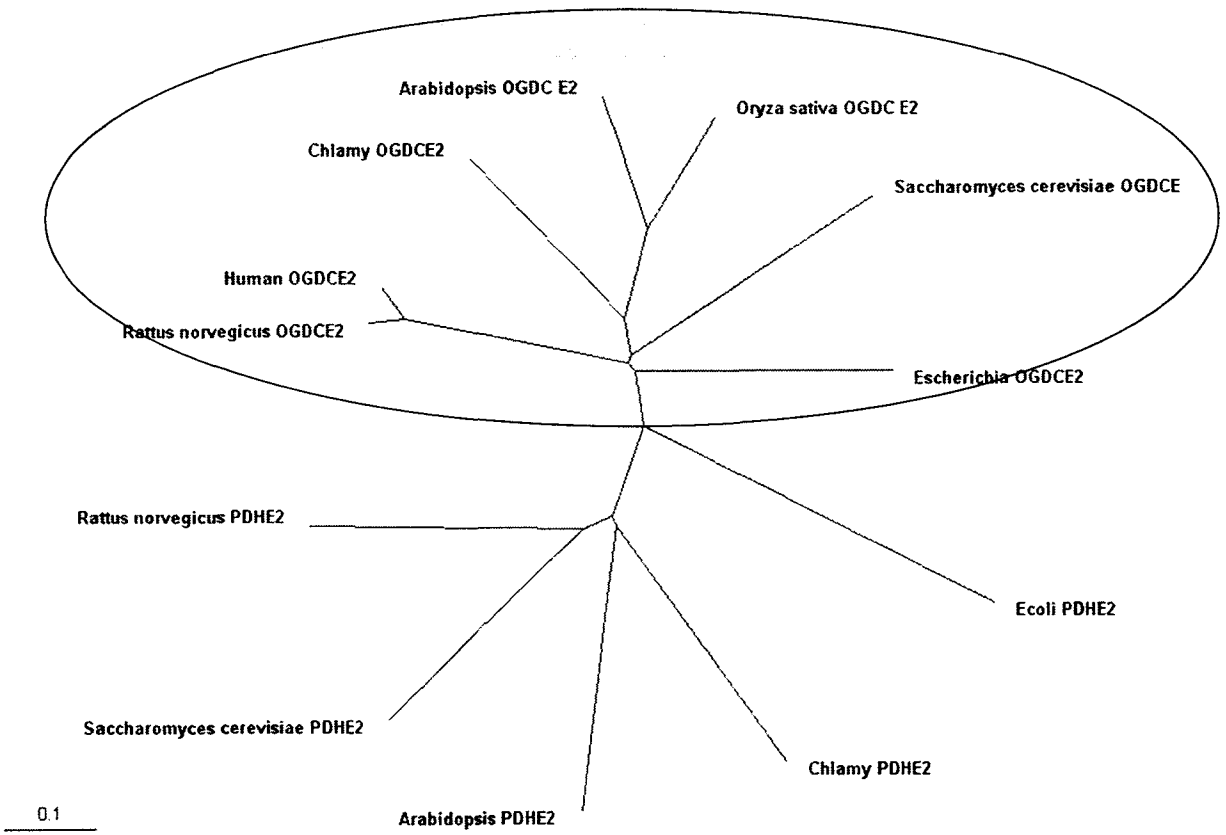
Arabidopsis OGDC E2
Oryza sativa OGDC E2
Chlamy OGDCE2
Saccharomyces cerevisiae OGDCE
Human OGDCE2
Rattus norvegicus OGDCE2
Escherichia OGDCE2
Rattus norvegicus PDHE2
Ecoli PDHE2
Saccharomyces cerevisiae PDHE2
Chlamy PDHE2
0.1
Arabidopsis PDHE2

Figure 3B.

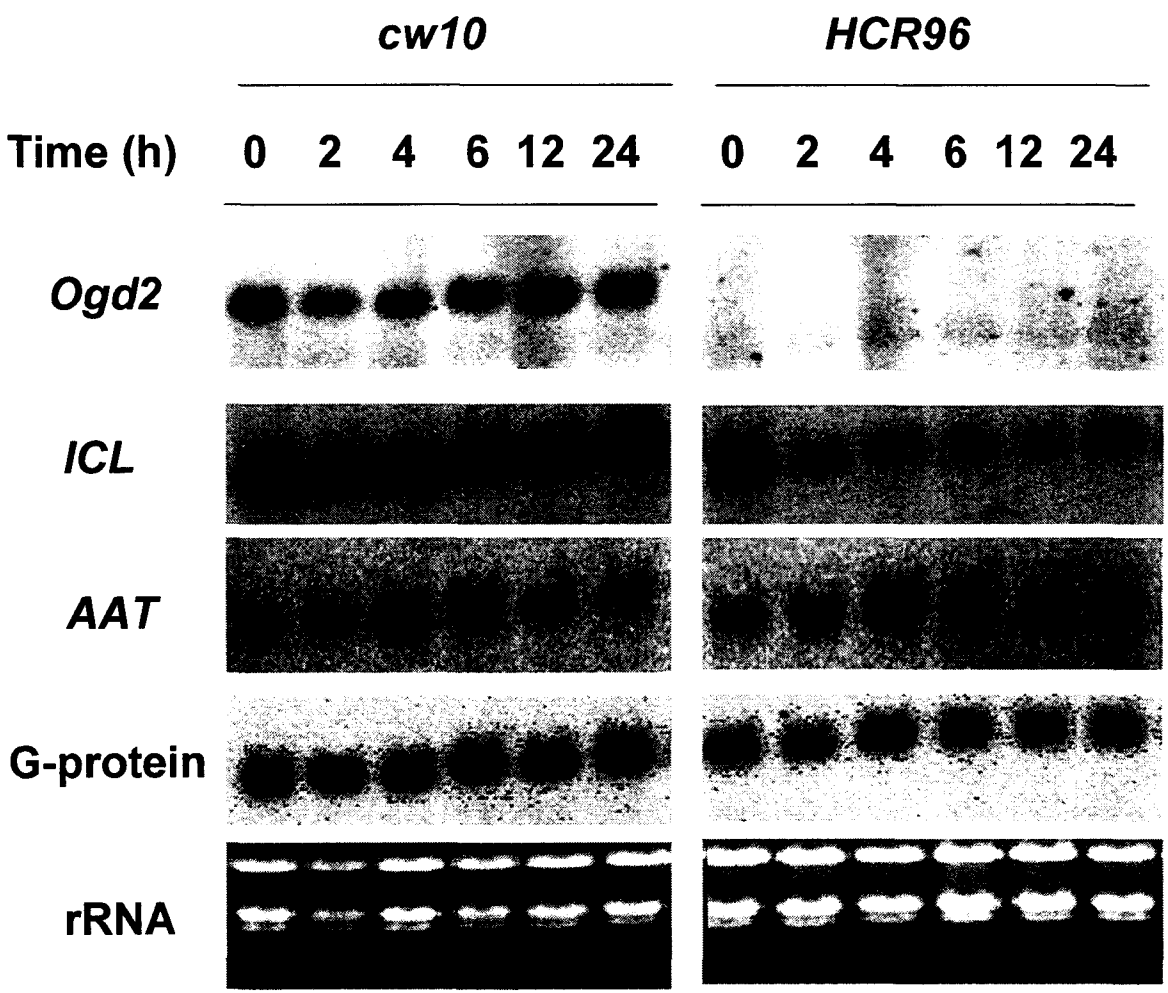
cw10
HCR96
Time (h)
0 2 4 6 12 24
0 2 4 6 12 24
Ogd2
ICL
AAT
G-protein
rRNA

Figure 4A.

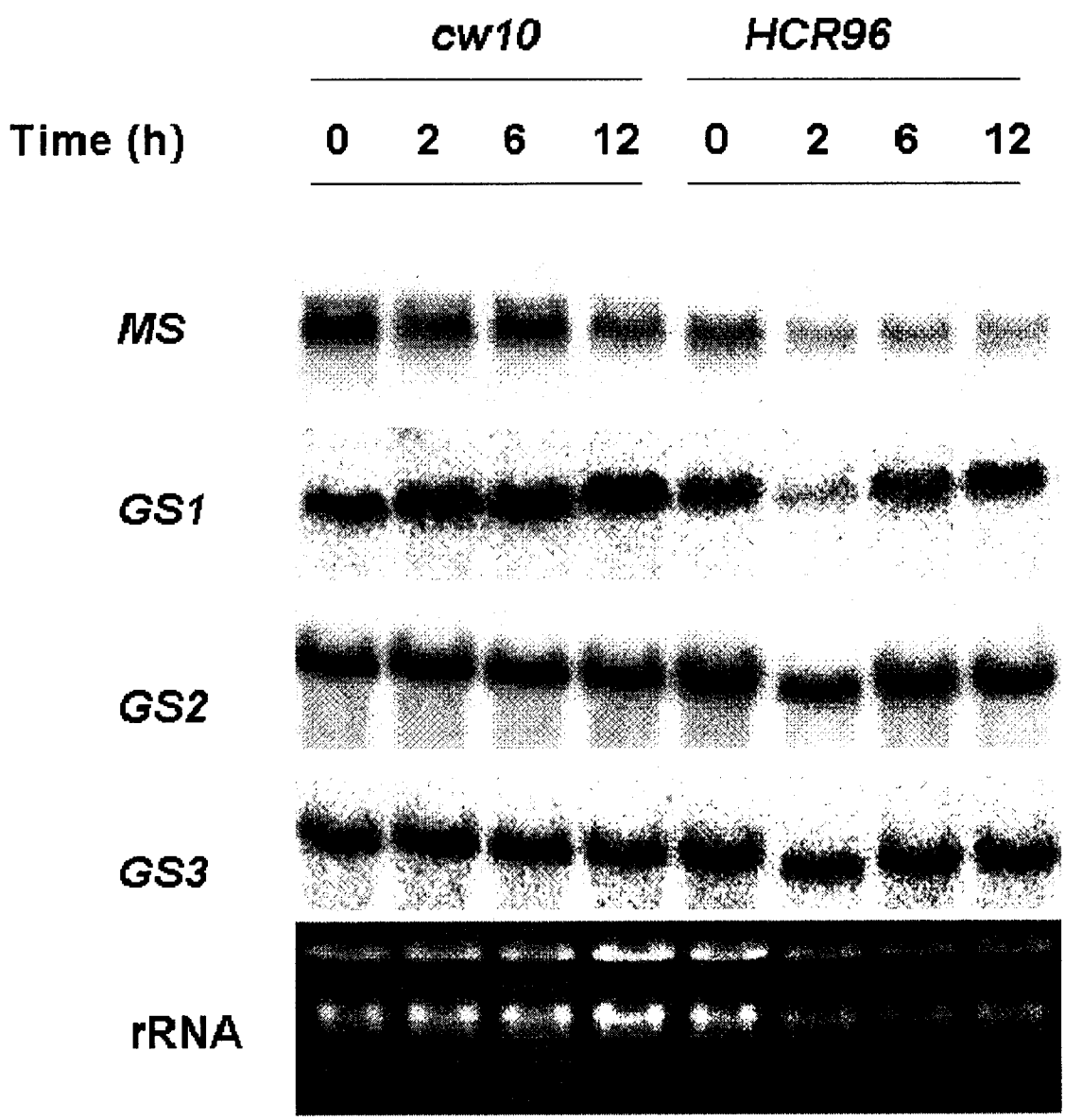

Figure 4B.

Chapter 4. Insertional mutagenesis and identification of gene defects in High CO_2 Requiring mutants of *Chlamydomonas reinhardtii.*

Abstract

Many aquatic photosynthetic organisms exhibit a variety of acclimation responses to limiting CO_2 availability, including induction of CO_2 concentrating mechanisms (CCMs), which represent adaptations to environments with changing and, at times, limiting CO_2 availability. Although the characteristics and regulation of limiting CO_2 acclimation, including the CCM, in the green alga *Chlamydomonas reinhardtii* have been well demonstrated by physiological and biochemical studies, much less information is available at the molecular level with regard to the components involved in the CCM and other parts of the limiting CO_2 acclimation response. We have applied insertional mutagenesis and genetic approaches in an effort to identify the components of the CCM, other pathways essential to limiting CO_2 acclimation and of the regulatory pathways controlling limiting CO_2 acclimation responses. Many mutants defective in acclimation to limiting CO_2 environments were generated by gene tagging, and, although all the identified mutants are phenotypically high-CO_2-requiring, they can be distinguished by differences in their ability to survive under various levels of CO_2, by differences in heterotrophic growth, and by differences in the gene expression patterns of CO_2 responsive genes. Using the insertional tags, several mutant genes have been identified or confirmed, including the transcription factor *Cia5*, 2-oxoglutarate dehydrogenase (*Ogd2*), heat shock proteins and *LciB*, which encodes an unknown protein apparently involved in inorganic carbon transport. The identity of defective genes in the mutants suggests that they are located in various metabolic or regulatory pathways, including the CCM, mitochondrial respiration, chloroplast photoprotection and some not yet identified regulatory pathways. In addition, it has become evident that analysis of insertional mutants in *C. reinhardtii* is rarely straightforward, even when there is a single insert that co-segregates with the mutant phenotype. We hope that this endeavor will continue to provide information useful in elucidating the networks associated with the limiting CO_2 acclimation response and its regulation.

Introduction

Most photosynthetic microorganisms are capable of acclimating to CO_2 limited environments by induction and operation of a CO_2 concentrating mechanism (CCM). This process involves active inorganic carbon (Ci) transport, interconversion between CO_2 and HCO_3^-, and dehydration of HCO_3^- in chloroplasts at sites of Rubisco. During the past several decades, the CCM has been extensively studied in *Chlamydomonas reinhardtii*, a unicellular green alga. A suite of adaptive responses has been demonstrated in *C. reinhardtii* during limiting CO_2 acclimation, including induction of active Ci transport, alteration in gene expression and changes in cellular structural and biochemical events (Berry et al., 1976; Badger et al., 1980; Geraghty and Spalding, 1996; Spalding et al., 1991; for review, see Spalding, 1998). Evidence indicates that the responses to limiting CO_2 signal(s) are controlled by different signal transduction pathways. Some key signals commonly involved in cellular differentiation and development, such as blue light and cAMP, have been demonstrated or implicated as being involved in signaling in limiting CO_2 acclimation. Furthermore, different metabolic events, cell cycle, and circadian rhythm all have been reported to contribute to the regulation of the expression of CO_2 responsive genes.

Although the characteristics and regulation of limiting CO_2 acclimation, including the CCM, in *C. reinhardtii* have been well demonstrated by physiological and biochemical studies, most components functioning in limiting CO_2 acclimation have not been identified at the molecular level. Neither the nature of the signal triggering the acclimation responses to changing CO_2, nor the signal transduction pathway associated with the CCM is well understood. For unraveling the mechanism of limiting CO_2 acclimation and its signal transduction pathways, identification and characterization of limiting CO_2 non-acclimating mutants are invaluable, and, to date, many such mutants have been identified in *C. reinhardtii* and have provided significant insight into the CCM and limiting CO_2 acclimation. Several important identified CCM mutants include: *pmp1*, a mutant deficient in Ci transport (Spalding et al., 1983); *cia3* and *ca1-1*, mutants deficient in a chloroplast localized carbonic anhydrase (CA), *Cah3* (Funke et al., 1997; Karlsson et al., 1995; Karlsson et al., 1998); *pgp1*, a phosphoglycolate phosphatase (PGP) mutant (Suzuki et al., 1990); *gdh1*, a glycolate dehydrogenase (GDH) mutant (Nakamura et al., 2005); and *ccm1* and *cia5*, mutants lacking any low CO_2 inducible characteristics (Moroney et al., 1988; Spalding, 1999; Xiang et al.,

2001; Fukuzawa et al., 2001). These mutants can be categorized into several groups, with each group representing different individual key component in limiting CO_2 acclimation. Availability of *C. reinhardtii* genome sequence (ref) and the development of transformation techniques (ref) have opened the door to powerful new approaches for investigating the components involved in CCM and the signal transduction pathways associated with limiting CO_2 acclimation. Insertional mutagenesis has been widely applied in recent years for isolation and characterization of *C. reinhardtii* mutants with an impaired CCM, and has proved to be very productive (Van et al., 2001; Colombo et al., 2002; Thyssen et al., 2003). Several important genes involved in limiting CO_2 acclimation have been cloned through this approach, including *Cia5* (*CCM1*), *Gdh1* and *LciB* (*Pmp1/Ad1*) (Fukuzawa et al., 2001, Nakamura et al., 2005, Wang and Spalding, Chapter 2 in this work).

In the current study, we have applied insertional mutagenesis to identify components associated with limiting CO_2 acclimation. Many limiting CO_2 non-acclimating mutants were identified, which lack some or all limiting CO_2 acclimation characteristics. These mutations should represent a wide variety of defects in the functional CCM or in the signal transduction pathway associated with limiting CO_2 acclimation, which have been, or will be characterized by molecular genetics and physiological and biochemical analyses.

Results

Isolation of mutants with defects in limiting CO_2 acclimation

Efficient nuclear transformation for *C. reinhardtii* has been developed and applied to investigate the mechanism of limiting CO_2 acclimation. Mutagenesis by gene tagging in *C. reinhardtii* can be achieved by transforming *C. reinhardtii* cells with exogenous DNA carrying a selectable marker. Once entering the nucleus, the tag DNA can integrate into the nuclear genome at a random location and cause a mutation, which could theoretically disrupt any gene in the *C. reinhardtii* genome. By screening the transformants, which either cannot grow under limiting-CO_2 conditions (High CO_2 requiring, HCR phenotype) or show abnormal expression patterns of CO_2 responsive genes, one can identify the components functioning in limiting CO_2 acclimation.

In the current study, two selective screening systems were applied to generate transformants with a tagged exogenous DNA insert (or inserts in case of multiple insertion events).

(1) A plasmid carrying the *arg7* structural gene (pARG7.8, DeBuchy et al., 1989; or pJD67, Davies et al., 1994) was used to transform a wall-less *arg7* mutant (CC425, with arginine requiring phenotype) to complement the *arg7* mutation. The transformants were selected for arginine prototrophic growth.

(2) A plasmid carrying the Ble^R gene (pSP124s, Lumbreras et al., 1998) was used to transform a wall-less, wild type strain, *cw10* (CC849). The Ble^R gene originates from the tallysomycin-producing actinomycete species *Streptoalloteichus hindustanus*, and encodes a small (13.7 kDa) protein conferring resistance to tallysomycin and related antibiotics including bleomycin, phleomycin and zeocin. The transformants were selected on plates containing zeocin.

After transformation, cells were grown in an elevated CO_2 condition. Once transformed colonies were visible on selective plates, they were transferred to duplicate plates and subjected to high level *vs.* limiting level CO_2 screening. High CO_2 was obtained by supplementing air with 5% CO_2. Two different levels of limiting CO_2 were used: (1) Air containing atmospheric level of CO_2 (350-400 ppm) is termed low CO_2. (2) CO_2-depleted air containing 50-200 ppm CO_2 is termed very low CO_2 and was obtained by passing air through a concentrated sodium hydroxide solution and re-mixing with normal air. The expected mutants either were not able to survive, or showed poor growth in limiting CO_2 levels (HCR phenotype).

Table 1 summarizes the new mutants with defects in limiting CO_2 acclimation that have been generated by insertional mutagenesis. All mutant lines were initially identified by their slow growth in limiting CO_2. Most mutants under investigation were unable to survive (stringent phenotype) or show very poor growth in a limiting CO_2 environment (either low CO_2, 350-400 ppm, or very low CO_2 level, 50-200 ppm), and therefore designated as high CO_2 requiring (HCR) mutants. However, a variety of growth phenotypes among these mutants could be still distinguished by their growth under different CO_2 concentrations in spot tests. Figure 1 shows the spot tests of several selected mutants. Some mutant lines showed a leaky HCR phenotype in their growth in limiting CO_2 environments: exhibiting reduced growth in limiting CO_2 but with a small proportion of the cells surviving. Several mutants generated with leaky HCR phenotypes, such as *HCR99* and *HCR103*, were recently observed to recover to a growth nearly as high as wild type in limiting CO_2 after many generations.

Within the mutants showing a stringent HCR growth phenotype, their growth still could be distinguished by their sensitivity to different levels of CO_2. While some lines were sensitive to both low CO_2 (air level) and very low CO_2, other strains exhibited sensitivity to only very low CO_2 with normal or nearly normal growth in air, similar to in the phenotype of the *cia5* mutant (Figure 1A and Figure 1 B).

We also identified several mutants which showed normal growth in both high CO_2 and very low CO_2, but surprisingly, die in the intermediate low level of CO_2 (air level). This group of mutants was named as *Air Dier* (*ad*) mutants (Figure 1B), including *ad1* and *ad3*. In addition, some HCR mutants, including *HCR92* and *HCR96*, showed an acetate sensitive phenotype. In addition to spot growth tests, physiological and biochemical characteristics, such as photosynthesis and respiration, were studied in selected mutants.

Characterization of mutants defective in regulation of the signal transduction associated with limiting CO_2 acclimation

To identify mutants with defects in components that regulate the expression of CO_2 responsive (CO_2-R) genes, we monitored the expression of several CO_2-R genes in the HCR mutants, including *Cah1* (encoding the periplasmic carbonic anhydrase, pCA1), *Ca1/Ca2* or *Mca1/Mca2* genes (encoding the mitochondrial CAs, mtCA1 and mtCA2), and the *Lip36*, or *Ccp1/Ccp2* genes (encoding two, nearly identical plastid envelope polypeptides of unknown function). Mutants with defects in signal transduction were expected to exhibit expression patterns different from wild type, either in expression level or timing.

Most mutants were unlikely to regulate CO_2R genes, as judged by their similarity to wild type CO_2-R gene expression, but several mutants showed either quantitative or times alterations in the expression patterns of the CO_2-R genes (Figure 2). Among these, two mutants, *p34*, and *209*, lacked expression of all tested CO_2-R genes (Figure 2A, expression of CO_2-R genes in *209* not shown), a phenotype similar to that of the *cia5* mutant with defects in CCM associated signal transduction. Two other mutants, *HCR99* and *HCR103*, showed a reduced expression level of all three tested CO_2-R genes (Figure 2B). However, in the mutant *HCR99*, genes normally showing constitutive expression under different CO_2 conditions, e.g., the gene encoding a putative G-protein subunit, and *RbcS* gene, a gene encoding the Rubisco small subunit, also showed reduced expression compared to that in wild type, suggesting a defect in general transcription or RNA stability, rather than a defect specific for

regulation of CO_2-R genes. Two other mutants, *HCR95* and *HCR92*, exhibiting altered patterns of expression in the tested CO*2*-R genes, were described in more detail in a previously published paper (Van et al., 2001).

In addition to the CO_2-R genes mentioned above, several genes encoding putative Ci transporters have been identified recently, including *LciA, Mrp1* and *LciB/LciC* (ref). The expression of these genes has been reported to be abolished in the *pmp1* mutant, although more recent data demonstrate the expression to be decreased but not abolished (Chap. 2). In the *air dier* mutant, *ad1*, *LciA* and *Mrp1* also exhibited a much reduced expression, similar to that observed in *pmp1*. A detailed characterization of *ad1* is described in Chapter 2.

Genetic analysis and physiological characterization

Southern blots were used to analyze the integration of the tag DNA into the genome, and the presence of the inserted selectable marker was verified in all the mutants tested (data not shown). Although most mutants have only a single insert, several were shown to have multiple inserts (Table 1). Genetic analyses, including tetrad analysis and allelism tests, were performed on the identified mutants to verify whether the insertions co-segregate with the HCR phenotype and whether new mutants are allelic to any known mutation. To confirm whether the HCR phenotype co-segregated with the selectable marker (and thus with the insert), the HCR mutants were crossed with wild type strains, and tetrad analyses were performed to investigate the relationships among the progeny's phenotypes. For some wall-less mutants, separation of tetrads was difficult because of the fragility of the progeny. In that case, random progeny analyses were carried out instead of tetrad analyses.

Mutants generated by *arg7* complementation were crossed with a strain carrying an *arg7* mutant allele, CC1068, to evaluate co-segregation of insert with the HCR phenotype. If an HCR phenotype was caused by the integration of a selectable marker, all progeny inheriting the HCR phenotype should also carry the selectable marker and be arginine prototrophic. The HCR phenotype and arginine prototrophic growth co-segregated in most of the HCR mutants tested, indicating linkage of the HCR mutation and the DNA tag (Table 1). Mutants generated with the *ble* insert were crossed with CC620 strain, and the resistance to zeocin was used to evaluate co-segregation of the HCR phenotype with the Ble^R selectable marker in the progeny.

The co-segregation of the *HCR* (or *ad*) phenotype was verified in several mutants, including *HCR86, HCR92, P14, P34, 3510*, and *ad1*. However, in some mutants, the selectable markers were shown not to co-segregate with the HCR phenotype, indicating either spontaneous or transformation-induced mutations occurred that were far separated from the insertion site. Once co-segregation between the HCR phenotype and the insert was confirmed, each mutant was further investigated, including cloning of the genomic sequence flanking the insert.

For mutants with multiple inserts, such as *103* and *201*, crosses between the mutant and wild type strain CC1068 were performed in an effort to segregate the inserts linked with the HCR phenotype. However, these efforts were not successful. For mutant *103* (3 inserts), the leaky HCR phenotype made it difficult to differentiate the HCR progeny from wild type cells, and therefore to separate mutant cells from wild type cells. For mutant *201*, several HCR progeny were recovered after crossing with wild type strains, but southern blots showed that all progeny with HCR phenotypes still retained multiple inserts (data not shown), suggesting tandem arrays or at least linked arrays of inserts might occur in this mutant.

A small proportion of cell fusions produce stable diploids rather than zygotes when two haploid cell strains are mated, and phenotypic complementation of recessive mutations will occur in the diploid cells if the two mutations are not allelic. In addition, of course, unlinked mutations will sort during meiosis to generate phenotypically wild-type progeny. To test allelism, mating between two mutants was used to generate recombinant progeny and vegetative diploids in order to test whether any phenotypically wild-type colonies (either progeny or diploids) were generated, thus demonstrating the new HCR mutants are not allelic to each other or to any previously characterized HCR mutants, such as *cia5, ca1, pmp1* and *pgp1*. In crosses where no wild-type colonies were found, it is likely the two mutations are allelic. In this way, *p34* and *209* were shown to be allelic to *cia5*, a mutant defective in induction of any responses to limiting CO_2. Similarly, *ad1* was demonstrated to be an apparent allele of *pmp1*, a mutant deficient in Ci uptake. Most mutants, however, were shown not to be allelic to each other or to any previously characterized mutants.

Cloning of the genomic sequence flanking the insertion in mutants

The DNA flanking the insert in a mutant generated by insertional mutagenesis can be cloned by several strategies, such as plasmid rescue, PCR based cloning, or screening a genomic

library generated from the mutant. These strategies were applied to clone the sequences flanking the integrated DNA tags from the HCR mutants. We found that plasmid rescue, when possible, was the most efficient way to clone the sequence flanking the vector side of the insert. Flanking sequences from nine HCR mutants were cloned by this approach. However, plasmid rescue requires the presence of a nearly full length vector sequence (pBluescriptKS or pBR329 in most of our mutants) in the integrated selectable marker. In many mutants some part of the vector sequence essential for plasmid amplification was missing, therefore, other approaches, such as inverse PCR, TAIL-PCR and genomic library screening, were applied instead. These approaches also were necessary to clone the sequence flanking the *arg7* end or *ble* end in the inserts. So far we have cloned flanking sequence for at least one side of the insert from most of the identified mutants (Table 1).

The majority of the *C. reinhardtii* genomic sequence recently became available in the DOE Joint Genome Institute (JGI) database, and we found that all flanking sequences recovered from the HCR mutants could be mapped on the scaffolds in this database. Also, we found that an insertion event frequently was accompanied by a deletion, with the deletions identified so far ranging in size from 4kb (in mutant *HCR86*) to 36kb (in mutant *ad1*). Since a large deletion causes the deletion of several genes in the insertion site, it is necessary to identify which of the deleted genes is responsible for the mutant phenotype. The flanking sequences also were used to search the *C. reinhardtii* EST and protein databases to identify candidate gene causing the HCR phenotype. Details are given in the individual cases described below.

Mutants characterized in our current research

P34* and *209: Both mutants were demonstrated as *cia5* alleles by genetic analysis. Like the *cia5* mutant, both *P34* and *209* appeared defective in induction of CO_2-R genes (Figure 2A). Flanking sequences from both mutants were cloned by inverse PCR and plasmid rescue, respectively, and sequence comparisons indicated that the insertion in each mutant disrupted the *Cia5* gene. A more detailed characterization of *P34* and *209* has been published, including complementation with the *Cia5* gene (Van et al., 2001; Spalding et al., 2002). Since Cia5 has been identified as an important transcription factor regulating limiting CO_2 acclimation (Xiang et al., 2001; Fukuzawa et al., 2001), we used yeast-two-hybrid analysis in an attempt to identify proteins that interact with Cia5. Interacting proteins could be

regulatory elements in the Cia5 transduction pathways, either upstream modifying Cia5 proteins upon changing CO_2 concentrations, or functioning together with Cia5 to transduce a signal to downstream events. The HybriZAP2.1 two-hybrid vector system (Stratagene) was used to screen for genes encoding proteins that interact with *Cia5*. A cDNA library was generated from mRNA isolated from the cells grown in different conditions (including high CO_2 and low CO_2 photoautotrophic growth, and heterotrophic growth) and inserted into the activation domain (AD) vector. Four bait plasmids containing *Cia5* fused with the DNA binding domain (BD) were constructed (Figure 3). They include either full length *Cia5* or different lengths of 3' *Cia5* cDNA coding region (*Cia5* cDNA was provided by Dr. Don Weeks). However, some of these bait plasmids were able to activate reporter genes in the absence of the AD vector. Only the construct carrying 400bp of 3' *Cia5* coding region, pBDcia1780, did not result in such activation. From about a hundred colonies in the initial screening, 15 putative interacting colonies were identified as activating expression of both reporter genes (HIS3 and lacZ) using the Cia5 C-terminal fragment pBDcia1780 as bait. However, most putative interacting proteins were later confirmed as being false positives, either because they were unable to activate reporter genes when reintroduced with pBDcia1780 or because they were found to be in the incorrect reading frame in the target plasmids. Attempts to identify any Cia5 interacting proteins have not been successful so far.

***Air dier* mutants**: As described above, we identified two mutants, *ad1* and *ad3*, showing the *air dier* phenotype. Mutant *ad1* is described in more details in Chapter 2, and *ad3* is still under current investigation. *ad3* showed growth in high CO_2 and very low CO_2 although slower compared to wild type, but died in low CO_2. However, it appears that *ad1* and *ad2* are not allelic, because, unlike with *ad1* (see Chapter 2) several putative transporter genes, including *LciB*, exhibited normal expression in *ad3* (Figure 4). It appears that Ad3 represents an additional essential component associated specifically with the low (air level) CO_2 acclimation.

HCR96: This mutant displays a stringent HCR phenotype, and shows very slow growth in both air and very low CO_2. It appears that *HCR96* is sensitive to acetate because its mixotrophic growth was inhibited by acetate under high CO_2 conditions. A more detailed characterization of *HCR96* is described Chapter 3.

HCR3510: This mutant can grow in low CO_2, but shows very poor growth in very low CO_2. It has a single insert that co-segregates with the HCR phenotype and is not allelic to any previously characterized HCR mutant. DNA flanking the insertion was recovered by inverse PCR, sequenced and found to correspond to a sequence on a single scaffold in the *C. reinhardtii* genome. The insert appears to disrupt a gene encoding a putative chloroplast heat-shock protein, here named the *3510* gene (Figure 5A). Analysis of the genomic sequence shows that this gene is organized "head to head" (~200 bp apart) with another putative chloroplast heat-shock protein previously identified as hsp22 (Grimm et al., 1989; Ish-Shalom et al., 1990). Alignment of the predicted 3510 protein and hsp22 protein sequences shows that the two proteins are very similar (Figure5B), and EST sequences indicate that the transcript of each gene has a long 3' non-coding region. In the mutant, one end of the insert is located in the 3' non-coding region of *3510*, but it is not known whether the insertion affects the hsp22 gene as well. Future experiments will clone the DNA flanking the other side of the insert and determine whether any of the multiple BAC clones identified with the flanking sequence are able to complement *3510*.

316: This mutant exhibits a stringent HCR phenotype. It grew normally in high CO_2 but could not survive either in low or very low CO_2 (Figure 1). No obvious difference in the expression of CO_2-R genes was observed between wild type and *316* (Figure 6).
The flanking sequences from both ends of the insertion were recovered by plasmid rescue and inverse PCR, respectively. A deletion of approximately 26 kb was found at the site of the insertion, which covers several predicted genes, including a retinitis pigmentosa-2 similar protein and several proteins of unknown function. The putative promoter of an *iojap* like protein is 2kb from the insert, but Northern blot analysis could not confirm whether this gene has normal expression (data not shown). Several BAC clones overlapping the site of the insertion have been identified, and will be used for complementation.

91: This stringent HCR mutant has a single insert. Flanking DNA was cloned by plasmid rescue and sequenced. The sequence of flanking DNA can be located to a region of the *C. reinhardtii* genome that lacks a gene model but is represented by ESTs. The insert is near a putative histone gene, but it is not clear if the histone gene is disrupted in any way.

***HCR92*:** This mutant was previously investigated as *HCR90* (Van et al., 2001), and showed a recessive HCR phenotype that co-segregates with the *Arg7* insert and does not show allelism to any known HCR mutants. It showed decreased expression of several CO_2 regulated genes. The 5' and 3'sequences flanking the *Arg7* insert were obtained. Both sequences are located on a single scaffold but are separated from each other by 500kb on the wild type genome, and the 5'end of the original insert is flipped on the genome to the 3' side of the original insert (Figure 7). It seems likely that the original insert might be separated into two pieces, but a large deletion or rearrangement also may have occurred. One end of the insert appears to have disrupted a putative protein kinase gene, and the other end has disrupted a gene of unknown function. However, if the region in between has been deleted, many additional genes might have been lost. Genomic BAC clones from a *C. reinhardtii* BAC library were identified by using both flanking sequences as probes. However, attempts to complement HCR92 using DNA from BAC clones or subcloned fragments were not successful so far.

Discussion

Identification of mutants with defects in low CO_2 acclimation

Limiting CO_2 acclimation in photosynthetic microorganisms requires signaling systems and a complex metabolic network interconnecting the Ci accumulation, carbon fixation and many other metabolic pathways. In *C. reinhardtii*, several systems have been demonstrated as essential for limiting CO_2 acclimation, including Ci transport systems, enzymes catalyzing inter-conversion of different Ci species, an energy supply system, a signaling system for sensing CO_2 availability and/or metabolic status, and several metabolic pathways associated with at least photosynthesis and photorespiration. However, the linkages of most specific gene products with physiological and biochemical functions in limiting CO_2 acclimation are not well established in this organism. In the current study, we have applied an insertional mutagenesis approach to identify components essential for limiting CO_2 acclimation, and to dissect the signaling network linking limiting CO_2 conditions with the corresponding acclimation responses. Defects in genes crucial for limiting CO_2 acclimation cause various non-acclimating phenotypes in the mutants.

Based on the physiological and biochemical characterization of the mutants, or the amino acid sequences of cloned genes, we can classify these genes into three major categories: (1) components apparently involved directly in Ci concentrating; (2) components of the metabolic pathways needed for low CO_2 acclimation; or (3) regulatory components apparently involved in signal transduction.

Regulatory mutants

Several mutants showing abnormal expression patterns of CO_2-R genes can be categorized into the group of mutants with defects in regulatory components. Among them, *P34* and *HCR209* exhibited an HCR growth phenotype and abolished inducible accumulation of all tested CO_2-R gene transcripts, similar to observations with *cia5*. Complementation group analyses, insertion site identification and complementation with *Cia5* all demonstrated that *P34* and *HCR209* are allelic to *cia5*. *Cia5* was cloned independently by Fukuzawa et al. (2001) and Xiang et al. (2001), and its amino acid sequence suggests that it is a transcription factor regulating the global responses in limiting CO_2 acclimation. However, it is still not clear how this protein links the limiting CO_2 signal to the downstream regulation in the acclimation responses. As an array of regulatory proteins typically are involved in signal transduction events, it is necessary to identify other components in the Cia5 signaling pathways, and some of those components are likely to interact directly with Cia5 protein. Attempts to identify Cia5 interacting proteins by yeast two-hybrid approach were not successful in this study though. Because Cia5 is expressed in a very low abundance, its interacting proteins may also be present in very low abundance, which may make it difficult to identify correct candidates from the cDNA libraries. Thus in addition to screening larger numbers of putative interacting proteins, other approaches to identify the components in the Cia5 signaling pathway may need to be applied. For this, one possible strategy is to isolate suppressor mutations of *cia5*. Since the *Cia5* gene in the *cia5* mutant carries a single point mutation, which is subject to reversion, *P34* and *HCR209* should be more useful than *cia5* in this application. Unlike *cia5*, both new mutants are insertional mutants, and not likely to generate revertants.

HCR103, *HCR90* and *HCR95* were identified as new mutants showing defects in regulation of CO_2-R gene expression. Unlike *cia5*, these mutants exhibited some induction of CO_2-R genes, but with altered patterns or levels than those observed in wild type. In *HCR90*, only

one set of CO_2-R genes, *Mca1* and *Mca2*, showed reduced expression, while in *HCR95*, expression of CO_2-R genes showed delayed and reduced accumulation of transcripts. In *HCR103*, all three tested CO_2-R genes exhibited much reduced transcript abundance. It therefore suggests that in all these mutants, at least part of a limiting CO_2 signal transduction pathway is impaired, possibly components downstream of Cia5. However, we can not exclude that the lesions only indirectly affect CO_2-R gene expression via changes in physiological or biochemical processes, and that the lesions are in functional components of the CCM rather than in the signal transduction pathway. Nevertheless, identifying the mutant genes will help clarify the functions of these components.

Components functioning in CCM and carbon metabolism

Cloning the defective genes in several mutants suggests that they are impaired in components functioning either in Ci uptake or other metabolic or biochemical processes instead of regulatory pathways. Among them, the *ad1* mutant exhibited defective Ci uptake and greatly decreased photosynthetic activity in low CO_2. A detailed analysis of *ad1*, which appears to be involved in Ci transport either directly or as a regulator, is described in Chapter 2. *HCR96* was confirmed to be a respiratory mutant (described in Chapter 3). As expected, non-regulatory mutants fall into broader categories, which represent defects in various metabolic processes.

In the mutant *HCR3510*, the insertion caused disruption of a gene encoding a protein with homology to heat shock proteins, and possibly also the disruption of another gene encoding a protein previously identified as Hsp22. Although not confirmed, defects in one or two putative heat-shock proteins in *HCR3510* may be responsible for its HCR phenotype. Hsp22 was identified as one of the major heat shock proteins induced by heat shock in *C. reinhardtii* (Kloppstech et al. 1985). It belongs to the plant low-molecular-mass heat-shock-protein family, and was shown to be transported into the chloroplast and localized in the grana lamellae (stacked thylakoid) (Schuster et al., 1988; Grimm et al., 1989). The transcription and translation of the *Hsp22* gene were shown to be regulated by light (Ish-Shalom et al., 1990), and reversible association of this protein with a chloroplast membrane-enriched fraction during heat shock and its release during recovery were also observed (Eisenberg-Domovich et al., 1994). Since *Hsp22* and its homologue, *3510*, are very similar in their nucleotide sequences and predicted amino acid sequences, it is not clear whether the

previous research on Hsp22 distinguished between these two gene products very well. The function of Hsp22 (possibly 3510 as well) was suggested to be protection against photo-inhibition during heat-shock (Schuster et al., 1988). Pre-incubation of *C. reinhardtii* for heat-shock resulted in a partial protection against photoinhibition. Both high light and limiting CO_2 can cause photoinhibition in photosynthetic organisms, including *C. reinhardtii*, as demonstrated by the overlap between some limiting CO_2 responsive genes and those induced by high light (Im & Grossman, 2001). Therefore, if Hsp22 and its homologue 3510 play a crucial role in limiting CO_2 acclimation, it seems likely that they may function at early stages against photo-damage before the CCM is fully induced.

Cloning and characterization of mutant genes

Although physiological and biochemical analyses of non-adaptive HCR mutants could provide important insights for understanding the functions of essential components involved in low CO_2 acclimation, identification of defective genes in the mutants will uncover the direct linkage between a gene product and its function. In the current research, we were able to successfully identify several defective genes in the mutants using a variety of different cloning strategies. However, identification and characterization of mutant genes was not always straightforward. Instead, it proved to be complicated in most cases. First of all, not all the inserts were linked with the HCR phenotypes, such as in *HCR95, HCR105, P33* and *HCR201*. In these cases, the inserted "tag" in the mutant was not useful for identification of the mutation responsible for the HCR phenotype. Other approaches therefore will need to be used instead, including mutant complementation with a pooled genomic library, or positional cloning. Complementation has been applied successfully to clone some essential genes in CCM, including the *Cah3* and *Cia5* genes (Funke et al., 1997; Xiang et al., 2001). Positional cloning also has been developed in *C. reinhardtii* in recent years and was successfully applied in cloning the *LF1* gene required for flagellar length control (Kathir et al., 2003; Rachel et al., 2005). With the availability of the *C. reinhardtii* genome sequence and fine mapping, this approach will become more important and efficient for cloning essential genes. Deletion or rearrangement of genomic DNA caused by integration of the inserted DNA also presents difficulties for cloning the mutant genes. In *HCR92*, the cloned DNA sequences flanking the two sides of the insert were shown to be far apart in the genome, making it difficult to identify the defective gene associated with the HCR phenotype. Another problem

associated with insertional mutagenesis is multiple independent integration events during transformation, such as the cases with *HCR103* and *201*. Although crossing a mutant containing multiple inserts with wild type strains can potentially separate the insert co-segregating with the mutant phenotype, it is time consuming and is not always successful. Finally, a leaky phenotype in an HCR mutant can complicate the genetic analysis and gene cloning. It makes it difficult to distinguish the mutant offspring from wild-type progeny and unmated wild type cells in random progeny analysis. In tetrad analysis, a leaky phenotype also makes it difficult to draw unambiguous conclusions. Direct selection of mutants complemented by wild type genes under limiting CO_2 conditions also is not possible when the phenotype is leaky; although this sometimes can be remedied by co-transformation of the wild type genomic DNA with a secondary selective marker.

Identifying functions of cloned genes

For components possibly involved in Ci transport or carbon metabolism, in addition to analysis of its amino acid sequence and homology search, the clarification of their function will depend on further physiological or biochemical characterization. Also, the expression pattern of candidate genes in different CO_2 levels, both at the mRNA and protein levels, will be useful to characterize the gene function. The expression of candidate genes also can be tested in several characterized mutant strains, especially the signal transduction mutants, such as *cia5*, to establish if the expression of the candidate gene is regulated by a known signal transduction pathway.

For the genes with possible regulatory functions, more work should be focused on their regulation of CO_2 responsive genes, including the interaction in double mutants generated between different regulatory mutants. Comparison of the compromised gene expression among different mutants and double mutants should help to elucidate the relationships among the different regulatory components in the signal transduction pathway. The interaction of different regulatory components also may be investigated by using systems for detecting protein-protein interaction, such as pull-down assays, or the yeast two-hybrid system. Although identification of the interaction of regulatory proteins associated with Cia5 by yeast two-hybrid analysis was not successful in the current study, we were able to identify proteins associated with LciB using the same system. A similar approach will be applied to other candidate genes either involved in the CCM or with regulatory functions.

Materials and Methods

Cell Strains, Culture Conditions and Genetic Analysis

Wild type *C. reinhardtii* strains, CC125, CC849, CC425, CC620 and CC1068 were obtained from the *Chlamydomonas* Genetics Center (Duke University), all strains were maintained on plates with CO_2 minimal medium as previously described (Geraghty et al., 1990). Liquid cultures were grown on an orbital shaker under aeration with 5% CO_2 in air (high CO_2-grown cells) or air aeration (low CO_2-acclimated cells). Cell cultures were switched from elevated CO_2 to limiting CO_2 for the induction at different durations as indicated in the experiments.

All genetic analyses were performed by crossing insertionally generated mutants with various strains according to the protocol of Harris (1989).

Generation of Mutants and Growth Spot Tests

Glass bead transformations were performed as described previously (Kindle, 1989). Linearized plasmids containing different selective markers were used to transform wild type cells as described in the results. Transformed colonies were screened by spot tests in different gas conditions for identifying limiting CO_2 non-acclimating mutants. Three gas conditions used in this study were: high CO_2 (5% CO_2 in air v/v) which was obtained by mixing compressed CO_2 gas with normal air; low CO_2 (normal air, 350-400ppm); and very low CO_2 (50-150 ppm) which was obtained by mixing normal air with either compressed CO_2 free air or air depleted in CO_2 by running normal air through a saturated sodium hydroxide solution. After transformant colonies were transferred to replica plates, each plate was placed in high CO_2, low CO_2 or very low CO_2 chambers.

Mutants identified in this primary screen as having high CO_2-requiring (HCR) or *air dier* (*ad*) phenotype were screened again using more careful spot tests. For spot growth tests, active growing cells were suspended to similar cell densities with minimal medium, spotted (3 μl) onto agar plates with different concentrations of CO_2 for 10 d (Harris, 1989).

DNA and RNA Blot Analysis

Genomic DNA was isolated from cells grown as patches on plates. Cells were resuspended in 250μL of H_2O, followed by addition of 250 μL lysis buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 80 mM Tris-HCl pH 8.0). After extraction twice with phenol:chloroform:isoamyl

alcohol (25:24:1 v/v), RNase was added and DNA samples were incubated in 37 °C for 30 minutes. The NaCl concentration was then adjusted to 0.7M by adding concentrated NaCl solution, followed by addition of 50 μL of 10% CTAB in 0.7M NaCl and incubation of the samples at 50 °C for 10 minutes. DNA was precipitated with ethanol following a final chloroform extraction.

Total RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (1987).

Southern and Northern analyses were performed as standard procedures (Sambrook et al.1989). RNA or DNA was transferred onto the Hybond-N^+ nylon transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and then covalently fixed by UV-cross linking (UV Stratalinker 1800, Stratagene). The probes were synthesized by random priming (Promega). The probe used for detecting gene expression is indicated in the results section. A constitutive probe, a fragment of the coding region of a G-protein β-subunit-like polypeptide (gift from Dr. Schloss; Schloss, 1990), was used as internal standard.

Cloning of the Genomic Sequence Flanking the Insertion

As outlined in the results, several different strategies were used in this study to identify the genomic sequence flanking the insert in the mutants.

Plasmid rescue: Southern blots analysis was performed to determine the restriction enzymes to be used in plasmid rescue and the size of the digested fragment containing the inserted vector and DNA flanking the insert. Genomic DNA (0.5 μg) isolated from the mutants was digested with appropriate restriction enzymes, and then circularized using 1 unit of T4 DNA ligase (Invitrogen) after phenol extraction. The circularized DNA was used to transformed *E. coli* strain DH5α. Purified plasmids were then verified by restriction enzyme digestion and sequenced.

Inverse PCR: Southern analyses were performed to determine which restriction enzymes produced a fragment with the proper a size (1- 2.5kb) that includes part of the inserted vector and its flanking genomic DNA. Digested genomic DNA (0.2 μg) from the mutant was circularized using 1 unit of T4 DNA ligase (Invitrogen) and then precipitated. The circularized product was used as template for inverse PCR using standard PCR procedures

with pairs of primers designed so that each pair of primers complements the sequence of the insert in the opposite orientations.

Thermal asymmetric interlaced (TAIL) PCR: TAIL-PCR was performed as described by Liu et al. (1995). To accommodate the high GC content of *C. reinhardtii* genomic DNA, an extra primer was designed (5'-SANTGNGASGNTGC-3') as the degenerated primer, together with primers AD1, AD2 and AD3 as described (Liu et al. 1995) to amplify the flanking sequence. Nested specific primers complementing the inserted vector were designed based on the sequence of different plasmids. Electrophoresis of the TAIL-PCR products was used to verify the size of PCR products and size differences with different pairs of primers.

Construction and Screening of Genomic Libraries: A genomic library of a mutant was constructed to rescue DNA flanking both 5'- and 3'-ends of the insert. Genomic DNA, size fractionated and partially digested by *BamH*I, was ligated into the *BamH*I site of ZAP EXPRESS (Stratagene). Positive clones, including regions flanking the insert, were identified using the fragment amplified from the vector (insert) as probe.

Identification of BAC Clones Containing the Wild Type Genes and Complementation of Mutants

DNA flanking the site of insertion was amplified by PCR based on the identified flanking sequence and the *C. reinhardtii* genome. The amplified DNA was used as the probe to identify the BAC clones containing the wild type DNA at the inserted region from a BAC library (Clemson University, http://www.genome.clemson.edu/groups/bac/).

All complementation was performed by the glass bead transformation procedure (Kindle 1990). After transformation, cells were kept in selective conditions (low CO_2 or very low CO_2) to observe wild type growth of complemented mutants. Cells transformed with either the empty vector or mock DNA were used as controls.

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Figure legends

Figure 1. Spot tests for growth of wild type and mutant strains in high CO_2 (5% CO_2), low CO_2 air; 350-400 ppm CO_2) and very low CO_2 (50-200 ppm CO_2). **A,** Wild type strains (*cw10*, CC125, CC400), mutants previously described (*cia5*, *cia5/cw10*, *pmp1* (165K)), and HCR mutants generated by *Arg7* insertion (*HCR86, HCR91, HCR92, HCR96* and *HCR3510*) were grown on plates with either CO_2 minimal medium or minimal medium with 0.01 M sodium acetate added. The key below the picture indicates the position of each strain. **B,** Growth of wild type strains, mutants *cia5* and *pmp1* (165K) and *air dier* mutants generated by *Ble* insertion. *ad1* and *ad2* were confirmed to be the offspring from a single transformant.

Figure 2. Expression of CO_2-R genes in HCR mutants. Total RNA (10 μg per lane) was isolated from high CO_2-grown (H) and air-adapted (L) cells. RNA loading levels are indicated by ethidium bromide staining of rRNA, and expression of *RbcS*, a *Chlamydomonas* gene with a constitutive expression, was used as a control. *Ca1/Ca21, Ccp1/Ccp2* and *Cah1* mRNA was probed with fragments corresponding to their cDNA coding sequences respectively. **A,** RNA gel blot of *Ccp1/Ccp2 (Lip36)* and *Cah1* expression in wild type (*cw10*), *P34* and HCR105 mutants. *P34* was demonstrated as a *cia5* allele. *LP34* is a *P34* revertant with somewhat slower growth than that of wild type and an intermediate expression level of CO_2-R genes. **B,** RNA gel blot of *Ca1/Ca2 (Lip21)* and *Ccp1/Ccp2 (Lip36)* expression in wild type (CC425) and *HCR99, HCR103* mutants.

Figure 3. Bait vector constructs for the identification of proteins interacting with Cia5 by yeast two-hybrid system. Bait vectors: pBDGal4cam plasmids containing different lengths of *Cia5* cDNA are shown.

Figure 4. Expression of several putative Ci transport genes in wild type and the *ad3* mutant. RNA gel blot analysis of *Lci A, LciB, LciC and LciD* expression in wild type (*cw10*) and *ad3* mutants. Total RNA (10 μg per lane) was isolated from high CO_2 (5% CO_2) grown cells and cells acclimated to low CO_2 (air; 400 ppm) for the durations indicated. RNA loading levels are indicated by ethidium bromide staining of rRNA. *LciB* , *LciC* and *LciD* mRNA was probed with PCR fragments specifically corresponding to *LciB, LciC* and *LciD* 3'UTR regions respectively, and *LciA* with a PCR fragment corresponding to its coding region.

Figure 5. Genomic organization of *3510* and *Hsp22* and sequence comparison.

A, Structure of the *3510* and *Hsp22* genes showing the opposite orientation of the two genes on the genome. Boxes represent the exons, and lines introns. The position of insertion in the *HCR3510* mutant is indicated by an arrow. **B,** Alignment of 3510 (2002-7061-2) and Hsp22 sequences.

Figure 6. Expression of *AAT* and *Ca1/Ca2* genes in the *316* mutant. RNA gel blot analysis of *AAT* and *Ca1/Ca2* expression was performed in wild type (*cw10*) and *316* mutants. Total RNA (10 μg per lane) was isolated from high CO_2 (5% CO_2) grown cells and cells acclimated to low CO_2 (air; 400 ppm) for the durations indicated. RNA loading levels are indicated by ethidium bromide staining of rRNA. *AAT, Ca1/Ca2* mRNA was probed with PCR fragments specifically corresponding to their coding regions respectively.

Figure 7. Two possible ways of insertion in *HCR92*. Upper: The insert was broken into two pieces during the integration into the genome, and inserted into different locations. Lower: The insert was intact, but caused two breaking points, inverting a large piece of genomic DNA.

A disruption of a putative protein kinase gene also is shown in the figure.

Table1. HCR mutants generated by gene tagging

mutants	Phenotype	Insert #[f]	Co-segregation of the insert and the HCR phenotype	sequence flanking the insert cloned	Altered expression pattern in CO_2-R genes
86[ab]	HCR^L	1	yes	yes	?
91[ab]	HCR^S	1	nd	yes	nd
92[ab]	HCR^L	1	yes	yes	yes
93[ab]	HCR^L	1	nd	yes	nd
95[ab]	HCR^L	1	no	no	yes
96[ab]	HCR^S	1	nd	yes	no
97[ab]	HCR^S	1	nd	no	nd
98[ab]	HCR^L	1	nd	yes	nd
99[ab]	HCR^L	1	nd	no	yes
102[ab]	HCR^L	1	nd	yes	nd
103[ab]	HCR^L	3	nd	no	yes
105[ab]	HCR^S	1	no	no	nd
106[ab]	HCR^L	1	nd	yes	nd
107[ab]	HCR^L	1	nd	no	nd
P14[ac]	HCR^L	1	yes	no	no
P33[ac]	HCR^S	1	no	yes	no
P34[ac]	HCR^S	1	yes	yes	yes
3510[ac]	HCR^S	1	yes	yes	no
201[ac]	HCR^S	>4	no	no	no
209[ac]	HCR^S	2	nd	yes	yes
219[ac]	HCR^L	3	nd	no	nd
220[ac]	HCR^L	nd	nd	no	nd
226[ac]	HCR^L	2	nd	no	nd
301[de]	HCR^S	1	nd	no	nd
315[de]	HCR^S	1	nd	no	nd
316[de]	HCR^S	1	nd	yes	nd
ad1[de]	Air $dier^S$	1	yes	yes	yes
ad3[de]	Air $dier^S$	nd	nd	no	nd

Wild type strains CC425 ([a]) or CC849 ([d]) were transformed with pArg7.8 ([b]), pJD67 ([c]) or pSP124s([e]). [L] leaky phenotype: growth of cells in low CO_2 or very low CO_2 was poor, but some cells could survive under tested conditions. [S] Stringent phenotype: spot test shows almost no growth under tested conditions. [f] Number of DNA inserts detected by Southern blot analysis. nd, not determined.

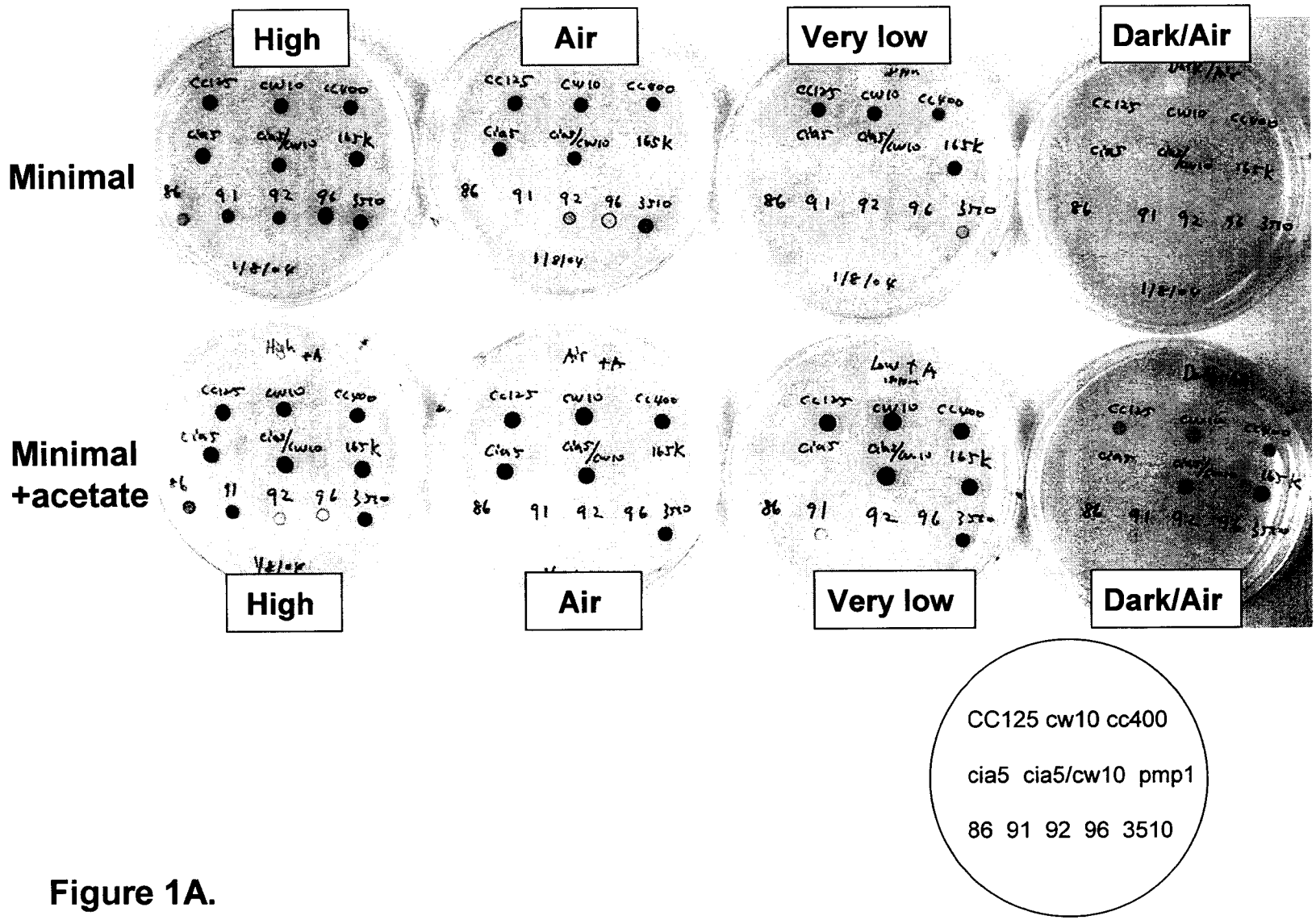

High
Air
Very low
Dark/Air
Minimal
Minimal +acetate
High
Air
Very low
Dark/Air
CC125 cw10 cc400
cia5 cia5/cw10 pmp1
86 91 92 96 3510

Figure 1A.

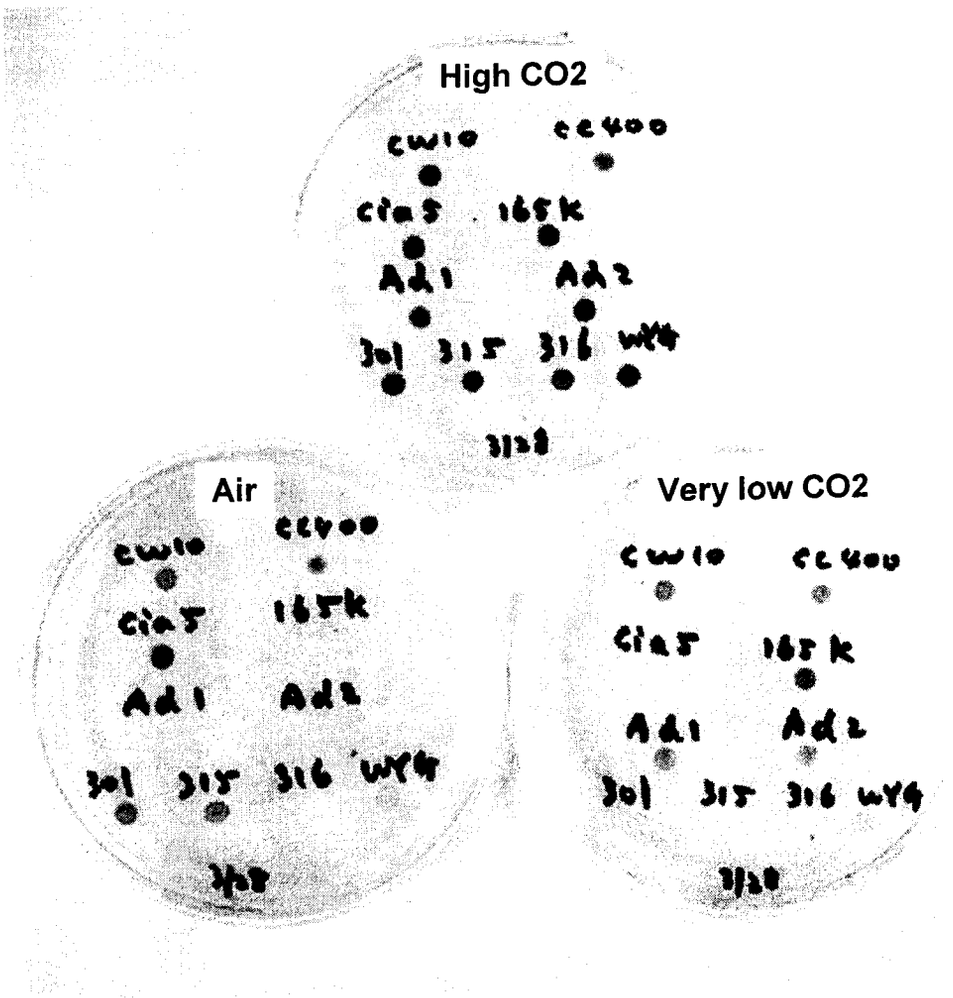
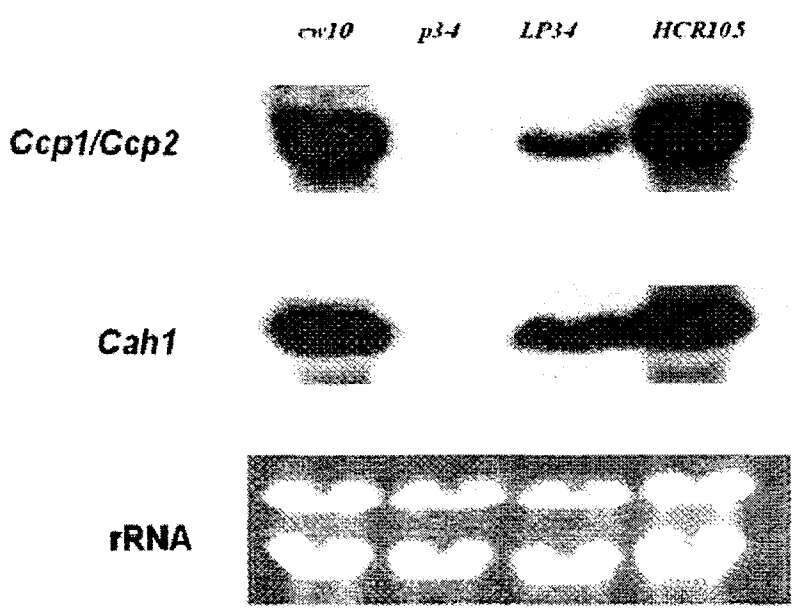
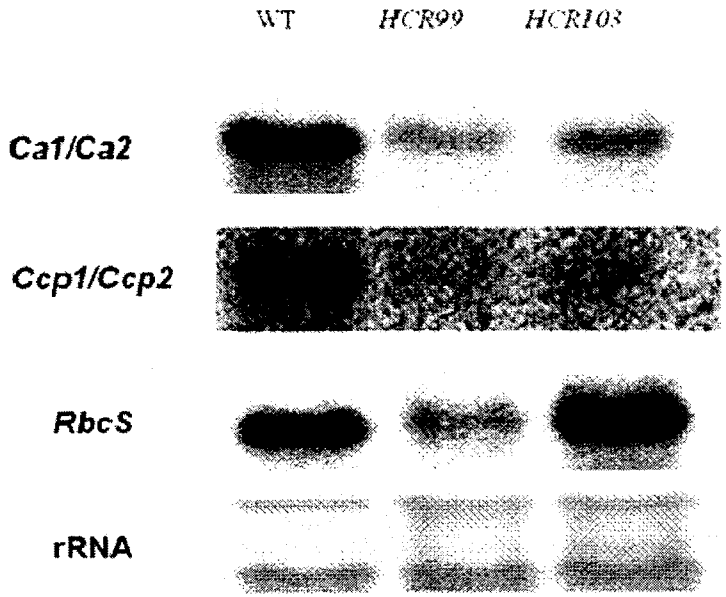


Figure 1B


cw10
p34
LP34
HCR105
Ccp1/Ccp2
Cah1
rRNA

A


WT
HCR99
HCR103
Ca1/Ca2
Ccp1/Ccp2
RbcS
rRNA

B

Figure 2.

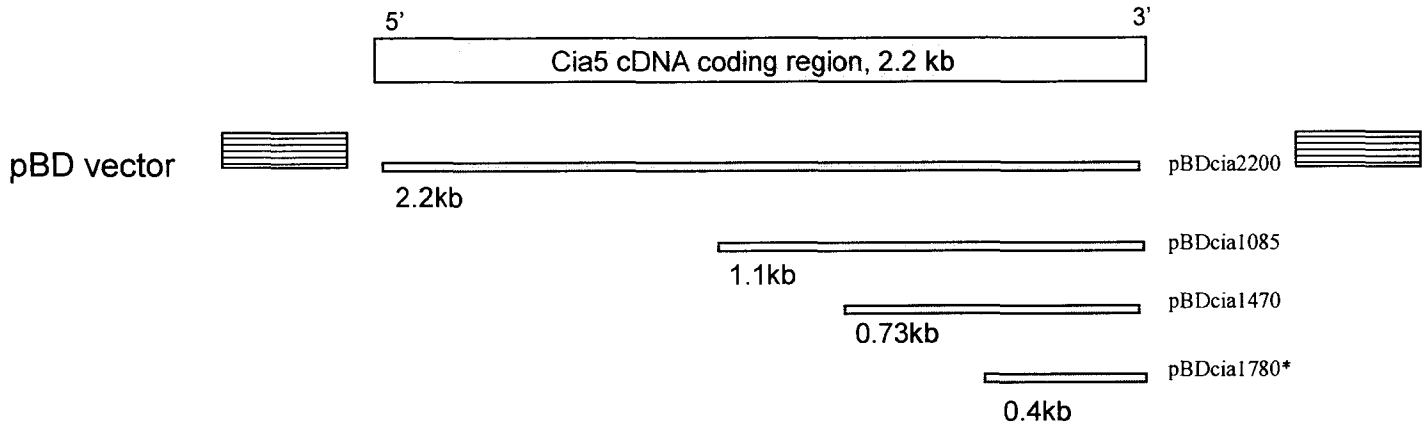

5'
3'
Cia5 cDNA coding region, 2.2 kb
pBD vector
pBDcia2200
2.2kb
pBDcia1085
1.1kb
pBDcia1470
0.73kb
pBDcia1780*
0.4kb

Figure 3.

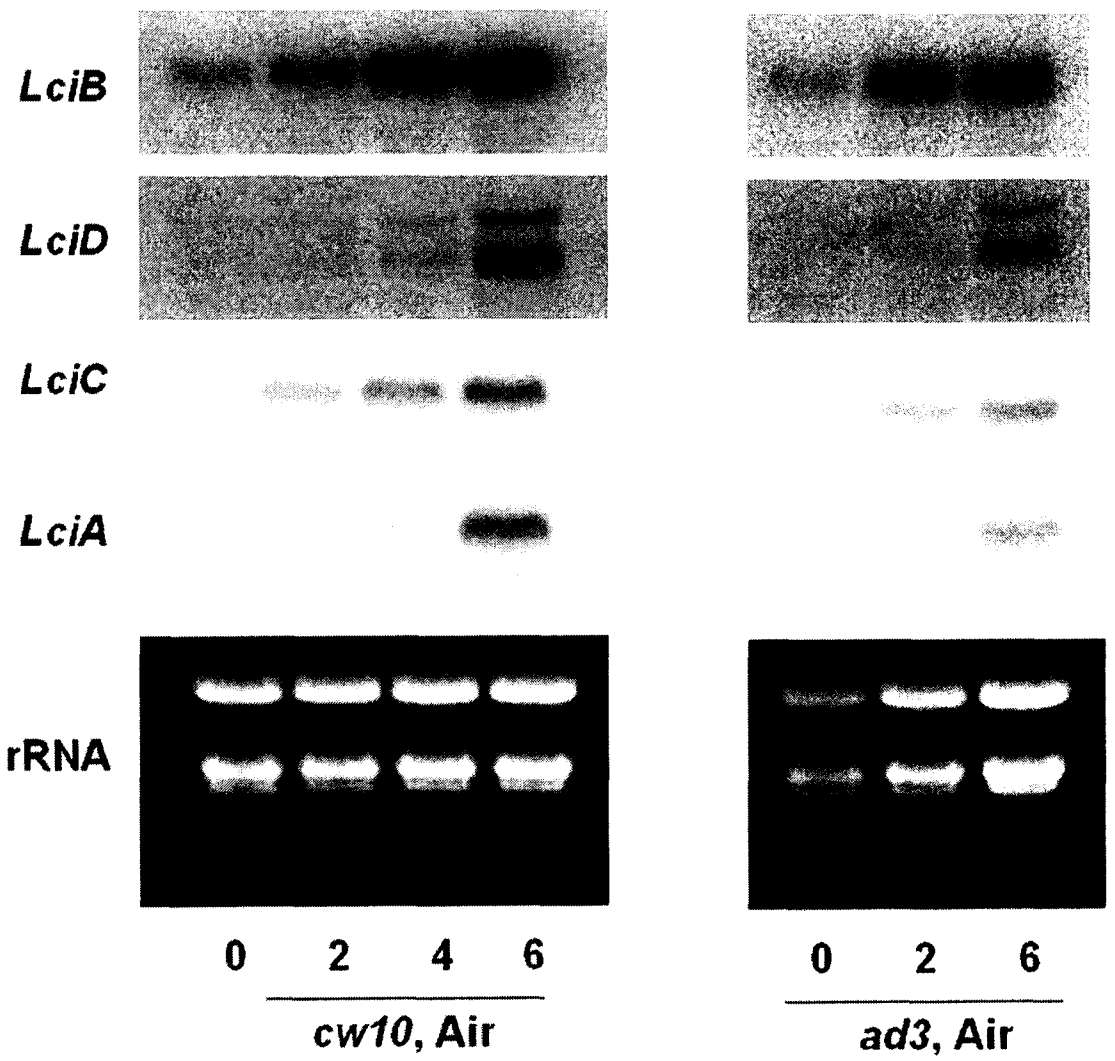


Figure 4.

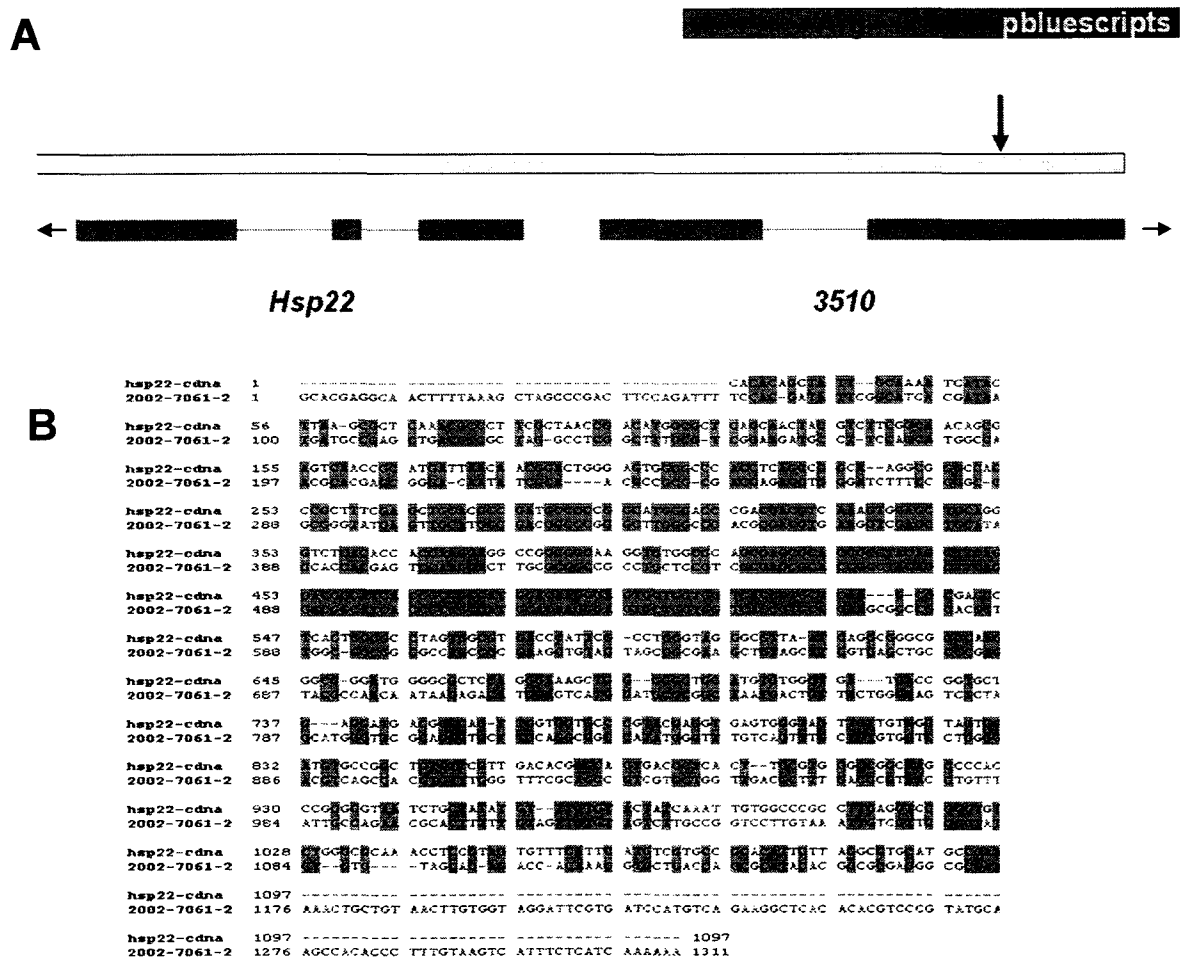


Figure 5.

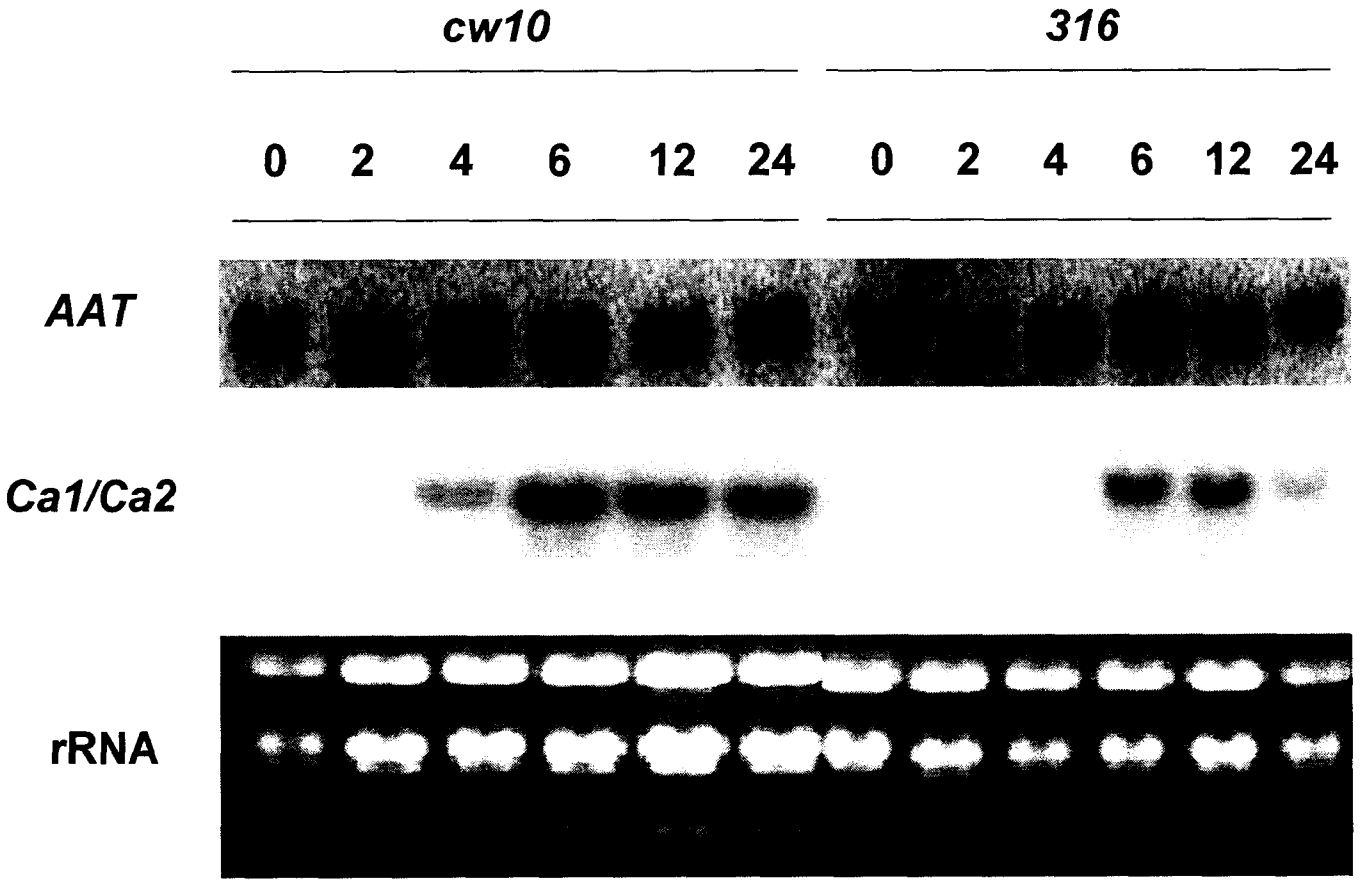


Figure 6

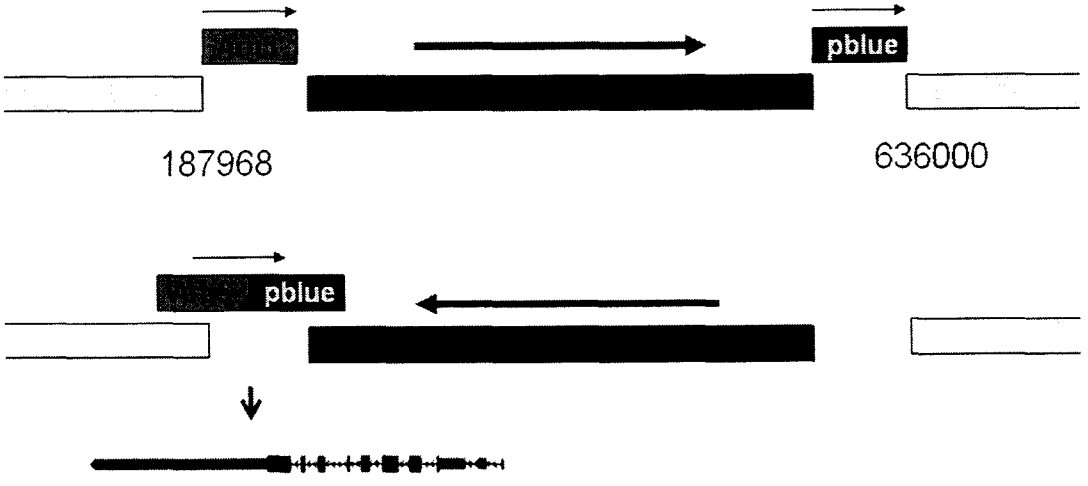


Figure 7.

Chapter 5. Regulation of chloroplast ribosomal proteins during limiting CO_2 acclimation in *Chlamydomonas reinhardtii*

Abstract

The inducible CO_2 concentrating mechanism (CCM) of microalgae represents an adaptation whereby a microalga can acclimate its metabolism to changes in its external environment. During limiting CO_2 acclimation in *Chlamydomonas reinhardtii*, many genes have been demonstrated to be either up- or down-regulated at the level of transcript abundance. Here, we report down-regulation of genes encoding chloroplast ribosomal proteins upon exposure of cells to limiting CO_2 concentrations. The transcript of a gene encoding a homologue of *E. coli* ribosomal protein L7/L12 decreased in abundance when cells were transferred from high CO_2 (5%) to low CO_2 (350 ppm) or very low CO_2 (100 ppm and 50 ppm) in wild-type *C. reinhardtii* but not in *cia5*, a mutant with a defect in an element regulating CO_2 responses. The extent of the decrease in message abundance was inversely correlated with the CO_2 concentration. In 350 ppm or 100 ppm CO_2, the decrease in transcript abundance was transient, showing either a full (350 ppm CO_2) or a partial (100 ppm CO_2) recovery after 12 hours. However, in a mutant, *pmp1*, with a deficiency in accumulation of internal Ci, the decrease in transcript abundance did not recover significantly. The regulation by CO_2 concentration of genes encoding chloroplast ribosomal proteins will be discussed with regard to potential signals and potential roles in the acclimation process.

Introduction

Most aquatic photosynthetic microorganisms can acclimate to limiting CO_2 environments by inducing CO_2 concentrating mechanisms (CCMs). The operation of the CCM and its related limiting CO_2 adaptive responses require concerted action of many gene products. Like other acclimations to changing environments, new polypeptides are induced in *Chlamydomonas reinhardtii* upon transition from high CO_2 to limiting CO_2 environments. In most cases, the genes with an expression induced or up-regulated by limiting CO_2 are among those specifically functioning in the CCM or metabolic pathways essential for acclimation to limiting CO_2 environments. In *C. reinhardtii*, many limiting CO_2 response genes have been

identified, including four genes encoding different carbonic anhydrases (CAs): two periplasmic CAs, pCA1 and pCA2, and two mitochondrial CAs mtCA1 and mtCA2 (Spalding, 1998). Other identified limiting CO_2 induced polypeptides include an alanine-α-ketoglutarate aminotransferase (AAT), two chloroplast proteins of unknown function encoded by *Ccp1* and *Ccp2* and several putative transporters, LciA, LciB/LciC and Mrp1 (Chen et al., 1996; Spalding, 1998; Im and Grossman, 2003; Miura et al., 2004).

While some limiting CO_2 inducible genes show no or a very low expression in high CO_2, but with a large increase in expression in limiting CO_2, many other genes maintain their transcripts in both high CO_2 and limiting CO_2, but exhibit up- or down-regulation with changing CO_2 concentrations. For example, the transcript level of the thylakoid lumen CA (*Cah3*) in limiting CO_2 is 2-fold higher than that in high CO_2 (Karlsson et al., 1998). The genes showing quantitative changes at varying CO_2 concentrations also include genes encoding phosphoglycolate phosphatase *(Pgp1)* and glycolate dehydrogenase (*Gdh1*) (Suzuki et al., 1990; Nakamura et al., 2005). Recent studies of differential gene expression profiles have revealed more genes changing their expression quantitatively under different CO_2 levels. In addition to regulation at the transcriptional level, transient up- or down-regulation at the translational level also has been reported, e.g., transient translational down-regulation of the large and small subunits of Rubisco by limiting CO_2 (Winder et al., 1992).

Since the carbon metabolism in photosynthetic organisms involves several cellular compartments and many different metabolic pathways, the induction of the CCM and the subsequent changes of carbon metabolism status therefore require a corresponding adjustment of other metabolic processes as well. As mentioned earlier, many genes involved in carbon metabolism, especially those in photosynthesis and photorespiration, can be up-regulated during limiting CO_2 acclimation, including *Pgp1*, *Gdh1* and the genes encoding Rubisco subunits (Suzuki et al., 1990; Nakamura et al., 2005; Winder et al., 1992). Moreover, limiting CO_2 as an environmental stress likely will trigger expression changes for genes which are responsive to general stress. However, this has not been extensively investigated in the past.

In this study, we demonstrated that expression of several chloroplast ribosomal proteins is down-regulated during limiting CO_2 acclimation. The extent of down-regulation was observed to be correlated with the CO_2 concentration. We propose that this global adjustment

of ribosomal protein expression reflects a general reaction in response to the stresses conferred by limiting CO_2 environments.

Results

CO_2 regulates expression of 15-178, a CO_2 response gene

A cDNA clone designated as 15-178 was identified accidentally as a CO_2 response gene during screening for Cia5 interacting proteins. Gene expression analyses indicated that this gene was a CO_2 response gene. As shown in the RNA gel blots in Figure1, the transcript abundance of 15-178 in wild-type *C. reinhardtii* cells decreased when cells were transferred from high CO_2 (5%) to low CO_2 (350 ppm) or very low CO_2 (100 ppm and 50 ppm). Quantification of the 15-178 mRNA abundance revealed that the extent of this decrease in transcript abundance appeared to vary inversely with the CO_2 concentration (~30% decrease at 350 ppm CO_2; >60% decrease at 50 ppm CO_2; Figure 2A). However, the decrease of 15-178 transcript level was not observed in *cia5*, a mutant lacking any acclimation associated characteristics in response to limiting CO_2. The expression of 15-178 in *cia5* maintained a nearly constant level during the high CO_2 to limiting CO_2 transition (Figure 1B).

Transient decrease in 15-178 transcript abundance

The decrease in transcript abundance for 15-178 was transient when cells were transferred into low CO_2 (350 ppm CO_2); a nearly full recovery was observed within 12 hours (Figure 2B). However, the decrease was less transient at 100 ppm CO_2; only partial recovery was observed within 12 hours. In an even lower CO_2 concentration, the decreased gene expression appeared relatively stable in that the decreased expression of 15-178 was stable for at least 24 hours in 50 ppm CO_2 (Figure 2B). The expression of 15-178 in *cia5* did not exhibit any detectable response to changing CO_2 concentrations (Figure 1B). In another mutant, *pmp1*, deficient in Ci uptake, the low CO_2 induced decrease in 15-178 transcript abundance appeared more stable compared to that in wild type (Figure 1B).

The 15-178 gene encodes a 70S chloroplast ribosomal protein

The deduced amino acid sequence of 15-178 was used to BLAST search for homologous proteins. The sequence of 15-178 exhibited high levels of amino acid sequence similarity with an *E. coli* ribosomal protein L7/L12. Figure 3 shows the comparison of 15-178 protein with L7/L12 proteins from other organisms. Like many other ribosomal proteins, L7/L12

appeared to be a very highly conserved protein. In addition, 15-178 protein was predicted to contain a chloroplast transit peptide, indicating this *C. reinhardtii* gene probably encodes a chloroplast 70S ribosomal protein.

Expression of genes encoding cytosolic ribosomal proteins

Cells of plants and algae contain both 80S ribosomes and 70S ribosomes that localize in the cytosol and the chloroplast, respectively. While 80S ribosomes represent eukaryotic ribosomes, the 70S chloroplast ribosomes are believed to originate from a photosynthetic prokaryote, and therefore resemble bacterial ribosomes in many aspects. To test whether the expression of ribosomal protein genes of the 80 ribosome also is regulated by CO_2, we monitored the transcript abundance of two cytosolic ribosomal protein genes, CL12 and CL10. RNA gel blots showed that the expression of neither cytoplasmic ribosomal protein gene was influenced by the CO_2 concentration (Figure 4). No obvious changes in transcript abundance were observed when cells were transferred from high CO_2 into various concentrations of limiting CO_2.

Discussion

In this study, we demonstrated that the transcription of chloroplast ribosomal protein L7/L12 was precisely regulated by the CO_2 concentrations. While the transcript abundance of this ribosomal protein gene was more abundant in high CO_2, it decreased when cells were transferred into limiting CO_2, and the transcript abundance was correlated with the CO_2 concentration. Recently, other research groups have performed large scale analyses of gene expression profiles in *C. reinhardtii* cells grown in different CO_2 and light conditions (Im and Grossman, 2002; Im et al., 2003; Miura et al., 2004), and their analyses have revealed that in addition to L7/L12, several other nuclear genes encoding chloroplast ribosomal proteins also exhibited changed expression in response to different levels of CO_2. These genes include the proteins from both large subunit and small subunit of the chloroplast 70S ribosomes, such as L1, L4, L13, L28, L6, L12, S10 and L17 etc., all of which exhibited higher expression in high CO_2, but decreased expression in limiting CO_2. Therefore, a global mechanism appears to exist to regulate the expression of chloroplast ribosomal proteins as a response to varying CO_2 conditions in *C.reinhardtii*.

The ribosome is a factory for protein synthesis and is one of the most essential components required to maintain routine functions in an organism. Like many other essential genes, genes for ribosomal proteins belong to a category of so-called house-keeping genes and need to be expressed all the time. Even so, a few lines of evidence have indicated that the expression of ribosomal proteins in some bacteria could be regulated under certain circumstances (Quiros et al., 1989; 1992; Blanco et al., 1994; 2001). Differences in the ribosomal protein pattern have been reported during the developmental cycle of *Streptomyces granaticolor* and *Streptomyces antibioticus* (Quiros et al., 1989; 1992). In the gram-positive bacteria *Streptomycetes*, the synthesis of two ribosomal proteins, L10 and L7/L12, exhibited a dramatic decrease as cells approached the stationary phase (Blanco et al., 1994).

During limiting CO_2 acclimation, expression of many genes involved in the CCM and its regulation have been demonstrated to be induced or up-regulated (Spalding, 1998). Recent research has shown that expression of at least two CO_2 responsive genes was regulated in a quantitative way which correlates with the ambient CO_2 concentrations (Vance and Spalding, 2005). The correlation between CO_2 concentrations and the transcript abundances of CO_2 responsive genes, including the ribosomal protein genes, indicates existence of a mechanism for precise regulation of gene expression during limiting CO_2 acclimation. It also is interesting to note the lack of L7/L12 transcript down-regulation in the *cia5* mutant, which indicates that the down-regulation is not simply the result of slower growth in limiting CO_2. It appears that a global regulation of chloroplast ribosomal protein expression by CO_2 availability exists, at least at the level of transcript abundance, and that this regulation is affected by Cia5-controlled events.

In *Escherichia coli*, ribosome proteins L7/L12 and L10 are located in the stalk of the ribosomal 50S large subunits and are involved in GTP hydrolysis. L7/L12, as well as other ribosomal proteins, therefore, has both structural and functional importance for translation. In contrast to many other CO_2 responsive genes up-regulated by limiting CO_2, 70S ribosomal protein genes appear to be down-regulated under limiting CO_2 conditions. Although we do not know to what extent the decreases in transcript abundance affect the protein level, we can speculate that this decrease in the transcript abundance of ribosomal proteins may cause an

overall decreased translation activity in the chloroplast. The down-regulation of plastid ribosomal proteins may reflect an acclimation response to limiting CO_2, in which overall protein synthesis can be decreased because photosynthesis and cell growth slow down in limiting CO_2. The recovery of L7/L12 transcript abundance after the transient decrease in low CO_2 acclimated cells may reflect a recovery of photosynthesis and growth rate after the CCM is fully induced.

In photosynthetic eukaryotic cells, while the majority of protein synthesis is performed by cytosolic ribosomes, the plant-specific, organelle ribosomes are responsible for the synthesis of proteins encoded in the plastid genome. The plastid translation system is unique from the nuclear translation system in many respects, and depends on the concerted action of the nuclear and chloroplast genetic systems. Although the plastid genome encodes only a relatively small number of proteins compared with the nuclear genome, the bulk of those synthesized in the chloroplast are important for the biosynthesis of the photosynthetic apparatus. However, because photosynthesis is not always required during all development stages in plants, and because photosynthetic activity needs to be tightly controlled with respect to environmental changes, the expression of the plastid genome must be precisely regulated in order to maintain optimal use of the cellular resources. In *C. reinhardtii* and many other eukaryotic photosynthetic organisms, translational regulation, combined with transcriptional and other post-transcriptional regulations, has been shown to play a significant role in regulating plastid gene expression (Hauser et al., 1998). Although the chloroplast ribosome is similar to that of prokaryotes, the regulation of chloroplast translation has been demonstrated to be quite different from the prokaryotic paradigm. The interactions between *cis*-acting sequences in the 5' untranslated regions and specific *trans*-acting nuclear gene products appear to mediate translation of plastid encoded mRNAs for specific genes (Hauser et al., 1998). The regulation of ribosomal protein expression demonstrated in this research could represent a new mechanism for regulation at the translational level in plastids, by which global modulation of gene expression in plastids can be achieved.

Methods and Materials

Cell Strains and Culture Conditions

Wild type C. reinhardtii strain CC849 was obtained from the *Chlamydomonas* Genetics Center (Duke University), *cia5* and *pmp1* were described previously (Spalding et al., 1983; Moroney et al., 1989). All strains were maintained on plates with CO_2 minimal medium as previously described (Geraghty et al., 1990). Liquid cultures were grown on an orbital shaker under aeration with 5% CO_2 in air (high CO_2-grown cells) or different limiting CO_2 concentrations. For limiting CO_2 induction, cell cultures were switched from elevated CO_2 to various limiting CO_2 concentrations for different time durations as indicated in the experiments.

In liquid culture, continuous gas flow was maintained through the culture flasks. Gas conditions used in this study were: high CO_2 (5% CO_2 in air v/v) which was obtained by mixing compressed CO_2 gas with normal air; low CO_2 (normal air, 350-400ppm); and very low CO_2 (50 ppm and 100 ppm) which was obtained by mixing normal air with either compressed CO_2-free air or CO_2 depleted air (air passed through a saturated sodium hydroxide solution).

Identification of 15-178 gene

The cDNA clone 15-178 was identified as a false positive Cia5 interacting protein from yeast two-hybrid screens (as described in Chapter 4). The 15-178 cDNA was around 1.5 kb, and appear to include the whole coding sequence for a protein similar to prokaryotic L7/L12 ribosomal proteins.

RNA Blot Analysis

Total RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (1987). RNA was transferred onto the Hybond-N^+ nylon transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and then covalently fixed by UV-cross linking (UV Stratalinker 1800, Stratagene). Radio-labeled DNA probes were synthesized by random priming (Promega). The probe used for detecting 15-178 expression was an *Xho*I/*Eco*RI fragment from the plasmid containing 15-178 cDNA. The control, constitutive expression probe was a fragment of the coding region of a G-protein β-subunit-like polypeptide (gift from Dr. Schloss; Schloss, 1990). Northern analyses were performed as standard procedures (Sambrook et al., 1989). After blotting, membranes were

scanned using a PhosphorImager (Storm). Expression level of individual genes on each blot was normalized to hybridization with the constitutive probe (gene for G-protein β-subunit-like polypeptide) using ImageQuaNT (Molecular Dynamics, Piscataway, NJ).

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Figure legends

Figure 1. Northern blot analysis of 15-178 transcript abundance in wild type and mutant *C. reinhardtii* cells during very low CO_2 (100 ppm) acclimation. The cells were grown in high CO_2 (5%), and then transferred into very low CO_2 (100 ppm). Total RNA was isolated after transfer of cells into very low CO_2 at times indicated. Transcripts were hybridized with 15-178 cDNA as probe, and the 15-178 transcript abundances were normalized to that of a constitutively expressed gene encoding a G protein β-subunit-like protein. **A,** 15-178 transcript abundance in cells from wild type (*cw10*) and from the limiting CO_2 signal transduction mutant *cia5* after transfer of cells to very low CO_2. **B,** Relative 15-178 expression level in wild type (*cw10*), *cia5* and *pmp1* mutants during very low CO_2 acclimation. After normalization with transcripts of the G protein β subunit-like polypeptide, the level of 15-178 transcript abundance from high CO_2 grown cells was set at 100%.

Figure 2. Expression of 15-178 in wild-type *C. reinhardtii* in response to different CO_2 concentrations. **A,** Relative *15-178* transcript abundance, normalized as described in Figure 1, as a function of CO_2 concentration. **B,** Time course for recovery of relative *15-178* transcript abundance after transfer of cells from high CO_2 into low (350ppm) or very low (100 ppm and 50 ppm) CO_2.

Figure 3. Similarity of 15-178 to L7/L12 ribosomal proteins from other organisms. Identical or similar amino acid residues are shaded with black boxes. Accession numbers of L7/L12 sequences are as follow: *Arabidopsis thaliana*, AAM64725; *Chlorella vulgaris*, NP_045915; *Escherichia coli*, 30042991; *Euglena gracilis*, NP_041932; *Lactococcus lactis* subsp., NP_267424; *Homo sapiens* (human), NP_002940; *Nicotiana tabacum*, CAA44226; *Porphyra purpurea*, NP_053949; *Prototheca wickerhamii*, CAB38448; *Synechocystis* sp. PCC 6803, NP_440736. Alignment was performed using CluxtalX.

Figure 4. Expression of *CL10* and *CL12*, two genes encoding cytoplasmic ribosomal protein. Total RNA (10 mg/lane) was isolated during 0-12 hours after wild type or *cia5* cells were transferred from high CO_2 to very low CO_2 (100 ppm or 50 ppm).

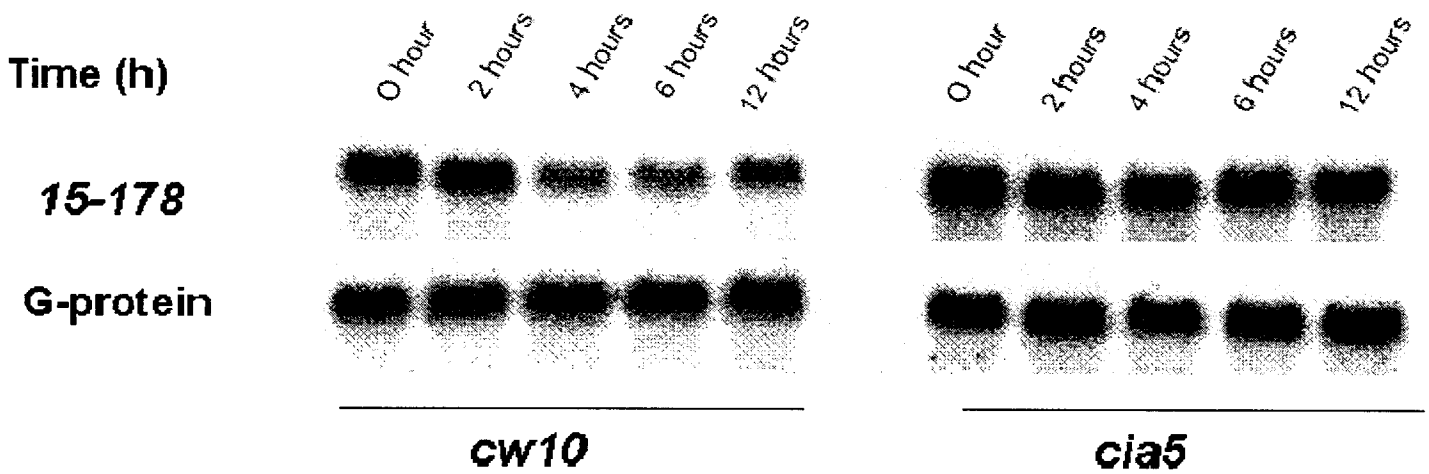
Time (h)
0 hour
2 hours
4 hours
6 hours
12 hours
0 hour
2 hours
4 hours
6 hours
12 hours
15-178
G-protein
cw10
cia5

Figure 1A.

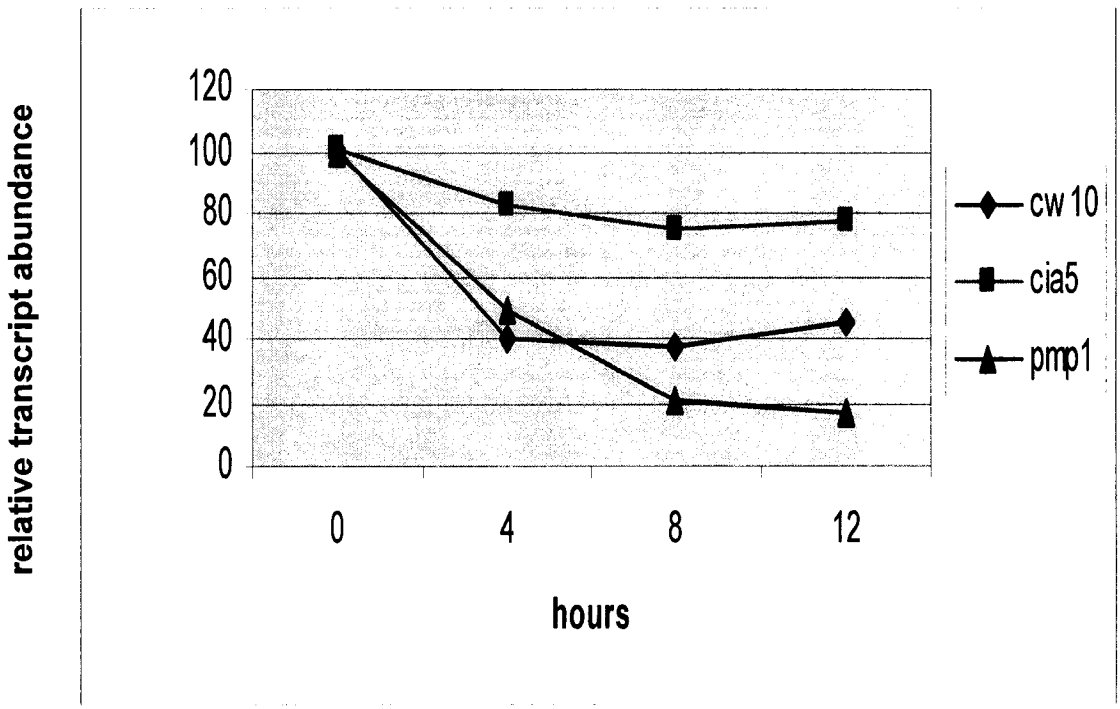
relative transcript abundance
120
100
80
60
40
20
0
0
4
8
12
hours
cw 10
cia5
pmp1

Figure 1B.

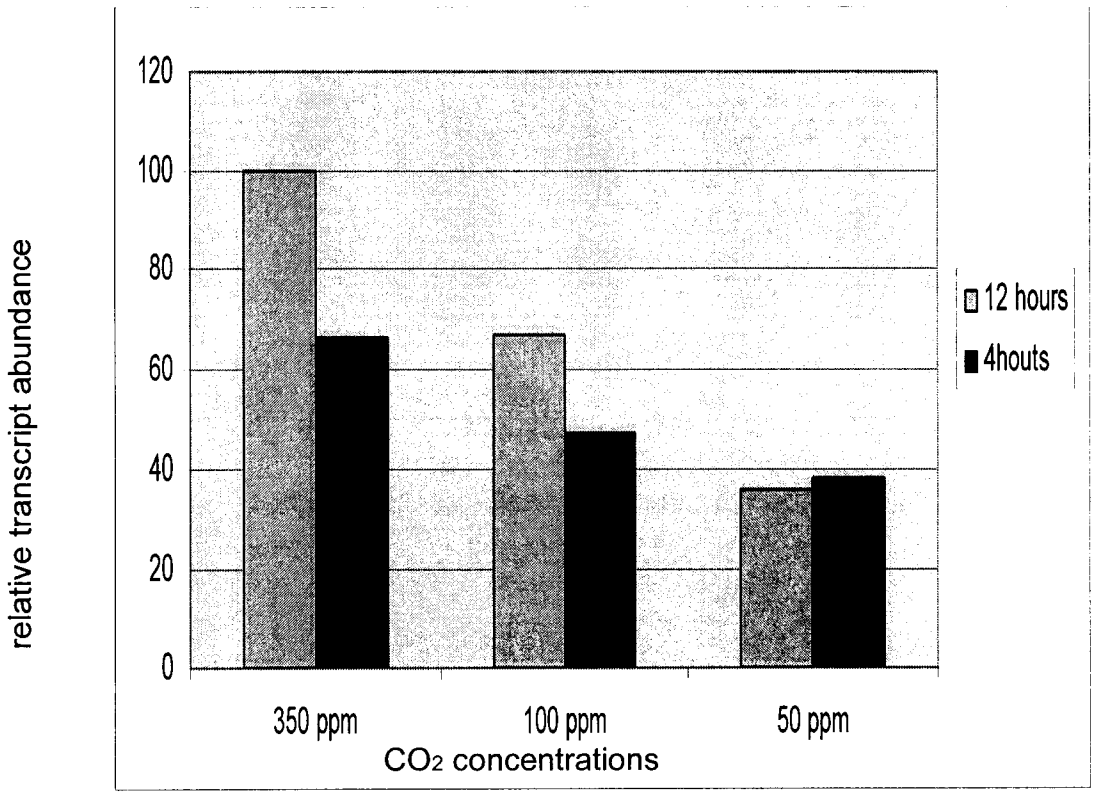
relative transcript abundance
120
100
80
60
40
20
0
12 hours
4houts
350 ppm
100 ppm
50 ppm
CO2 concentrations

Figure 2A.

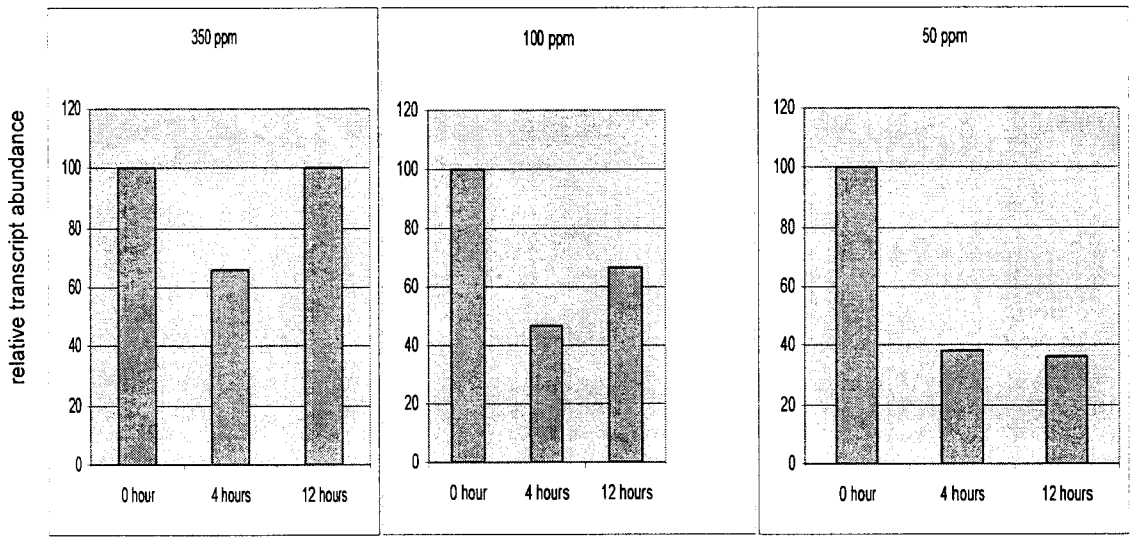
relative transcript abundance
350 ppm
100 ppm
50 ppm
120
100
80
60
40
20
0
0 hour
4 hours
12 hours

Figure 2B.

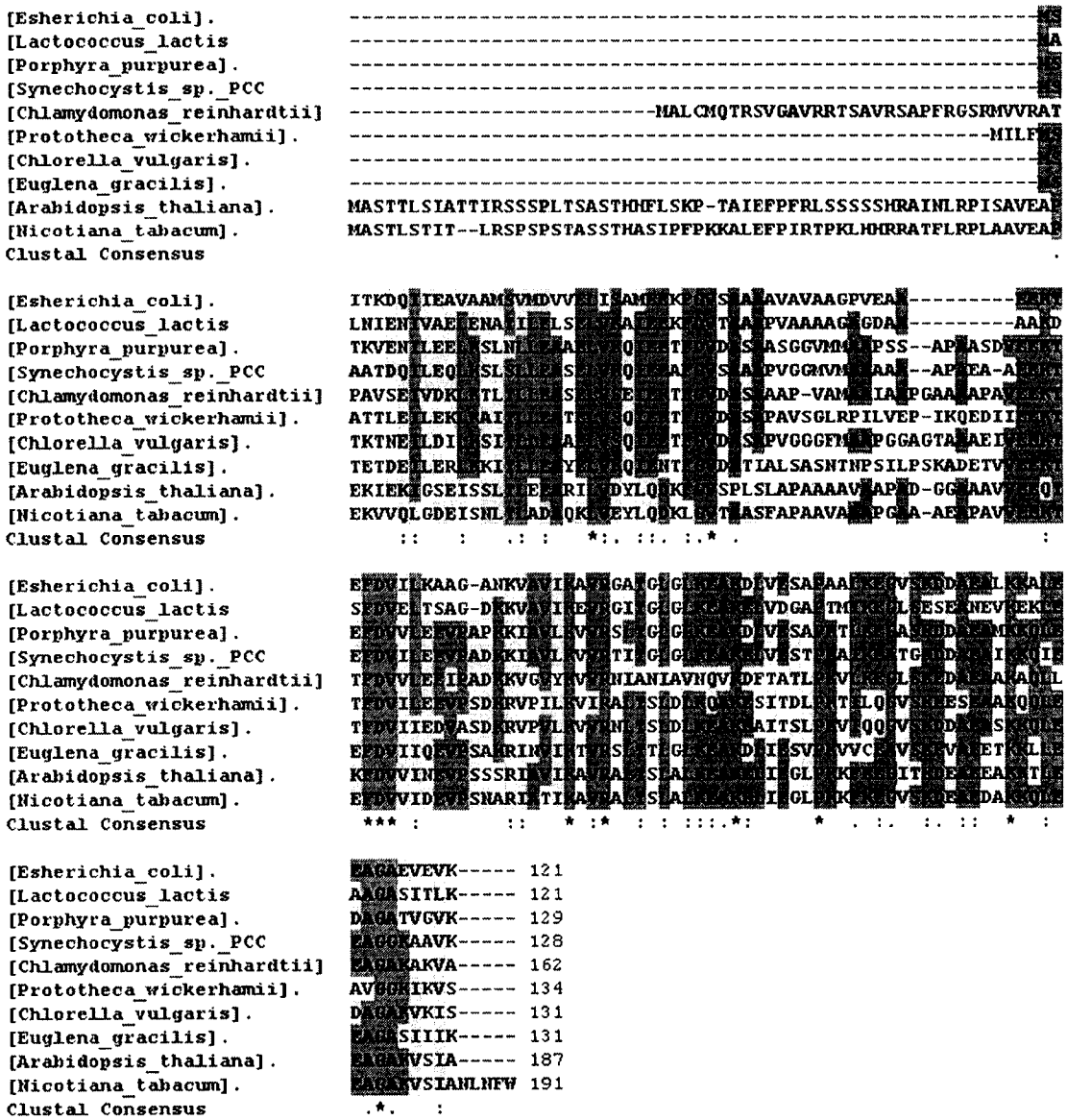
[Escherichia_coli].
[Lactococcus_lactis
[Porphyra_purpurea].
[Synechocystis_sp._PCC
[Chlamydomonas_reinhardtii]
[Prototheca_wickerhamii].
[Chlorella_vulgaris].
[Euglena_gracilis].
[Arabidopsis_thaliana].
[Nicotiana_tabacum].
Clustal Consensus
121
121
129
128
162
134
131
131
187
191

Figure 3.

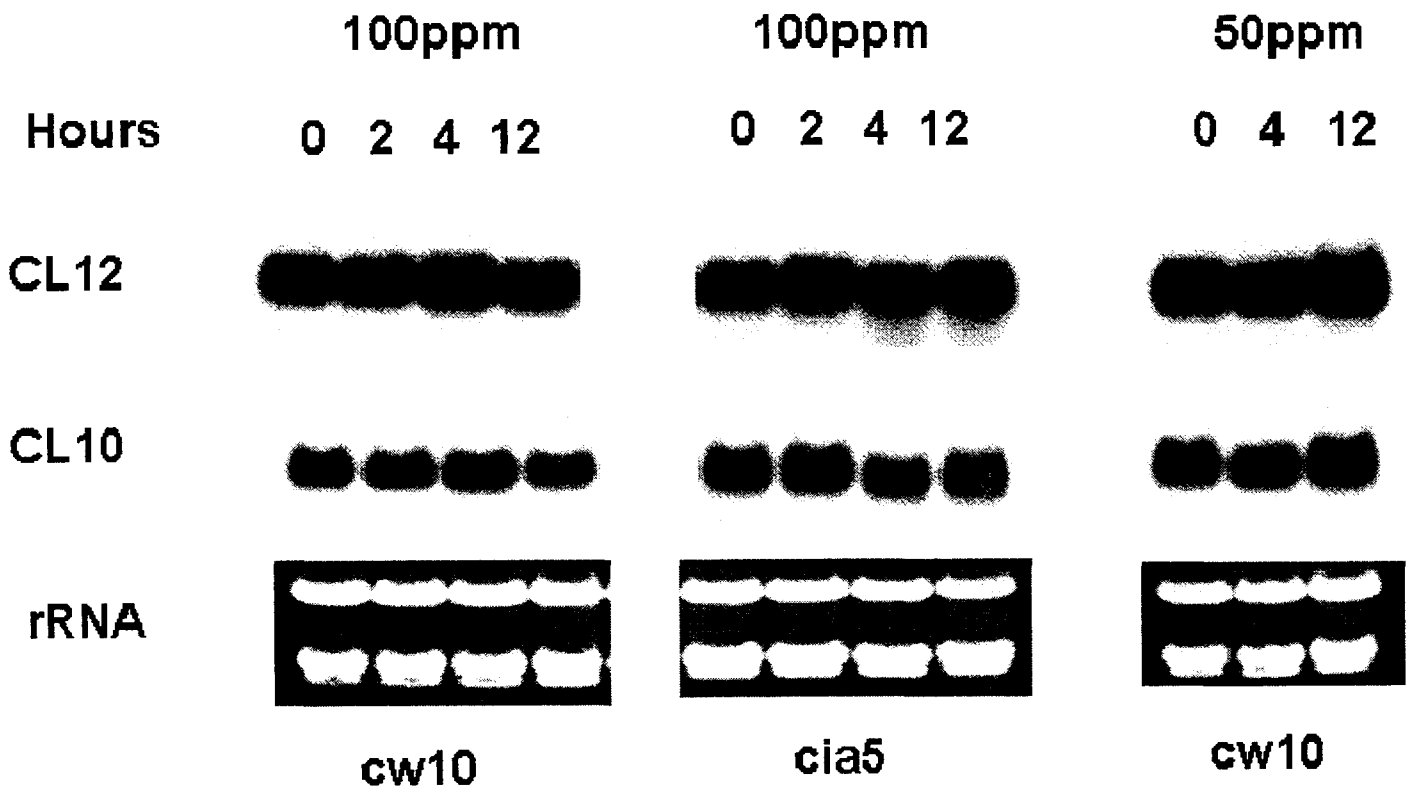
100ppm
100ppm
50ppm
Hours
0 2 4 12
0 2 4 12
0 4 12
CL12
CL10
rRNA
cw10
cia5
cw10

Figure 4.

Chapter 6. General summary

General conclusions

Photosynthetic algae and cyanobacteria play a predominant role in the carbon cycle on the earth by converting atmospheric CO_2 into organic compounds. As CO_2 is frequently limited in the aquatic environments these microorganisms inhabit, CO_2 concentrating mechanisms (CCMs) have evolved by which means these microorganisms can accumulate a large quantity of intracellular inorganic carbon (Ci) and maintain high rates of photosynthesis. Recently, with the aid of mutant studies and the availability of several cyanobacterial genomes, an integrated picture is emerging to reveal many of the molecular details in the cyanobacterial CCMs (Badger and Price, 2003). The major components involved in the CCM have been identified and characterized in cyanobacteria, including Ci uptake systems and enzymes catalyzing the interconversion between different species of Ci. Moreover, introduction of the cyanobacterial *ictB* gene, a gene proposed to be involved in Ci uptake, into *Arabidopsis* and tobacco plants has been reported to enhance photosynthesis and growth in the transgenic plants (Bonfil et al., 1998; Lieman-Hurwitz et al., 2003). These advances indicate that the CCM not only represents a good model system for studying the adaptive characteristics in an organism in response to a changing environment, but also promise a potential application to boost the photosynthetic potential in C3 crop plants, which normally exhibit a low affinity for CO_2.

Compared to those of cyanobacteria, the photosynthetic characteristics and carbon metabolism of eukaryotic algae are more similar to higher plants in many respects. However, our knowledge about the CCM in eukaryotic algae is far more limited than that in cyanobacteria. In order to gain better understanding of limiting CO_2 acclamation in eukaryotic microalgae, we chose *Chlamydomonas reinhardtii* as a model system to unravel the molecular network involved in the CCM operation and regulation, and have applied insertional mutagenesis, together with physiological and genetic analyses, to identify and characterize non-acclimating mutants. This approach has proved to be valuable for increasing our understanding of the CCM and limiting CO_2 acclimation at the molecular level.

The induction of the CCM requires the concerted operation of a diverse set of biochemical and physiological events involving many gene products. In agreement with this, our mutant studies have revealed that many defects, which fall into various categories, can result in non-acclimating characteristics in *C. reinhardtii*. As summarized in Chapter 4, defects in these mutants can be categorized into three major groups: (1), regulatory events; (2) Ci uptake systems; (3) other physiological processes related in carbon acquisition.

Among the mutants exhibiting defects in regulation of the CCM, *p34* and *HCR209* were demonstrated to be alleles of *cia5*, which lacks any limiting CO_2 inducible acclimating characteristics. In line with its proposed establishment as a master regulator upstream the signal transduction pathway associated with limiting CO_2 acclimation, Cia5 must require other components to form a regulatory network for control of the global responses to one or more limiting CO_2 signals. In attempts to identify Cia5 interacting proteins, we performed yeast two-hybrid screening. However, our efforts in this direction have not resulted in the identification of any bona fide interacting protein. This lack of success might be result from: (1) the scale of screening not being large enough to identify bona fide interacting gene products, e.g., gene products with low abundance in the cDNA library; (2) interacting domains of Cia5 itself were not included because of the self-activation of Cia5 in the screen; (3) a requirement for *C. reinhardtii* specific post-translational modification to facilitate protein-protein interaction that is absent in yeast system. Despite the difficulties encountered thus far, there still is more ground to cover, including bait constructs from other functional domains, and/or larger scale screenings.

Whilst Cia5 may represent a global regulator in limiting CO_2 acclimation, additional regulatory components may only affect part of the regulatory network. The mutation of these regulatory proteins therefore may result in partial defects in acclimation responses. In *HCR103, HCR92* and *HCR95*, either the expression of CO_2 responsive (CO_2-R) genes was reduced or exhibited aberrant timing compared to wild type or only some of the CO_2-R genes were induced by limiting CO_2. Characterization of these mutants and identification the defective genes will likely reveal some downstream components in the signal transduction pathway. In addition, it will be valuable to identify more regulatory mutants exhibiting altered gene expression in response to limiting CO_2 in order to dissect the entire regulatory network associated with limiting CO_2 acclimation.

Among the mutants categorized as defective in components of metabolic pathways required for limiting CO_2 acclimation, two mutants, *HCR96* and *HCR3510*, were detailed in Chapter 3 and Chapter 4, respectively. While *HCR3510* appears to be defective in gene products possibly involved in photoprotection, suggesting that limiting CO_2 acclimation relies on adjustments in photosynthesis, *HCR96* is defective in a subunit of 2-oxoglutarate dehydrogenase, a key enzyme in the tricarboxylic acid cycle. Although it is not yet conclusive whether the defect in 2-oxoglutarate dehydrogenase causes the HCR phenotype in *HCR96*, it is possible that its HCR phenotype is related to the impaired function of respiration under autotrophic growth conditions. Even with photosynthesis being able to satisfy the energy requirement, the essentiality of respiration may result from a reliance on its function in carbon metabolism to generate carbon skeletons for biosynthetic reactions, or in its interactions with photosynthesis to maintain the redox balance between different energy-generating processes. Nevertheless, future study of *HCR96* may provide clues regarding the function of respiration in a photosynthetic organism and the interactions between the photosynthesis and respiration.

Finally, we isolated a *pmp1* allele, *ad1,* which is defective in Ci uptake and exhibits an *air dier* phenotype. This mutant is especially interesting because of its dramatic shift in phenotype between low (air level) CO_2 and very low CO_2, suggesting it is defective specifically in the low CO_2 (air level) acclimation response. As detailed in Chapter 2, we have identified the defective gene in *ad1*, *LciB*, which was previously identified as a CO_2.R gene. Although the lack of similarity of *LciB* to any other proteins with known functions makes it difficult to predict its exact role in Ci uptake, *LciB* is the first gene unambiguously confirmed to function directly or indirectly in Ci uptake in *C. reinhardtii*. Therefore, our future research will focus more on the *LciB* gene and its gene products, because it provides a great opportunity to unravel the Ci uptake mechanism in photosynthetic eukaryotes and the multiple acclimation response to different levels of limiting CO_2.

Research plan in future

1. Identify the proteins in LciB complex.

Yeast two hybrid screens already have proved to be an efficient approach to identify LciB interacting proteins, as demonstrated by confirmation of the interaction between LciB/LciC and/or LciB/LciB. However, because *LciB* and *LciC* are expressed with high abundance, at

least at the message level, they may overshadow other genes with lower transcript abundance in the cDNA library. It may require a much larger scale screening in order to reveal interacting proteins other than LciC and LciB. In addition, as indicated in the discussion in Chapter 2, other LciB/LciC interacting proteins could be transmembrane proteins, therefore hydrophobic, making them difficult to identify in conventional yeast two hybrid systems. Therefore, we are planning to apply another approach, co-immunoprecipitation to identify additional interacting proteins. This approach also will be used to confirm the interaction between proteins previously identified in the yeast two hybrid system. Putative interacting proteins will be analyzed by MALDI-TOF and/or tandem MS/MS following 2D-PAGE separation of the co-immunoprecipitated proteins.

2. Down-regulation of expression of *LciB* family genes by RNA interference (RNAi)

As LciB has already been proven as essential in Ci uptake (maybe LciC as well), it would be interesting to identify the role(s) played by other members in this family, especially *LciD* and *LciE*. A reverse genetic approach, RNAi, will be applied to knock down the expression of *LciD* and *LciE*. As indicated by the phenotype exhibited in *ad1* and *pmp1*, members in *LciB* gene family do not appear to be functionally redundant, at least not with regard to LciB function. Therefore, down-regulating expression of individual members of this family may distinguish the distinct role(s) played by them, if any, in low or very low CO_2 acclimation.

3. Identification and characterization of more *air dier* mutants and their suppressors

Besides *ad1*, we have isolated another mutant with similar *air dier* phenotype, *ad3*. In *ad3*, most genes in *LciB* family, including *LciB*, as well as the putative Ci transporters *LciA* and *Mrp1*, exhibited expression similar to that in wild type, suggesting the defect in *ad3* is different from that in *ad1*. Therefore, *ad3* may represent another component essential for air level CO_2 acclimation. This protein might be a component of an LciB complex, or it may be located in another system essential for low CO_2 acclimation. Low (air level) CO_2 acclimation may require many different components, and, if so, identification of other *air dier* mutants may help identify others. Identification and characterization of *ad3* and other *air dier* mutants, or suppressors of these *air dier* mutants will provide a more integrated picture of low CO_2 acclimation.

4. Investigation of low CO_2 vs. very low CO_2 acclimation

The majority of past research on limiting or low CO_2 acclimation in *C. reinhardtii* has focused mainly on air level CO_2 acclimation, while targeted research on very low CO_2 acclimation has been limited. Since studies on *ad1* and *pmp1* reveal the existence of multiple discrete levels of limiting CO_2 acclimation, future investigation of these distinct acclimation states should definitely help fill the gap in our understanding of the multiple levels of CO_2 acclimation in *C. reinhardtii*. As physiological research already has provided some details in this issue, more future research should focus on the investigation at the molecular level. Like the value of *air dier* mutants for understanding low CO_2 acclimation, mutants unable to acclimate specifically to very low CO_2 would help unravel the mechanism of very low CO_2 acclimation. Several mutants in our collection exhibit this "*very low CO_2 dier*" phenotype, and the approaches mentioned in investigation of *air dier* mutants also will be used for these mutants.

In addition, large scale gene expression profiles and comparisons between cells grown in different CO_2 conditions is a very effective way to identify the molecular components associated with each of the different acclimation states, as well as components associated with both. In past large scale gene expression studies, different levels of limiting CO_2 have not been distinguished, making it difficult to interpret the results with regard to these distinct acclimation states. We have reasons to believe that application of large scale gene expression analyses (microarray analyses) of different acclimation states will provide a wealth of additional information regarding the mechanisms of limiting CO_2 acclimation in *C. reinhardtii*.

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