Isolation and characterization of two closely related beta-carbonic anhydrases of Chlamydomonas reinhardtii

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ISOLATION AND CHARACTERIZATION OF TWO CLOSELY RELATED BETA-CARBONIC ANHYDRASES OF CHLAMYDOMONAS REINHARDTII

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

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ABSTRACT

Aquatic photosynthetic organisms such as the green alga, *C. reinhardtii* respond to low CO₂ conditions by inducing a CO₂ concentrating mechanism (CCM). Important components of the CCM are carbonic anhydrases (CAs), zinc metalloenzymes that catalyze the interconversion of CO₂ and HCO₃⁻. *C. reinhardtii* now has nine carbonic anhydrases, three α-CAs and six β-CAs. This dissertation describes the identification and characterization of two closely related β-CAs, CAH7 and CAH8. These CAs are 63% identical.

CAH7 and CAH8 encode proteins of 399 and 333 amino acids, respectively. Both of these CAs are constitutively expressed at the transcript and protein level. Preliminary results of immunolocalization studies localized CAH7 in the chloroplast while CAH8 was localized in the periplasm. Both the CAH7 and CAH8 open reading frames (ORF) were cloned in the overexpression vector pMal-c2x and expressed as recombinant proteins. Activity assay demonstrated that CAH7 and CAH8 are active carbonic anhydrases. The proposed roles for CAH7 and CAH8 are discussed.

Previously, insertional mutants were generated to be able to isolate HCO₃⁻ transporters and other proteins that might be essential for a functional CCM. One of the generated insertional mutants is slc211, a mutant that requires high CO₂ for optimum growth. The mutant slc211 had an insertion in the novel gene designated as CIA7. RNA interference was successfully used to reduce the expression of CIA7. The resultant transformants had a growth phenotype similar to slc211 requiring high CO₂ for optimum growth. These results suggest that CIA7 is a gene that facilitates growth in *C. reinhardtii* under low CO₂ conditions. The possible functions of CIA7 are discussed.
CHAPTER 1
INTRODUCTION

Photosynthesis supports the majority of life on Earth and aquatic photosynthetic microorganisms account for almost 50% of the world’s photosynthesis (Field et al., 1998). *Chlamydomonas reinhardtii* is a unicellular eukaryotic alga that has been an excellent model organism to study the vital process of photosynthesis. Since *C. reinhardtii* is unicellular it is simpler than multicellular organisms and has a photosynthetic apparatus closely related to higher plants. The most significant attribute of *C. reinhardtii* as a model organism for the study of photosynthesis is its ability to grow heterotrophically using acetate as a sole carbon source which allows isolation of viable mutants unable to perform photosynthesis (reviewed in Dent et al., 2001).

Carbon dioxide, although present in minute quantities in the environment, has a significant impact to life, since it is a major substrate of photosynthesis. Quite a number of photosynthetic organisms face the challenge of surviving low CO$_2$ concentrations and thus mechanisms to increase levels of inorganic carbon higher than atmospheric levels are widespread among photosynthetic organisms. Aquatic photosynthetic organisms such as the green alga *C. reinhardtii* respond to low CO$_2$ conditions by inducing a CO$_2$ concentrating mechanism (CCM). The role of the CCM is to increase the concentration of CO$_2$ for Rubisco, the enzyme responsible for the initial fixation of CO$_2$. Unlike C4 and CAM photosynthesis in higher plants, the mechanism whereby aquatic photosynthetic algae concentrate and transport inorganic carbon has not been fully elucidated. Likewise, a number of components are yet to be identified although it is clear that one important component of the CCM are carbonic anhydrases, which are zinc metalloenzymes that catalyze the interconversion of CO$_2$ and HCO$_3^-$, and transport systems for inorganic carbon.
The general objective of this dissertation is elucidation of some of the components involved in the CO\textsubscript{2} concentrating mechanism in \textit{C. reinhardtii}. To understand the CO\textsubscript{2} concentrating mechanism in \textit{C. reinhardtii}, insertional mutagenesis was used to identify new genes that might be involved in acclimation to low CO\textsubscript{2} conditions (Colombo et al., 2002). Insertional mutants that show poor growth in low CO\textsubscript{2} conditions might have a gene required for growth under low CO\textsubscript{2} conditions disrupted by the insert. On the otherhand, the sequencing of the \textit{C. reinhardtii} genome led to the identification of two additional β-carbonic anhydrases, designated as \textit{CAH7} and \textit{CAH8}.

The specific objectives of this study include (1) identification and characterization of the β-carbonic anhydrases, \textit{CAH7} and \textit{CAH8}, (2) identification and characterization of the insertional mutant \textit{slc211} and its disrupted gene we designated as \textit{cia7}, and (3) the determination of the possible roles that the protein products of these genes play in photosynthesis and in the CCM. Chapters 4 and 5 discuss CAH7 and CAH8, respectively while chapter 6 discusses the insertional mutant \textit{slc211}. In general, the broader and longer term impact of the elucidation of the CCM components in the unicellular alga \textit{C. reinhardtii} can aid in the feasibility of some prospects of introducing CCM components from a more efficient CO\textsubscript{2} concentrating mechanism into higher plants for increased crop productivity.
CHAPTER 2
REVIEW OF LITERATURE

CARBON DIOXIDE CONCENTRATING MECHANISM

Many algae have adapted to limiting CO₂ conditions through the development of a CO₂ concentrating mechanism (CCM). The CCM is a biological adaptation to low carbon dioxide concentrations in the environment and is a variation of photosynthesis in which organisms raise the level of carbon dioxide in the cell many times over the environmental concentration of carbon dioxide (Moroney, 2006).

Why Do Microalgae Need the CCM?

First, the ability of photosynthetic organisms to use CO₂ for photosynthesis depends in part on the properties of ribulose bisphosphate carboxylase-oxygenase (Rubisco). Rubisco is an unusually slow enzyme with a low affinity for CO₂. At atmospheric levels of CO₂, Rubisco can only function at about 25% of its catalytic capacity. These conditions are further exacerbated by O₂ being a competitive substrate of CO₂. Second, the rate of diffusion of CO₂ in aqueous solution is ten thousand times slower than the diffusion of CO₂ in air. Thus the ability to scavenge CO₂ as quickly as it becomes available is highly advantageous to aquatic photosynthetic organisms. Third, algal environments are often subjected to fluctuations in inorganic carbon (Ci = CO₂ + HCO₃⁻) concentrations and pH that changes the availability of CO₂ and HCO₃⁻ for photosynthesis. At acidic pH, the vast majority of inorganic carbon is in the form of CO₂, while at alkaline pH, most Ci is in the form of HCO₃⁻ with CO₂ making up only a small fraction of the available inorganic carbon (Beardall, 1981; Gehl et al., 1987).

Types of Carbon Dioxide Concentrating Mechanisms

CCMs include biochemical mechanisms such as C4 photosynthesis and Crassulacean Acid Metabolism (CAM), on an active transport of Ci across membranes, or on processes
involving localized enhancement of CO₂ concentration by acidification of a particular cellular compartment (Giordano et al., 2005). The CCM increases the concentration of CO₂ at Rubisco, the enzyme responsible for the initial fixation of CO₂. While three different mechanisms will be discussed below, it is likely that aquatic photosynthetic organisms display a variety of ways to concentrate CO₂.

C4 and CAM Mechanisms. C4 photosynthesis and CAM in terrestrial higher plants were the first photosynthetic CCMs to be described in detail. They involve a spatial (C4) or temporal (CAM) separation of the fixation of CO₂ by PEP carboxylase to produce a four carbon dicarboxylic acid which is transported and decarboxylated, increasing the CO₂ available to Rubisco (Lawlor, 2001). In higher plants, the C4 CCM is dependent on a specialized operation and interaction of leaf mesophyll and bundle sheath photosynthetic cells.

The primary CO₂ fixation is through PEP carboxylase located in the cytosol of the mesophyll cells. PEP carboxylase uses HCO₃⁻ as its substrate for fixation of CO₂ into oxaloacetate, so CO₂ entering from the external environment must be hydrated rapidly by a carbonic anhydrase (CA) and converted to HCO₃⁻. Thus, in C4 plants the predominant CA activity is found in the mesophyll cell cytosol to make this HCO₃⁻, in contrast the highest levels of CA activity in C3 plants are associated with the stroma of mesophyll cell chloroplasts (Badger, 2003; Burnell and Hatch, 1988; Ku et al., 1996; Okabe et al., 1984). C4 dicarboxylic acids such as malate or aspartate formed in the mesophyll cell cytosol serve as the intermediate CO₂ pool.

The presence of C4 or C4-like metabolisms have been observed in submerged aquatic plants and algae. Examples include Isoetes howellii and Sagittaria subulata (Keeley, 1996) and the green ulvophycean benthic macroalga Udotea flabellum (Reiskind et al., 1988; Reiskind and Bowes, 1991) and the planktonic diatom Thalassiosira weissflogii (Reinfelder et al., 2000;
Reinfelder et al., 2004) grown under inorganic CO$_2$ limited conditions. Evidence of a CAM-like mechanism has also been proposed for brown macroalgae where high levels of PEP carboxykinase and fluctuations in titratable acidity and malate have been observed (Johnston, 1991; Raven, 1997b).

**Active Transport of Inorganic Carbon Mechanism.** Examples of active transport of HCO$_3^-$ come primarily from studies using cyanobacteria. Cyanobacteria have a sophisticated CCM which involves a variety of active CO$_2$ and HCO$_3^-$ uptake systems and an internal microcompartment, the carboxysome (Price and Badger, 1989; Badger et al., 2006). At least five distinct Ci transport systems are known in cyanobacteria. An interesting feature of cyanobacterial CCM is the induction of multiple transporters under Ci limitation. Cyanobacteria appear to utilize pairs of Ci transporters with complementary kinetics for the same Ci species.

For example, two complementary HCO$_3^-$ transporters are present in *Synechococcus* PCC7002. The BicA transporter has a relatively low transport affinity of around 38 µM but is able to support a high flux rate. It pairs with the SbtA transporter, which has a high transport affinity of 2 µM but possesses a lower flux rate (Price et al., 2004). This strategy of employing a high flux/low affinity transporter with a low flux/high affinity transporter appears to be a common theme in freshwater and estuarine cyanobacteria (Badger et al., 2006).

In cyanobacteria, the carboxysome is the specialized compartment in which accumulated HCO$_3^-$ is converted to CO$_2$ through the action of specific carboxysomal carbonic anhydrases (Fukuzawa et al., 1992). CO$_2$ is elevated due to diffusion restrictions on efflux by the carboxysome protein shell structure (Price and Badger, 1989; Kaplan and Reinhold, 1999; Kaplan et al., 2001). Thus the overall mechanism elevates HCO$_3^-$ in the cytosol of the cell and converts this accumulated Ci back to CO$_2$ in the carboxysome, the location of Rubisco.
**CO₂ Concentration Following Acidification in a Compartment Adjacent to Rubisco.**

A third type of CCM found in eukaryotic algae relies on the pH gradient set up across the chloroplast thylakoid membrane in the light. In the light, a large ∆pH is established across the thylakoid membrane; the chloroplast stroma has a pH of close to 8.0 and the thylakoid lumen has a pH of between 4 and 5. This pH differential is significant because the pKa of the bicarbonate to CO₂ interconversion is about 6.3 (Equation 1).

Equation 1. \( \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{CO}_2 + \text{H}_2\text{O} \)

Under these conditions HCO₃⁻ is the predominant species of Ci in the chloroplast stroma while CO₂ is the most abundant form of Ci in the thylakoid lumen. Any bicarbonate transported into the thylakoid lumen would be converted to CO₂, thus elevating the CO₂ concentration above ambient levels. This mechanism as first suggested by Semenenko, Pronina and colleagues requires a carbonic anhydrase in the acidic thylakoid lumen to rapidly convert the entering HCO₃⁻ to CO₂ (Pronina et al., 1981; Pronina and Semenenko, 1990). In addition, since HCO₃⁻ cannot rapidly cross biological membranes (Gutknecht et al., 1977), there must be a transport protein or complex that allows HCO₃⁻ to enter the thylakoid lumen. This model predicts that CO₂ accumulation would not occur in the dark as light-driven photosynthetic electron transport is required to set up these pH gradients.

**Acclimation to Changing CO₂ Concentration**

Acclimation to limiting CO₂ concentration involves induction of a set of genes required for a carbon dioxide concentrating mechanism. This acclimation is modulated by some mechanisms in the cell to sense CO₂ availability. In Chlamydomonas reinhardtii, expression of several genes has been shown to be regulated in response to changes in external CO₂
concentration. Levels of mRNAs of most proteins shown to be induced under low CO₂ show a rapid increase soon after transfer of cells to limiting carbon dioxide concentration. Previously identified CO₂ responsive genes include a periplasmic CA, CAH1 (Fukuzawa et al., 1990), the mitochondrial CAs, mtCA1 and mtCA2 (Eriksson et al., 1996), a chloroplast envelope protein, LIP-36 encoded by CCP1 and CCP2 (Chen et al., 1997), a chloroplast inner envelope protein, LCI1 (Burow et al., 1996), AlaAT, an alanine:α-ketoglutarate aminotransferase (Chen et al, 1996) and four photorespiratory genes; GDH1 (glycolate dehydrogenase), PGP1 (phosphoglycolate phosphatase), HPR (hydroxypyruvate reductase), and SGAT (serine glyoxylate aminotransferase) (Mamedov et al., 2001; Tural and Moroney, 2005). The development of the chloroplast pyrenoid is likewise modulated by the supply of CO₂ (Kuchitsu et al., 1991). Recently, by comparing expression profiles of cells grown under CO₂ rich conditions with those grown on CO₂ limiting conditions using a cDNA membrane array containing 10,368 expressed tags, 51 low CO₂ inducible genes were detected in C. reinhardtii (Miura et al., 2004).

The environmental signal controlling the expression of the CCM and CCM-related transcripts has not yet been identified. On the basis of observed photosynthetic performance at various ambient CO₂ and pH levels, it has been postulated that CO₂ concentration is sensed at the plasmalemma by specific receptors (Matsuda et al., 1998). Modified acclimation behavior of C. reinhardtii mutants impaired in phosphoglycolate phosphatase activity (Suzuki et al., 1990) and of a Synechococcus sp. PCC7942 mutant overexpressing this enzyme (Kaplan et al., 1998) support the notion that metabolites in photorespiration may play an important part in the transduction pathway. These metabolites may be directly involved or might be sensed as starvation signals. Photorespiration has been shown to be necessary for acclimation to CO₂ limiting stress using the high CO₂ requiring mutant, pgp1-1 (Suzuki, 1995). Seven of the 51 low
CO₂ inducible genes detected in cDNA microarray were found to have significant similarities with photorespiratory genes (Miura et al., 2004). In cyanobacteria results of a number of physiological studies (Badger and Gallagher, 1987; Mayo et al., 1986) were interpreted as a hypothesis in which the induction of the CCM is affected by direct sensing of external Ci levels. However, other modes of sensing are also possible, such as sensing of redox stress, sensing of internal inorganic pool or sensing photorespiratory intermediates (Kaplan and Reinhold, 1999). Work by Woodger et al. in 2003 forwards the notion that CCM induction in *Synechococcus sp* PCC7942 involves either the sensing of a low internal Ci carbon pool or the sensing of photorespiratory intermediates arising from the Rubisco oxygenase reaction. Since inorganic carbon transport requires energy and is light dependent, it has been suggested that light can regulate the expression of CCM in cyanobacteria and in microalgae. Light is a prerequisite under low Ci conditions for the expression of CCM related transcripts in *Synechocystis* PCC6803 (McGinn et al., 2003). In these studies a cooperative requirement for both low levels of Ci or CO₂ and sufficient photon flux were required for significant CCM expression.

**THE CARBON DIOXIDE CONCENTRATING MECHANISM IN *C. REINHARDTII***

**The CCM in *C. reinhardtii***

A model for concentrating CO₂ in *C. reinhardtii* is shown in Figure 2.1. In this model the CCM can be divided into two phases. The first phase involves acquiring inorganic carbon from the environment and delivering CO₂ and HCO₃⁻ to the chloroplast. The components of this part of the CCM would include carbonic anhydrases in the periplasmic space (CAH1 and possibly CAH8), a carbonic anhydrase in the cytoplasm (CAH9), as well as HCO₃⁻ transporters and CO₂ channels on both the plasma membrane and the chloroplast envelope. The second part of the proposed model entails the generation of elevated levels of CO₂ in the chloroplast stroma utilizing the pH gradient across the thylakoid membrane. This part of the CCM includes the
carbonic anhydrase located in the chloroplast stroma (CAH6), the carbonic anhydrase located within the thylakoid lumen (CAH3) as well as a proposed HCO₃⁻ transporter on the thylakoid membrane.

**Physiological Evidence for Ci Uptake in C. reinhardtii**

The physiological evidence that *C. reinhardtii* can accumulate Ci and enhance CO₂ fixation is two-fold. First *C. reinhardtii* has the ability to efficiently fix CO₂ even when the external CO₂ concentration is well below the Km (CO₂) for Rubisco (Badger et al., 1980; Spalding et al., 1983a; Spalding et al., 1983b; Moroney and Tolbert, 1985). For example, whole cell photosynthesis rates are saturated at about 2-3 µM CO₂, while the Km (CO₂) of *C. reinhardtii* Rubisco is about 20 µM (Jordan and Ogren, 1981). In addition, Ci uptake has been measured directly in a number of laboratories (Badger et al., 1980; Moroney and Tolbert, 1985; Sültemeyer et al., 1989; Sültemeyer et al., 1991; Asamiziu et al., 2000) and the Ci concentration inside the cell is higher than can be accounted for by diffusion alone.

In this model for Ci uptake the pH gradient across the thylakoid membrane is an essential part of the CCM. Since light-driven electron transport is required to set up the ∆pH, Ci uptake should only occur in the light. To date, inorganic carbon concentration in eukaryotic algae has only been observed in cells or chloroplasts exposed to light. The strongest evidence in support of the light requirement comes from the pioneering work of Spalding and Ogren (1982) who showed that electron transport inhibitors as well as mutants in the electron transport chain also inhibited the CCM in *C. reinhardtii*. While this work does not prove that a pH gradient across the thylakoid membrane is required for the CCM to operate, their data are fully consistent with this model. Another requirement of this model is a carbonic anhydrase in the thylakoid lumen to rapidly convert the bicarbonate entering the lumen to CO₂. In *C. reinhardtii* CAH3 has been localized to the thylakoid lumen and mutations in the *CAH3* gene result in cells with a
non-functional CCM (Karlsson et al., 1998). Another requirement of this model is that the CO₂ generated in the thylakoid lumen becomes available to Rubisco before being converted back to HCO₃⁻ in the basic environment of the chloroplast stroma. The pyrenoid of the chloroplast might serve to separate Rubisco from the carbonic anhydrase in the stroma of the chloroplast. The pyrenoid is a proteinaceous structure where most of the Rubisco is located. The pyrenoid undergoes a dramatic morphological change when cells are switched from a high to a low CO₂ condition (Rawat et al., 1996). When the CCM is functional a starch sheath appears around the pyrenoid and 90% of Rubisco is present in the pyrenoid (Morita et al., 1997; Borkhsenious et al., 1998). Notably almost all eukaryotic photosynthetic algae have pyrenoids, while pyrenoids are almost absent from the chloroplasts of terrestrial higher plants.

Finally, a carbonic anhydrase located in the chloroplast stroma would also be required for the operation of this type of CCM. This stromal carbonic anhydrase would serve two functions: first, to convert CO₂ entering the chloroplast to HCO₃⁻ in the basic environment of the chloroplast stroma and, second, to recapture the CO₂ coming from the thylakoid lumen before it diffuses from the chloroplast. In *C. reinhardtii* most of the required features of this type of CCM have been identified. There are carbonic anhydrase isoforms in the thylakoid lumen (Karlsson et al., 1998) and the chloroplast stroma (Mitra et al., 2004). What has not been established is how bicarbonate is transported across the thylakoid membrane. However, a number of proteins have been identified as potential HCO₃⁻ transporters and these transporters are discussed below.

**Components of the CCM**

**Putative Transporters.** While a number of CAs have been shown to be part of the CCM in *C. reinhardtii*, no transporter has been definitively linked to the CCM. However, some of the promising candidate genes and proteins have been identified and it is likely that one of the
Figure 2.1. Model of the Carbon Dioxide Concentrating Mechanism of *Chlamydomonas reinhardtii*. The size of the lettering depicts the concentrations of bicarbonate and carbon dioxide within the chloroplast and pyrenoid relative to the external environment. CAH1, CAH3, CAH6, CAH8 and CAH9 stand for specific carbonic anhydrase isoforms. PGA stands for 3-phosphoglyceric acid, PM for plasma membrane, CE for chloroplast envelope and TM for thylakoid membrane. The filled circles indicate possible bicarbonate (or Ci) transporters and the closed diamonds the photosynthetic electron transport chain.
following proteins may participate in Ci uptake in *C. reinhardtii*. The candidate proteins are CCP1, CCP2, LCI1, NAR1.2 (LCIA), LCIB, HLA3, RH1 and YCF10. All of these proteins are nuclear encoded with the exception of YCF10, which is encoded by the chloroplast genome. Most of these proteins, or the corresponding genes, were first identified because the protein or mRNA dramatically increases in abundance when *C. reinhardtii* is grown under limiting CO\(_2\) growth conditions. For example, *CCP1, CCP2, LCI1, NAR1.2, LCIB* and *HLA3* are all strongly induced when *C. reinhardtii* is making a functional CCM (Chen et al., 1997; Burow et al., 1996; Miura et al., 2004; Im and Grossman, 2001). In addition, mutations in the putative transcription factor CIA5/CCM1 (Fukuzawa et al., 2001; Xiang et al., 2001) reduce the expression of many of these proteins (Moroney et al., 1989; Chen et al., 1997; Miura et al., 2004; Mariscal et al., 2006).

Very few mutants have been found that affect the expression of the genes encoding putative Ci transport proteins. The mutant *pmp1* does have a mutation in *LCIB* and this mutant is defective in Ci transport (Spalding et al., 1983b; Vance and Spalding, 2005). Recently, the allelic mutant *ad1*, air dier1, was also described and this strain also cannot grow in low CO\(_2\) (350 ppm) but can grow either in high CO\(_2\) (5% CO\(_2\)) or in very low CO\(_2\) (200 ppm). The fact that *pmp1/ad1* fails to grow on air levels of CO\(_2\) but manages to survive on very low levels of CO\(_2\) has been interpreted as indicative of the existence of multiple Ci transport systems in *C. reinhardtii* corresponding to multiple CO\(_2\) level-dependent acclimation states (Spalding et al., 2002; Wang and Spalding, 2006). This would be similar to the multiple Ci uptake systems seen in cyanobacteria. *PMP1/AD1* is identical to the previously identified CO\(_2\) responsive gene, *LCIB* (Miura et al., 2004). *LCIB* does not have any significant homology to proteins from other organisms, but its predicted amino acid sequence has similarity with the predicted amino acid sequence of three genes, *LCIC, LCID* and *LCIE* in the *C. reinhardtii* genome. *LCIC* and *LCID* are also upregulated under low CO\(_2\) conditions. While these observations point to a role for
LCIB in the adaptation to low CO₂, it is unlikely that LCIB is a transport protein by itself as it lacks any hydrophobic transmembrane domains. Therefore, LCIB more likely has either a regulatory role or might be part of a complex that transports Ci (Van et al., 2001).

Another promising candidate protein to be an Ci transporter is LCI1. The gene encoding LCI1 was first identified as being very highly expressed in cells growing under low CO₂ conditions (Burow et al., 1996). LCI1 contains four predicted transmembrane helices and also shows very little homology to any other protein in the NCBI database. Recent work with strains showing reduced expression of LCI1 due to the presence of an LCI1-RNAi insert show reduced growth on low CO₂ (Mason and Moroney, unpublished observations), but the physiological role of LCI1 remains to be determined.

Two other genes encoding putative Ci transport proteins are CCP1 and CCP2. These genes encode the low CO₂ inducible proteins LIP-36 G1 and LIP-36 G2 (Geraghty and Spalding, 1996). These two proteins are 96% identical, have six transmembrane helices, are localized in the chloroplast envelope (Moroney and Mason, 1991; Ramazanov et al, 1993) and have a high degree of similarity to the mitochondrial carrier family of proteins (Chen et al., 1997). When the abundance of CCP1 and CCP2 messages were reduced using RNAi, the resultant strains grew poorly on low CO₂ levels but normally on elevated levels of CO₂ (Pollock et al., 2004). However, Ci uptake was normal in these strains (Pollock et al., 2004). This might indicate that CCP1 and CCP2 are transporters of metabolic intermediates of photorespiration, transporters of other metabolic intermediates (Pollock et al., 2004), or that these proteins are part of a redundant system of Ci transport, as seen in cyanobacteria.

Another putative Ci transporter, LCIA, was also first discovered using expression analysis (Miura et al., 2004). LCIA is also called NAR1.2. LCIA/NAR1.2 was first annotated as a nitrite transporter and it has strong similarity to the bacterial nitrite/formate family of transporters.
NAR1.2 belongs to a gene family consisting of six NAR genes in C. reinhardtii and, surprisingly, these genes have no obvious homolog in Arabidopsis. The expression of NAR1.2 is induced in low CO₂ conditions and is partially under the control of CIA5, a transcription factor that is required for the expression of other CCM genes (Miura et al., 2004). NAR1.2 is predicted to be localized to the chloroplast thylakoid or chloroplast envelope and has six transmembrane domains. The functional expression of NAR1.2 in Xenopus oocytes has shown that the presence of NAR1.2 increased bicarbonate entry into the oocytes by 2-fold compared to that of the control (Mariscal et al., 2006). These features suggest that NAR1.2 is an attractive candidate to be a bicarbonate transporter.

Three other proteins suggested to be part of the Ci uptake system include HLA3 (Im and Grossman, 2001), RH1 (Soupene et al., 2002) and YCF10 (Rolland et al., 1997). HLA3, was first identified as a gene expressed when C. reinhardtii cells were exposed to high light. Subsequent work showed that HLA3 expression is also controlled by CO₂ concentration. HLA3 has strong sequence similarity to an ABC transporter and HLA3 was first predicted to be localized to the chloroplast membrane (Im and Grossman, 2001). However, more recent versions of the prediction servers give much less clear predictions as to the location of HLA3. HLA3 might be a potential transporter in the acclimation of cells to low CO₂ or might be involved in redox control and only indirectly involved in the control of CCM expression (Im and Grossman, 2001). Another chloroplast envelope protein that has been implicated in Ci uptake is the product of the ycf10 gene. The ycf10 gene product can form two to three transmembrane domains, and has been localized in the inner chloroplast envelope membrane (Sasaki et al., 1993). Disruption of its ORF with the chloroplast aadAd expression cassette by biolistic transformation affected uptake of inorganic carbon (Rolland et al., 1997). These observations raise the possibility that this protein is a Ci transporter. However, subsequent experiments
provided evidence that YCF10 may not directly be involved in Ci uptake but rather regulate the Ci transport system. YCF10 could be associated with a system in the chloroplast envelope involved in HCO$_3^-$ and/or CO$_2$ uptake (Rolland et al., 1997).

RH1 has been implicated in CO$_2$ transport as it is very similar to bacterial proteins shown to be ammonia and/or CO$_2$ channels (Soupene et al., 2002). However, the expression of this protein is not consistent with its being part of the CCM as RH1 is expressed at high levels when cells are grown on elevated CO$_2$ and not when cells are grown on low CO$_2$. In addition, when RH1 expression is reduced by mutation, *C. reinhardtii* still can grow on low levels of CO$_2$ but shows reduced growth on elevated levels of CO$_2$ (Soupene et al., 2004). Likewise, RH1 is not regulated by CIA5 (Wang et al., 2005). The possible role of this protein is facilitating CO$_2$ entry into the cell when the CO$_2$ level is high. The role of RH1 in CO$_2$ transport remains a very interesting question in this field.

**The Pyrenoid.** An additional requirement of the model for CO$_2$ acquisition by *C. reinhardtii* is that the CO$_2$ generated in the thylakoid lumen becomes available to Rubisco before being converted back to HCO$_3^-$ in the basic environment of the chloroplast stroma. The pyrenoid of the chloroplast might serve to separate Rubisco from the carbonic anhydrase in the stroma of the chloroplast. The pyrenoid is a proteinaceous structure where most of the Rubisco is located. The pyrenoid undergoes a dramatic morphological change when cells are switched from a high to a low CO$_2$ condition, (Rawat et al., 1996). When the CCM is functional a starch sheath appears around the pyrenoid and 90% of Rubisco is present in the pyrenoid (Morita et al., 1997; Borkhsenious et al., 1998; Ramazanov et al., 1994).

Notably most eukaryotic photosynthetic algae have pyrenoids (Bold and Wynne, 1985), while pyrenoids are almost absent from the chloroplasts of terrestrial higher plants. Exceptions include some strains of *Chloromonas* which exhibit CCM but are demonstrated to lack pyrenoids.
(Morita et al., 1998). However, pyrenoid-less CCM containing strains of *Chloromonas* were demonstrated to have small Ci pools, 24-31 µM in comparison with the large Ci pools, 231-252 µM in algae exhibiting typical (dense with high concentration of Rubisco) pyrenoids. It has been speculated that the formation of large intracellular Ci pool in algae with a CCM is correlated to the presence of typical pyrenoids containing a high concentration of Rubisco molecules. Algae such as Chloromonas lacking pyrenoids are often found in harsh environments and perhaps the presence of a pyrenoid is not necessary as other factors besides CO₂ fixation are limiting growth (Morita et al., 1999).

**Carbonic anhydrases.** The adaptation to limiting CO₂ has been correlated with increased levels of carbonic anhydrases (Badger et al., 1980; Spalding et al., 1983a; Coleman and Grossman, 1984; Aizawa and Miyachi, 1986; Fukuzawa et al., 1990). As of this time, the *Chlamydomonas* genome contains genes for nine putative CAs, which has led to questions about what roles are played by these CAs and which ones are critical to the functioning of the CCM. Five of these CAs, (*CAH1, CAH3, CAH4, CAH5, CAH6*) have possible roles in the CCM. The *C. reinhardtii* carbonic anhydrases will be further discussed in the subsequent sections.

**Regulatory Components.** The isolation of high CO₂ requiring mutants, *cia5* and C16 (Moroney et al., 1989; Fukuzawa et al. 1998) paved the way for the identification of the master regulatory element which controls the transcription of quite a number of low CO₂ induced genes. The gene which has been designated *CIA5* consists of 6481 bp (Xiang et al., 2001). The same gene has been cloned by a different group using the insertional mutant C16 and has been designated as *CCM1* (Fukuzawa et al., 2001). Both groups have shown that abundance of 5.1 kb transcript and its 76 kD protein product were not affected by a change in CO₂ level. Both groups have both proposed that this gene is a putative transcription factor.
The mutant *cia5* has a single point mutation from T to C and as a consequence of this base substitution His-54 in the putative zinc finger motif of the protein is replaced by Tyr in *cia5*. This mutation strongly suggests that His-54 in the CCM1 plays a critical role in the regulation of the CCM, including the induction of a set of CCM-related genes and pyrenoid development. Likewise, CCM1 contains a Gln-repeat stretch, which is reported to be necessary for the regulatory functions of several eukaryotic transcription factors. CCM1 may interact directly with DNA or regulatory proteins through putative zinc finger domain(s) and activate the transcription of CCM-related genes under low CO$_2$ conditions.

Although the coding region for this putative zinc-finger domain of CCM1 of *C. reinhardtii* is expressed in the mutant, the CO$_2$ responsive regulation of CCM is impaired (Fukuzawa et al., 2001). When *cia5* mutants were transformed with a truncated *CIA5* gene, transformants exhibited constitutive synthesis of mRNAs from CO$_2$ responsive genes in the light under both high and low CO$_2$ conditions. These results suggest that post-translational changes to the C-terminal domain control the ability of CIA5 to act as an inducer and directly or indirectly control the transcription of low CO$_2$ induced genes. Thus the C-terminal region of CCM1 is essential to transduce low CO$_2$ signal in addition to the putative zinc finger domain.

CCM1 was designated as master regulator of the CCM that controls almost all the low CO$_2$ inducible genes detected in wild type using gene expression profiling. Of 51 low CO$_2$ inducible genes, the expression ratios of 47 genes were reduced in *ccm1* (Miura et al., 2004).

Information on the DNA elements that may confer responsiveness of gene expression to CO$_2$ concentration has so far been limited to the silencer and enhancer cis-elements identified via the analysis of the periplasmic CA, *CAH1* (Kucho et al., 1999). *CAH1* has been shown to be controlled by two regulatory regions: a silencer region, which represses transcription under high CO$_2$ conditions or in the dark, and an enhancer region, which activates it under low CO$_2$.
conditions in light (Kucho et al., 1999). These two enhancer elements, EE-1 and EE-2 contain a 7-bp consensus motif named EEC, GANTTNC which are recognized by both the EE-1- and EE-2 binding proteins. EEC binding proteins were observed to be present regardless of changes in CO$_2$ concentration and light illumination, even when CAH1 is not activated. Likewise, the formation of DNA-protein complexes was not affected by CO$_2$ concentration or light illumination and thus it was suggested that the EEC sites may be important cis-acting elements that constitutively bind one or more proteins that serve to assist in the regulated transcription of the CAH1 gene (Kucho et al., 2003).

Likewise, a regulatory gene of CAH1 that has been described is LCR1. LCR1 is a transcription factor that functions in amplification and maintenance of CAH1 mRNA levels in response to limiting CO$_2$. The expression of LCR1 under low CO$_2$ conditions is regulated by CCM1. The lcr1 mutant shows relatively slower growth rate and reduced affinity for Ci under low CO$_2$ conditions indicating that LCR1 is essential for full induction of CCM. LCR1 may regulate genes that play a significant role in the CCM such as those encoding Ci transporters. Gene array analysis and RNA gel blot analyses indicated that three low CO$_2$ inducible genes, CAH1, LCI1 and LCI6 are regulated by LCR1. LCR1 may interact with other transcription factors and together regulate expression of low CO$_2$ inducible genes. Kucho et al., (2003) have proposed a possible mechanism by which expression of CAH1 is induced in response to CO$_2$ limiting stress.

When cells are exposed to CO$_2$ limiting stress, the CO$_2$ limiting signal is transmitted to CCM1, which is constitutively expressed regardless of CO$_2$ conditions. Then CCM1 is post transcriptionally modified and induces initial expression of both LCR1 and CAH1 via interactions between activated CCM1 and EEC binding proteins. Newly synthesized LCR1 is transported to the nucleus. Imported LCR1 interacts with unidentified enhancers in the CAH1
promoter region, then amplifies the magnitude of CAH1 induction and maintains the mRNA levels under continuous low CO2 conditions.

**CARBONIC ANHYDRASES**

Carbonic anhydrase (carbonate dehydratase, carbonate hydro-lyase; EC 4.2.1.1) is a zinc metalloenzyme that catalyzes the interconversion of CO2 and HCO3− (Khalifah, 1971). The first CA was purified from bovine erythrocytes in 1933 (Meldrum and Roughton, 1933). Carbon dioxide (CO2) and bicarbonate (HCO3−) are substrates and products of many different metabolic reactions in the cells. Thus, carbonic anhydrases (CAs) are involved in a broad range of biochemical processes that involve carboxylation or decarboxylation reactions such as photosynthesis and respiration (Moroney et al., 2001), pH homeostasis and ion transport (Tashian, 1989) and catalysis of key steps of pathways for the biosynthesis of physiologically important metabolites (Mitsuhashi et al., 2003).

Carbonic anhydrase proteins and genes have been identified in a wide range of archaebacteria, eubacteria, algae, plants and animals. There are clearly three distinct classes of CAs, α-, β-, and γ-CAs. These classes of proteins have no significant identity to each other, both at the amino acid sequence level and folding structure of the proteins and has underscored the conclusion that CAs have evolved multiple times quite independently of each other (Badger, 2003).

Two other classes of carbonic anhydrases, δ and ζ (Lane et al., 2005) have been reported to be present in the marine diatom *Thalassiosira weisflogii*. The *T. weisflogii* δ-CA, similar to the other known CAs, coordinates zinc (Cox et al., 2000) while the *T. weisflogii* ζ-CA coordinates cadmium (Cox et al., 2000). The presence of these two classes of CAs has so far have only been restricted in *T. weisflogii*. Whether these two CAs belong to new classes of CA is not yet well defined.
Despite primary sequence and structural differences, all three types of CAs are Zn\(^{2+}\) metalloenzyme and all appear to share a similar catalytic mechanism (Lindskog, 1997; Christianson and Cox, 1999). Kinetic studies indicate that all three classes employ a two step mechanism (Northrop and Simpson, 1998). The first step is the nucleophilic attack of a zinc bound hydroxide on CO\(_2\) (Eqn.1). The second is the regeneration of the active site by ionization of the zinc-bound water molecule and a removal of a proton from the active site (Eqn. 2). The reaction is reversible and the overall net reaction for the interconversion of CO\(_2\) and HCO\(_3^-\) is given in Eqn. 3.

\[
\begin{align*}
\text{E-Zn}^{2+}\text{OH}^- + \text{CO}_2 & \rightleftharpoons \text{E-Zn}^{2+} + \text{HCO}_3^- \quad \text{(Eqn. 1)} \\
\text{E-Zn}^{2+} + \text{H}_2\text{O} & \rightleftharpoons \text{E-Zn}^{2+}\text{OH}^- \quad \text{(Eqn. 2)} \\
\text{CO}_2 + \text{H}_2\text{O} & \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad \text{(Eqn. 3)}
\end{align*}
\]

In the absence of a catalyst, hydration-dehydration reactions are slow, whereas the dissociation reactions are considered instantaneous (Moroney et al., 2001). Carbonic anhydrase speeds up the hydration of dissolved CO\(_2\) in solution, thereby increasing the rate at which forms of inorganic carbon interconvert in solution. The equilibrium between the inorganic forms of carbon is pH dependent. At normal intracellular ionic strength, when the pH level is below the first dissociation constant (pK\(_1\) ~ 6.4) CO\(_2\) predominates; at pH between 6.4 and about 10.3 (pK\(_2\)) HCO\(_3^-\) predominates; whereas above pH of 10.3, CO\(_3^{2-}\) predominates.

**The Carbonic Anhydrase Gene Families**

\(\alpha\)-CAs have been reported in animals, plants, eubacteria and viruses and thus are widely distributed. The \(\alpha\)-class is the best characterized with 11 isozymes identified in mammals. Several isozymes are implicated in various diseases whereby the application of sulfonamides that inhibit carbonic anhydrase activity has been the frequent treatment (Tripp et al., 2001). Vertebrate \(\alpha\)-CAs has been divided into two distinct groups: (1) the soluble isoforms and (2) the
membrane-associated and secreted CAs. The membrane-associated CAs are characterized by hydrophobic C-terminal extensions. In photosynthetic organisms the few α-CAs identified include the cyanobacterium α-CA (Soltes-Rak et al., 1997) and three α-CAs from C. reinhardtii (Karlsson et al., 1998; Fujiwara et al., 1990; Rawat and Moroney., 1991). Previously, it has been reported that Arabidopsis contained sequences that align with six α-CAs (Moroney et al., 2001). Recently, Arabidopsis has been reported to contain eight α-CAs (Atα-CA1-8) and analyses of ESTs from the TAIR database indicated that only three of the Atα-CAs are expressed whereby two of these Atα-CAs respond to CO2 (Fabre et al., 2007).

All of the enzymatically active α-CAs have three histidines coordinating the zinc atom. X-ray crystallographic studies show the zinc atom liganded to the side chains of three histidines and a hydroxyl ion (Christianson and Cox, 1999). Most α-CAs are active as monomers of about 30 kD. An exception is the C. reinhardtii periplasmic CA, CAH1 which is a heterotetramer with two 37 kD sub-units and two 4 kD sub-units held together by disulfide bonds (Kamo et al., 1990). In addition, the CA gene of Dunaliella salina which encodes a protein of about 63 kD appears to have two active sites, a possibility of a gene duplication/fusion events (Fisher et al., 1996).

β-CAs were first recognized as CAs in photosynthetic organisms (Burnell, et al., 1990; Fawcett et al., 1990) and were initially composed solely of enzymes from plants but were later been found in eubacteria (Hewett-Emmett and Tashian, 1996), archaebacteria (Smith and Ferry, 1999), cyanobacteria, microalgae, yeast (Götz et al., 1999) and higher plants. One type of β-CA localized to the cyanobacterial carboxysome has been described (Fukuzawa et al., 1992; Yu et al., 1992) and the loss of this CA led to a high CO2 requiring phenotype (Price and Badger, 1989). Six β-CAs have been identified in the unicellular green alga C. reinhardtii. The symbiotic alga Coccomyxa has a cytosolic β-CA (Hiltonen, et al., 1998) and the red alga, P.
*Purpureum* has one β-CA described to date (Mitsuhashi et al., 2000). The most thermostable CA reported to date is a β-CA from *Methanobacterium thermoautotrophicum* (Smith and Ferry, 1999). In C3 plants, the chloroplast stroma has the highest levels of CA activity and that activity is due to a β-CA (Okabe et al., 1984). *Arabidopsis thaliana* contains six β-CAs (*At*β-CA1-6) which are targeted to specific sub-cellular compartments: CA1 and CA5 are targeted to the chloroplast, CA2 and CA3 to the cytosol, CA4 to the plasma membrane and CA6 to the mitochondria (Fabre et al., 2007). The C4 plants *Zea mays* (Burnell et al., 1999) and *Urochloa panicoides* (Ludwig and Burnell, 1995), also have β-CAs. Maize has a 74 kD and 60 kD β-CAs that appears to be a fusion of two monomers due to its large size and each contains two sets of active site residues (Burnell et al., 1999). These polypeptides were described as being unique among higher plants and their close relatives since the cDNAs of *Urochloa panicolata* and *Flaveria bidentis*, both C4 plants, were typically sized β-CAs having deduced molecular weights of between 24 and 30 kD. The fused duplicated gene seen in maize was likewise observed in the red alga, *P. purpureum* β-CA. The *P. purpureum* protein has two active sites per polypeptide instead of the one found in most β-CAs (Mitsuhashi et al., 2000).

Compared to α-class and γ-class enzymes which are strictly monomers and trimers respectively, members of the β-class are dimers, tetramers, hexamers and octamers which suggests a dimer as the basic building block. The pea CA, for example, is an octamer in which dimers form tetramers which form octamers. X-ray absorption spectroscopy studies on spinach β-CA (Bracey et al., 1994; Rowlett et al., 1994) and β-class crystal structures (Mitsuhashi et al., 2000; Kimber and Pai, 2000) have shown that a histidine and two cysteine residues are the zinc coordinating residues. In addition, it has been found that the three dimensional structure of the active site of the β-CA is a mirror image of that of the active site of the α-CA (Kimber and Pai, 2000).
γ-CA was first discovered in the archaeabacterium *Methanosarcina thermophila* (Alber and Ferry, 1994) and it is the only active γ-CA reported to date. The crystal structure of *M. thermophila* γ-CA showed that γ-CA’s structure is remarkably different from α-CAs and β-CAs. The γ-CA functions as a trimer of identical subunits which contains three zinc atoms, each at three subunit interfaces and each monomer is a left-handed β-helix (Kisker et al., 1996). As in α-CAs, three histidines and a water molecule coordinate the zinc atom but the histidines are provided by two subunits. In *M. thermophila*, two histidines are provided by one subunit and the other histidine is provided by another subunit. In spite of the fact that the active site is at the subunit interface, architecturally the active site of γ-CA resembles that of the α-CA (Kisker et al., 1996).

*Synechococcus PCC7942* has a γ-CA homologue CcmM. This γ-CA lacks a detectable CA activity but is required for growth in low CO₂. The mutation of this gene resulted in a mutant that cannot grow on air levels of CO₂. This suggests that CcmM might be an essential part of the cyanobacterial CCM (Price et al., 1993). The *C. reinhardtii* EST database has two ESTs that align well with *M. thermophila* γ-CA. Genomic sequences were obtained for both these gamma CA-like protein genes (*GCLP1* and *GCLP2*). GCLP1 has 30-40% similarity with *M. thermophila* γ-CA and the CcmM of cyanobacteria and contains the characteristic three histidines as zinc coordination residues seen in the enzymatically active γ-CA of *M. thermophila*. GCLP1 was successfully overexpressed in *E. coli* cells but no CA activity was seen in the crude extracts (Mitra et al., 2005). Two additional genes in *C. reinhardtii* genome align well with *GCLP1* and these genes were annotated as subunits of the mitochondrial NADH dehydrogenase complex. However, these genes do not have the three histidines found in *M. thermophila* protein nor in the cyanobacterial CcmM protein. It is therefore unlikely that the proteins encoded by these genes have CA activity (Mitra et al., 2005).
Mitochondrial NADH dehydrogenase (complex I) of *Arabidopsis* includes five structurally related subunits representing γ-type carbonic anhydrases, CA1, CA2, CA3, CAL1 and CAL2 (Parisi et al. 2004; Perales et al., 2005). Computer modeling using the crystal structure of *M. thermophila* γ-CA revealed that CA1, CA2 and CA3 have conserved γ-CA’s active sites (Parisi et al., 2004). Furthermore, Arabidopsis CA2 and CA3 knock-out lines showed a phenotype not distinguishable from wild type which might be a consequence of redundant activities of the five related complex I subunits. However, suspension cell cultures of these mutants had a reduced growth rate and respiration. In addition, complex I levels were clearly reduced, indicating that the CA subunits are important for complex I assembly (Perales et al., 2005). Unique to *Arabidopsis* complex I assembly not observed in bacteria, fungi nor animal complex I is an extra spherical matrix exposed domain attached to the central part of its membrane arm (Dudkina et al., 2005). This domain was concluded to be the γ-CA, CA2, which is anchored through its C-termini to the hydrophobic arm of complex I (Sunderhaus et al., 2006).

**Physiological Roles of Carbonic Anhydrases**

There is an observed wide occurrence of different carbonic anhydrases, however the functional roles of CAs in the physiology of various organisms is still poorly understood. The connection between CAs and photosynthesis is perhaps the best understood role (Badger, 2003). Carbonic anhydrases play critical roles in the photosynthetic CO₂ concentrating mechanism. For example, carbonic anhydrases located external to the cell membrane such as the *C. reinhardtii* α-CA, CAH1, play key roles enabling the supply of CO₂ to the cell surface, particularly with HCO₃⁻ as the predominant species at high pH (Moroney et al., 1985). The function of periplasmic α-CAs identified in *Anabaena* and *Synechococcus* (Soltes-Rak et al., 1997) is still unclear but it is possible that their function is similar to *C. reinhardtii* CAH1 (Moroney et al., 2001). Carboxysomal β-CA of the cyanobacteria *Synechococcus* and *Synechocystis* converts...
accumulated $\text{HCO}_3^-$ to $\text{CO}_2$ for Rubisco in the carboxysome. The $\gamma$-CA analogue, CcmM, is required for correct carboxysome assembly and for optimal growth on low levels of $\text{CO}_2$ (Price et al., 1993).

The roles of chloroplast $\beta$-CAs in higher plants range from modulation of the pH of stroma to facilitating diffusion of $\text{CO}_2$ across the chloroplast envelope. It is also proposed that the $\beta$-CA replenishes the $\text{CO}_2$ supply in the stroma from $\text{HCO}_3^-$ which is more abundant in the alkaline stroma (Moroney et al., 2001). Transgenic tobacco plants were made containing antisense CA contracts to study the function of the $\beta$-CA. Results of one study reported no deleterious effects of the reduction of CA activity in the mutant (Price et al., 1994). On the other hand, in a different study, antisense plants compensated for the decrease in CA with an increase in stomatal conductance leading to an increase in water loss (Majew et al., 1994). The drought sensitivity has been attributed to the possibility that a reduction in photosynthesis due to a decrease in the delivery rate of $\text{CO}_2$ to Rubisco caused the leaf stomata to open. Another possibility is the disruption of the signal for the plant to close the stomata as a result of lowering the chloroplast $\beta$-CA (Moroney et al., 2001). Antisense approach done by Kim and Bartlett was also used to reduce CA activity in *Arabidopsis* leaves. Similar to the observations in antisense tobacco plants, no phenotype was evident when seedlings were grown in soil, suggesting that the chloroplast CA has little effect on photosynthesis in C3 plants. The transgenic plants on the other hand when grown on agar died whereas control plants were normal, which suggests that the chloroplast $\beta$-CA is required at least when levels of carbon dioxide are limiting (Kim, 1997).

In C4 plants where most of the CA is located in the cytosol of the mesophyll cells (Ku et al., 1996), the CA is required to supply adequate level of $\text{HCO}_3^-$ to phosphoenolpyruvate carboxylase which produces C4 acids (Hatch and Burnell, 1990). The C4 acids diffuse to and are decarboxylated in the bundle sheath cells providing $\text{CO}_2$ to Rubisco. Three cDNAs encoding...
putative β-CAs (CA1, CA2 and CA3) have been identified from *Flaveria bidentis* leaf cDNA library (von Caemmerer et al., 2004). Using an antisense strategy to modulate the expression of CA3, the main putative cytosolic CA, it was shown that CA activity is essential to maximize C4 photosynthesis (von Caemmerer et al., 2004).

In non-photosynthetic tissues, the α-CA found throughout the young soybean root nodules but mainly in the cortical region of old root nodules suggests that the CA recycles CO₂ during early nodule development while it facilitates diffusion of CO₂ from the nodule during the later stage of nodule development (Kavroulakis et al., 2000). A “trapping” role has been proposed for plastidial CA whereby its metabolic role was described to be an indirect one, wherein CA improves the efficiency of fatty acid synthesis by rapidly cycling inorganic carbon for acetyl CoA carboxylase (Hoang and Chapman, 2002).

Mammalian CAs also have a wide range of functions. Examples include the cytosolic CAII and membrane associated CAIV as being essential to renal HCO₃⁻ absorption, CAIV expressed in the pancreas which may form mutually complementary system with CAII regulates the luminal pH of the human pancreatic duct system and CAIV in the heart plays significant role in cardiac pH regulation (cited in Alvarez et al., 2003). More recently, interaction of bicarbonate transporters with intracellular and extracellular carbonic anhydrases has been suggested to be a universal component of bicarbonate transport physiology, as these CAs play a role in maximizing transmembrane HCO₃⁻ gradient local to the bicarbonate transporters and thus activating their transport rate (Alvarez et al., 2003).

In the yeast, *Saccharomyces cerevisiae*, the β-CA NCE103 provides the inorganic carbon for the bicarbonate-dependent carboxylation reactions catalyzed by pyruvate carboxylase, acetyl-CoA carboxylase and carbamoyl phosphate synthetase (Aguilera et al., 2005). It has been reported that the tobacco salicylic acid binding protein 3 (SABP3) is a chloroplast β-CA, which
exhibits antioxidant activity and plays a role in the hypersensitive defense response (Slaymaker et al., 2002).

In *Escherichia coli*, cyanate induces the expression of *Cyn* operon. The *Cyn* operon includes the gene *CynS* which encodes a cyanase that catalyzes the reaction of cyanate with HCO$_3^-$ to give ammonia and CO$_2$. The *cynT* gene which encodes for a β-CA, is a part of the *Cyn* operon. This β-CA recycles the CO$_2$ produced in the cyanase reaction back to HCO$_3^-$ which would have diffused out of the cell (Guilloton et al., 1993). In *Corynebacterium glutamicum*, β-CA was found to be essential for the bacteria’s growth under atmospheric conditions, a growth defect that was restored under elevated CO$_2$ conditions and introduction of the red alga, *P. purpureum* β-CA into the deletion mutants (Mitsuhashi et al., 2003).

In *Methanosarcina thermophila*, an organism that obtains energy for growth via conversion of methyl groups of acetate, methanol or methanolamines to methane, a metabolic switch from methanol to acetate has shown an elevation of γ-CA activity. It has been proposed that CA may be required for a CH$_3$CO$_2^-$/$H^+$ symport system or for efficient removal of cytoplasmically produced CO$_2$ and thus this γ-CA is important for growth on acetate (Alber and Ferry, 1994). In the cyanobacterium *Microcoleus chthonoplastes*, a different role of extracellular CAs was its possible participation in extracellular carbonate precipitation (Kupriyanova et al., 2007). Thus, aside from CAs wide occurrence in nature, CAs also exhibit a wide variety of physiological roles.

**THE *C. REINHARDTII* CARBONIC ANHYDRASES**

As of this time, nine different α- and β-CA genes have been identified in *C. reinhardtii* genome. This plethora of CA genes has led to questions about what roles are played by these CAs and which ones are critical to the functioning of the CCM. A number of these proteins are implicated to have possible roles in the CCM.
The role of the periplasmic α-CA, CAH1, is to facilitate entry of carbon dioxide into the algal cell. At pH above 6.3, HCO$_3^-$ is the predominant inorganic carbon species. This form of Ci, being an anion, cannot readily cross the plasma membrane (Gutknecht et al., 1977; Moroney and Somanchi, 1999). CAH1, one of the first α-CAs reported in a photosynthetic organism, converts HCO$_3^-$ to CO$_2$. Two lines of evidence have been presented for this physiological role of CAH1. First, membrane impermeant CA inhibitors have a strong inhibitory effect on photosynthetic CO$_2$ fixation at high pHs where HCO$_3^-$ predominates but a less pronounced effect at lower pHs, where most of the inorganic carbon is already in the form of carbon dioxide and activity of periplasmic CA is no longer required (Moroney et al., 1985). This view of the role of CAH1 was challenged by Van et al. (1999) who found no evidence of growth inhibition in a mutant missing CAH1. The presence of other CA isoforms in the periplasmic space, namely CAH2 (Rawat and Moroney, 1991; Tachiki et al., 1992) and possibly CAH8 makes the interpretation of these results more complicated. CAH1 biosynthesis is strongly regulated by changes in environmental CO$_2$ concentration as well as light. CAH1 is very strongly induced under limiting CO$_2$ conditions, where CCM is operational (Fukuzawa et al. 1990).

CAH1 has been shown to be controlled by two regulatory regions: a silencer region, which represses the transcription under high CO$_2$ conditions or in the dark, and an enhancer region, which activates it under low CO$_2$ conditions in the light (Kucho et al., 1999). These sites may be important cis-acting elements that constitutively bind one or more proteins that assist in the regulated transcription of CAH1 (Kucho et al., 2003). LCR1, has also been identified as a regulatory gene of CAH1. LCR1 is a myb transcription factor that functions in amplification and maintenance of CAH1 mRNA levels in response to limiting CO$_2$ (Yoshioka et al., 2004).

CAH2 is also a periplasmic α-CA but is not thought to have a role in the CCM. CAH2 is an active CA (Rawat and Moroney, 1991; Tachiki et al. 1992) but is poorly expressed. In fact
CAH2 expression is down-regulated under limiting CO₂ conditions, the growth condition under which the CCM is operational (Fujiwara et al., 1990). CAH2 is only 1.4 kb bases away from the CAH1 gene (Fujiwara et al., 1990) and may be the result of a recent gene duplication.

CAH3, the third CA gene described in C. reinhardtii, codes for an α-CA that has a leader sequence consistent with targeting CAH3 to the thylakoid lumen (Karlsson et al., 1995; Funke et al., 1997). Immunoblot studies using antibodies raised against CAH3 demonstrated that CAH3 is associated with the thylakoid membrane (Karlsson et al., 1998). More specifically, immunolocalization studies indicated that CAH3 is localized on the lumenal side of the thylakoids and inside the pyrenoid tubules (Mitra et al., 2005). The evidence that CAH3 plays an essential role in the CCM is persuasive. C. reinhardtii strains defective in CAH3 cannot grow in air levels of CO₂ even though they grow normally on elevated levels of CO₂ (Spalding et al., 1983a; Moroney et al., 1986; Pronina and Semenenko, 1992; Karlsson et al., 1998). Putting the wild-type CAH3 gene back into these strains restores normal photosynthesis (Funke et al., 1997; Karlsson et al., 1998). Strains defective in CAH3 also accumulate large pools of Ci but are unable to use Ci efficiently for photosynthesis (Spalding et al., 1983a; Moroney et al., 1986). Therefore CAH3 appears to convert accumulated HCO₃⁻ to CO₂, the form of Ci that Rubisco can use. Its location suggests that CAH3 catalyzes the formation of CO₂ from HCO₃⁻ in the acidic lumen of thylakoids and that this CO₂ diffuses through the thylakoid membrane to the pyrenoid where the CO₂ will be fixed by Rubisco (Moroney and Mason, 1991; Badger and Price, 1994; Raven, 1997a; Moroney and Somanchi, 1999; Park et al., 1999). CAH3 is expressed under both high and low CO₂ growth conditions, although there is a two-fold increase in message abundance under low CO₂ conditions.

CAH3 has also been proposed to be associated with PSII and help to stabilize the PSII manganese cluster and catalytic function of PSII reaction centers (Park et al., 1999; Villarejo et
C. reinhardtii contains identical mitochondrial β-CAs (mtCAs), CAH4 and CAH5, that exhibit a pattern of expression which correlates with the expression of the CCM. The genes encoding CAH4 and CAH5 are adjacent to each other in the C. reinhardtii genome (Eriksson et al., 1998). They are highly induced at both the transcriptional and translational levels under low CO2 conditions (Eriksson et al., 1996; Geraghty and Spalding, 1996; Eriksson et al., 1998; Miura et al., 2004) and may have an important role in the acclimation of C. reinhardtii to low CO2 conditions. However, the exact role of these CAs is still not clear. One suggested function of mitochondrial CAs is to buffer the mitochondrial matrix, since prior to the complete induction of the CCM, photorespiratory glycine decarboxylation produces equivalent amounts of NH3 and CO2. The mtCA might serve to catalyze the hydration of CO2 producing H+ which would prevent alkalinization in the mitochondrial matrix as a result of the generation of NH3 by glycine decarboxylation (Eriksson et al., 1998). Alternatively, the mtCAs have been proposed to play a role in converting the CO2 generated by respiration and photorespiration to HCO3-. This would effectively “recapture” the CO2 generated by the photorespiratory pathway (Raven, 2001). More recently, it has been shown that even at low CO2 conditions, but with increasing NH4+ concentrations in the growth medium, the expression of mtCAs decreases both at the transcriptional and translational levels. Thus, it has been proposed that mtCAs are involved in supplying HCO3- to PEP carboxylase for NH4+ assimilation under certain conditions (Giordano et al., 2002). This hypothesis is reinforced by the evidence that under low Ci concentrations, the cah3 mutant, cia3, is impaired in maintaining high rates of electron transport and/or coupling the residual electron transport to ATP formation (Van Hunnik and Sültemeyer, 2002). However, subsequent studies with C. reinhardtii cah3 mutant have shown that as CO2 becomes limiting the chloroplast RuBP pool is increased compared with wild type which indicated a CO2 supply limitation rather than a PSII energy supply defect (Hanson et al., 2003).
al., 2003). As of this writing there are no mutants of *C. reinhardtii* missing these mitochondrial CAs.

CAH6 is a constitutively expressed β-CA in the chloroplast stroma (Mitra et al., 2004; Mitra et al., 2005). This CA might be involved in recapturing CO$_2$ as it effluxes from the thylakoid lumen and helping to maintain a high concentration of inorganic carbon in the stroma. Likewise, it might be another CA responsible for supplying CO$_2$ for Rubisco. It might be shuttling HCO$_3^-$ to CO$_2$ in the stroma as CO$_2$ is depleted by the action of Rubisco. This is the same role proposed for chloroplast CAs of higher plants. The generation of mutants of *CAH6* could help to confirm the physiological role of CAH6 in photosynthesis and CCM.

Three more additional β-CAs have been identified CAH7, CAH8 and CAH9. CAH7 and CAH8 will be further discussed in Chapters 4 and 5, respectively.
CHAPTER 3

MATERIALS AND METHODS

CELL CULTURES

Wild type C. reinhardtii 137+ was obtained from Dr. R. K. Togasaki, of Indiana University, Bloomington. The strain D66 (nit2-, cw15, mt+) was obtained from Rogene Schnell, University of Arkansas-Little Rock. Strain cia3 was generated by Dr. J. V. Moroney’s laboratory group (Moroney et al., 1986). To start cultures, cells from Tris AcetatePhosphate (TAP) medium plates were inoculated into 100 mL of TAP medium (Sueoka, 1960) and grown with continuous shaking and light (300 µE m⁻²s⁻¹) for two days. An aliquot of the culture was then transferred to 1.5 L of minimal medium (Sueoka, 1960) and bubbled with high CO2 (5% CO₂ in air) until it reached a cell density of about 2 x 10⁶ cells/mL. The culture was diluted with an equal volume of fresh medium and split into two flasks. One was bubbled with high CO₂ and the other with low CO₂ (0.035% CO₂ in air). The time of low CO₂ acclimation varied from 2 to 12 hours. The high and low CO₂ acclimated D66 cells were used for RNA isolation, measurement of chlorophyll content and western blots.

DNA PREPARATION, SEQUENCING AND HOMOLOGY ANALYSIS

There were three different DNA protocols employed. The first method was Quick and Easy Genomic Prep from http://www.chlamy.org/methods/quick_pcr.html (Pollock, 2003) for screening C. reinhardtii transformants. The second method was Total DNA Isolation (Newman et al., 1990). The cells grown as patches on TAP plates were resuspended in disruption buffer containing sodium dodecyl sulfate (SDS) and then the nucleic acids were extracted using phenol/chloroform. The aqueous phase was extracted a second time with chloroform. The nucleic acids were then precipitated with an equal volume of ethanol and washed twice with 70% ethanol. The third method was a genomic preparation which gives cleaner DNA which was
useful when subjecting DNA for further enzymatic reactions. One hundred mL of cells were centrifuged at 3000 rpm for 5 min and the precipitate was resuspended with 0.72 mL of H2O. To this solution 1.44 mL sodium dodecyl sulfate extraction buffer (SDS-EB) and 0.4 mg of proteinase K were added. This solution was incubated at room temperature for one hour. After the incubation three extractions were performed with 2.2 mL of phenol chloroform isoamyl alcohol (25:24:1 v/v/v). Chloroform isoamyl alcohol (24:1 v/v), 2.2 mL, was added to the final aqueous layer. Centrifugations were done at 3000 rpm for 15 min. Nucleic acids were precipitated from the aqueous layer by the addition two volumes of 100% ethanol and left in the freezer (-20°C) overnight. The pellet was collected after centrifugation at 11,500 rpm for 30 min. The pellet was air-dried, resuspended in 200 µL Tris-EDTA (TE) (pH8.0), supplemented with 1 µL of RNAse and was incubated at 37°C for 30 min. Extraction with equal volumes of phenol chloroform isoamyl alcohol (200 µL) was done three times, collecting the aqueous portion after every extraction. Chloroform isoamyl alcohol (200 µL), was added to the final aqueous layer. Centrifugations were done at 15000 rpm for 5 min. The aqueous layer was added with 20 µL of 3M Na acetate (pH 5.0) and two volumes (400 µL) of 100% ethanol. The precipitate was collected by centrifugation at 14,000 rpm for 10 min. The pellet was air-dried and resuspended with TE.

Plasmid and cosmid DNAs were purified using a combination of the standard ethanol precipitation method (Sambrook et al., 1989) followed by the purification method using the spin columns from a commercial kit (QIAGEN, Chatsworth, CA). The cDNA and genomic PCR products were purified from the DNA gel. Cut DNA bands from the gel were treated with 6M sodium iodide at 55°C to melt the gel piece. DNA was purified from the liquefied gel using the mini spin columns from the commercial kit mentioned above. DNA was sequenced using the ABI dye terminators (for some PCR fragments and cosmids enriched in the GC content, the use
of dGTP-BigDye generated better sequences than BigDye). DNA of 10 and 40 ng were used to sequence PCR products (100-500 bp) and PCR products (500-1000 bp) respectively. Primers of 3.2 pMol and 1.0 µL of Big Dye were used per 10 µL sequencing reaction.

The following PCR profile was used: 30 cycles at 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Homology searches (against *Chlamydomonas* EST and the full database) were performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997). Exon/intron splice sites and open reading frames were identified manually (Silflow, 1998). Signal peptide analysis and molecular weight and pI calculations were done using different protein prediction servers like ChlorP, TargetP and SortP which had hyperlinks in the Expasy server (http://ca.expasy.org/tools/#translate).

**cDNA LIBRARY PREPARATION**

A cDNA core library (amplified bacteriophage libraries) was obtained from the *Chlamydomonas* Culture Collection at Duke University. The core library was made from cDNAs prepared from CC-1690 cells grown to mid-log phase in TAP medium in the light, TAP medium in the dark, HS (minimal) medium in ambient levels of CO₂ and HS medium bubbled with 5% CO₂. The cDNAs were cloned into the lambda Zap II (Stratagene, La Jolla, CA) in the EcoRI (5') and XhoRI (3') sites. The lambda ZAP II vector is designed to allow simple efficient in vivo excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert.

In vivo excision involving the ExAssist interference–resistant helper phage along with the SOLR strain of *E. coli* was used. The ExAssist helper phage contains an amber mutation that prevents the replication of the phage genome in a nonsuppressing *E. coli* strain like SOLR. This allows efficient excision of the pBluescript phagemid from the Lambda ZAP II vector while eliminating the problems associated with helper phage co-infection.
Fifty mL overnight cultures of XL1 Blue MRF’ and SOLR cells were grown in Luria Bertani (LB) broth at 30°C. Harvested cells were resuspended in 25 mL of 10 mM MgSO4. The concentration of cells in 10 mM MgSO4 was adjusted to a concentration of 1 x 10^8 cells mL^{-1}. The amplified lambda bacteriophage library was mixed with the XL1 Blue cells in the ratio of 1:10. This was done to excise 10 to 100 fold more lambda phage than the size of the primary library to ensure statistical representation of the excised clone. ExAssist helper phage was added at a 10:1 helper phage to cells ratio to ensure that every cell was co-infected with the lambda phage and the helper phage.

The mixture was allowed to incubate at 37°C for 15 minutes to allow the phage to attach to the cells. Twenty mL of fresh LB broth was added to the mixture and allowed to incubate for 2- 3.5 hours at 37°C with shaking. The mixture was then heated to lyse the phage particles and the cells. The lysed mixture was spun at 1000 g for 10 minutes to pellet the cell debris and supernatant containing the excised phagemid was collected. The titer of the excised phagemid in the supernatant was determined and was used to calculate the number of colonies that were needed for the statistical representation of the excised clones. Colonies from all the culture plates were pooled by mass scraping and resuspension in 3 mL of LB. Plasmids were purified from these cells using the spin column method (QIAGEN, Chatsworth, CA).

AMPLIFICATION OF CAH7 GENOMIC DNA FROM THE COSMID LIBRARY

To amplify a portion of CAH7 genomic region containing the unknown sequence (N region in the database) primers Bf 5’-tct gaa gga cgg tct gat gc-3’ and Br 5’-ccg taa tca cgc ctg tta tg-3’, generated an approximately 1.5 kb fragment that spanned the N region, a PCR-based screen of an indexed cosmid library was used. An indexed cosmid library was constructed using a cosmid library from Saul Purton, University of London (Purton and Rochaix, 1994). Briefly, 7680 different E. coli cells carrying single cosmids were grown in LB media on 80 different 96-
well microtiter plates having 8 rows (A to H) and 12 columns (1-12). Using this indexed library, 80 pools of cells, each containing 96 single cosmids, were generated. DNA from each pool, obtained by common alkaline lysis procedures, was used to create 10 superpools (each containing 768 about single cosmids) that were suitable for PCR. Using the sequence information obtained from the EST database of *C. reinhardtii*, primer sets were designed and used to screen the superpools. Once a plate carrying the correct cosmid was identified, a new set of pools was generated (12 pools, each containing 8 single cosmids). Finally, a new PCR reaction was performed with the single cosmids from the positive pool described above. Using this protocol, after 4 rounds of PCR, cosmid 37-A-7 containing the CAH7 gene was isolated from the cosmid library. The 1.5 kb DNA fragment was used as template to generate different sized fragments that were used for sequencing the unknown region of CAH7.

**TOTAL RNA ISOLATION**

All the glassware used was either baked at 180°C overnight or soaked with DEPC (diethyl pyrocarbonate)-treated water and autoclaved for two hours. Total RNA was extracted from *C. reinhardtii* using Trizol reagent (Invitrogen, Carlsbad, CA). Fifty mL of cells were harvested, transferred to 50 mL orange capped tube and spun for 5 min, at 3000 rpm, 4°C. The pellet was resuspended using the very minimal amount of media left after the supernatant was decanted. One hundred µL of the resuspended pellet was transferred into 1.5 mL Eppendorf tube and one mL of Trizol was added. This was mixed by vortexing for several seconds. After incubation for one hour at room temperature (RT), 200 µL of chloroform was added, vortexed for several seconds to mix, incubated at RT for 5 min and was centrifuged at 10,000 rpm for 15 min at 4°C.

The aqueous phase which was approximately 600 µL was transferred into a fresh 1.5 mL Eppendorf tube and was added with 0.5 mL of isopropanol, was incubated for 30 min and was
centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was decanted and the RNA pellet was washed with one mL 75% ethanol. The tube was flicked to loosen the pellet and was centrifuged at 8,000 rpm for 5 min. The supernatant was carefully decanted and the pellet was air dried for 45-60 min, to ensure that the pellet is free from ethanol which might interfere with DNAses treatment. The RNA was treated with Ambion Turbo DNase following the manufacturer’s protocol followed by an RNA clean-up using Qiagen’s RNeasy Clean-up Kit. Total RNA concentration was determined by absorbance at 260 nm, using the conversion 1 OD_{260} = 44 µg of RNA•mL^{-1}.

5’ RLM-RACE (RNA LIGASE MEDIATED RAPID AMPLIFICATION OF cDNA ENDS)

5’RLM-RACE used to establish the 5’ ends of mRNAs was performed by using the First Choice RLM-RACE Kit (Ambion, Austin, TX) as instructed by Ambion.

PCR PROTOCOLS

Colony PCR Protocol for E. coli/C. reinhardtii transformants

This protocol was adapted from Gussow and Clackson (1989). Individual E. coli colonies or C. reinhardtii cells were selected with a sterile toothpick and transferred into a 250 µL Eppendorf tube containing 50 µL of 10 mM sodium EDTA. The tube was then placed in a thermocycler set at 100°C for 5 min. The lysate was vortexed briefly and spun at 14000 rpm for one min. A 1.5 µL supernatant was used for PCR.

Inverse Polymerase Chain Reaction (iPCR)

iPCR was performed as described by Gasch et al., (1992). Briefly, approximately 500 ng of genomic DNA from a mutant in a volume of 500 µL was digested with 10 units of a restriction endonuclease for 12 to 16 hours. The digested genomic DNA was then diluted to a concentration of 10 ng•µL^{-1} in 500 µL and circularized with 400 Units of DNA ligase for 12 to 16 hours at 16°C.
The restriction endonucleases chosen for this step cut within the multiple cloning site of the vector. Primers used to amplify the flanking DNA using conventional PCR protocols were Ble5-1, a reverse complement of the 5’ region of the \( Ble^R \) coding sequence 5’-CGC TGA TGAACA GGG TCA C-3’ and Ble3-1, was in the 3’ region of the \( Ble^R \) coding sequence 5’-AGT GGT CGG AGG TCG TGT C-3’. Nested PCR reactions were performed using the following primers: Ble5-2, 5’-GGT CG G TCC AGA ACT C-3’ and Ble3-2, 5’-CGA GGA GCA GGA CTA A-3’; and Ble5-3, 5’-AGA TGT TGA GTG ACT TCT CTT-3’ and Ble3-3, 5’-GAA GAT ACT GCT CTC AAG TG-3’.

**Adaptor Mediated PCR**

This method is the modified version by Pollock (2003) of the Genome Walker™ Kit (Clontech, Palo Alto, CA). The DNA was digested with restriction endonucleases that recognize sequences that are not present in the \( Ble^R \) insertion; the two enzymes AfeI and PmlII cleave at six bp recognition sequences that occur on average every 4 Kb in the *Chlamydomonas* genome creating blunt-ended fragments. A blunt ended adaptor consisting of 48 bp (+) strand (GTAATACGACTCATAAGTACCGTGTCGGACGGCCTGCTG) and a 10 bp (-) strand (/5Phos/ACCAGCCCCG/3AmMC7/), which is 5’-dephosphorylated and capped with a 3’-methyl group, was ligated to the digested DNA.

A series of three sequential PCRs using 3 \( Ble^R \) specific primers and 2 adaptor specific primers are used to amplify the region from the known \( Ble^R \) sequence to the adaptor. The adaptor primers are complementary to the (-) strand of the 48 bp strand of the adaptor; such binding sites will only exist after polymerase, using \( Ble^R \) specific primers, has extended the 10 bp (-) strand. By using primer sets directed out of the 5’ or 3’ ends of the \( Ble^R \) insert it is possible to amplify DNA on both sides of the insert. The adaptor specific primers are AP1 5’-GTA ATA CGA CTC ACT ATA GAG T-3’ and AP2 5’-ACT ATA GAG TAC GCG TGG T-3’.
RT (Reverse Transcriptase) PCR

RT-PCR was done using 2 µg total RNA using Qiagen One-step RT-PCR System (Qiagen, Valencia, CA). PCR primers were designed either to span from one exon to the other to prevent amplification of residual genomic DNA or to straddle exons so that amplified fragments from residual DNA would be larger and can be differentiated from the amplified products from the RNA. Primers used for RT-PCR are given in Appendix B.

Quantitative RT (Real Time) PCR

Total RNA of 3 µg was used as template for synthesis of cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) according to manufacturer’s instruction. One tenth aliquot from the synthesized cDNA was used as template with Takara Bio Sybr Green Premix Ex Mix (Takara-Bio, Madison, WI) for quantitative PCR in an ABI Prism 7000 sequence detection system following the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The primers used for all RT-PCRs are listed in Appendix B. Primers specific for the gene CBLP (Chlamydomonas beta subunit-like polypeptide) were used as control primers for equal loading. CBLP was shown not to change in expression due to changes in CO₂ concentration (Im and Grossman, 2001).

CONSTRUCTION OF OVEREXPRESSION CONSTRUCTS

CAH7 AND CAH8 were cloned in the pMal-c2x overexpression vector. All the genes were cloned downstream from the MalE gene which encodes maltose-binding protein (MBP). The vector (6648 bp) has an exact deletion of the MalE signal sequence (bases 1531-1605) resulting in the cytoplasmic expression of the fusion protein. The vector contains the inducible $P_{\text{lac}}$ promoter, which is a hybrid of Trp promoter and LacUV5 promoter. $P_{\text{lac}}$ is positioned to transcribe a Male-LacZα gene fusion (Fig. 3.1). The LacI⁰ gene codes for the Lac repressor and has a promoter mutation which increases intracellular concentration of LacI repressor. It turns
Figure 3.1 Diagram Showing Some Features of the pMal-c2x Expression Vector. *LacI*<sup>q</sup>, promoter with a mutation which increases intracellular concentration of LacI repressor, Ptac, hybrid of Trp promoter and LacUV5 promoter, PCS, polylinker cloning site, Gene X, any gene cloned into the vector, *LacZ*<sub>α</sub>, β-galactosidase gene and *Amp<sup>r</sup>*, β-lactamase gene.
off transcription from $P_{\text{lac}}$ until IPTG is added (Fig. 3.1). The polylinker cloning region provides restriction endonuclease sites to insert the gene of interest, fusing it to the $\text{MalE}$ gene (Fig. 3.2). Insertion of the desired gene in this site interrupts the $\text{LacZ}$α allowing a blue-white selection on LB + Amp + X-Gal + IPTG plates. The vector also has $\text{Amp}^r$ gene which codes for the $\beta$-lactamase gene that confers ampicillin resistance. This allows additional screening of transformants on LB + Amp plates. The fusion protein can be purified by one-step affinity chromatography using an amylose column. The vector has a spacer sequence coding for 10 asparagine residues, between the $\text{MalE}$ sequence and the polylinker sequence (Fig. 3.2). This allows insulation of MBP from the protein of interest, increasing the chances that a particular fusion protein will bind tightly to the amylose resin during purification. The vector also includes a sequence coding for the recognition site of a protease, Factor Xa, which allows the desired protein to be cleaved from MBP after purification, without adding any vector derived residues to the protein.

The carbonic anhydrases $\text{CAH7}$ and $\text{CAH8 ORF}$ cDNA sequences were each cloned in the pMal-c2x overexpression vector. Primers, CAH7-Xmn1F 5’-GGG AAG GAT TTC GAT GCA GGA GTG CCC GAA TA- 3’ and CAH7-HindIIIR 5’- CCC AAG CTT AC GGT GGC ATC CAG CTA- 3’ were used to amplify a 1.5 kb CAH7 ORF while primers, CAH8-Xmn1F 5’-GGG AAG GAT TTC GAT GGC ACC GTC GTC TGA G- 3’ and CAH8-HindIIIR 5’-CCC AAG CTT GGG CTT CCA AGT GCT CTC A-3’ were used to amplify a 1.2 kb CAH8 ORF. The pMal-c2x vector was double digested with HindIII and XmnI and ligated to the CAH7 or to CAH8 PCR product. A high fidelity DNA polymerase (Pfx from Invitrogen) was used for all PCRs. This was used to prevent any missense/null mutation in the protein sequence during PCR and generated blunt ended amplified products. The vector was double digested with the blunt end cutter XmnI at the 5’ end and a sticky end cutter HindIII at the 3’ end. Amplified cDNAs
Figure 3.2 Partial Sequence of the Polylinker Cloning Site of pMal-c2x. The Factor Xa recognition amino acid sequence is shown in red. The Factor Xa cleavage site is denoted by an arrow. MalE codes for the maltose binding protein (MBP) and Lac Zα codes for the β-galactosidase α-fragment. Restriction enzyme sites in the polylinker cloning region are shown on top.
were purified from the DNA gel using QIAGEN spin columns and were digested with XmnI and HindIII. Ligation of the insert to the overexpression vector pMal-c2x vector was performed following the protocol in the NEB technical manual. Transformations of DH5α cells were done following the protocol in Sambrook et al., (1989). Although transformants were isolated by screening both on LB + Amp (100 µg/mL) and LB + Amp (100 µg/mL) + IPTG (0.1mM) + X-Gal (80 µg/mL) plates, they were picked only from LB + Amp plates.

Because of the strength of the P_tac promoter, transformants taken from a plate containing IPTG can contain mutant plasmids that have either 1) lost part or all of the fusion gene or 2) no longer express the protein at high levels. ampicillin and IPTG used for the experiment were filter sterilized. The clones containing the insert were determined by PCR and restriction enzyme digests. In-frame insertion of CAH7 and CAH8 with the sequence of MBP in the recombinant clone was verified by DNA sequencing.

OVEREXPRESSION AND PURIFICATION OF MBP FUSION PROTEINS

Selected clones of CAH7 and CAH8 were grown at 37°C in 100 mL LB (0.2% glucose) + Amp cultures on a water bath shaker. Glucose is necessary in the growth medium to repress the maltose genes on the chromosome of the E. coli host, one of which is amylase which can degrade the amylose on the affinity resin that is used for purification. The cells were induced for 4 hours with 1 mM IPTG for CAH7 and 0.6 mM for CAH8 at 37°C when the culture OD_{600} was between 0.6-0.7.

Both induced and uninduced cells were harvested and resuspended in 1X column buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA with / without 10 mM 2-mercaptoethanol and ruptured in a prechilled French pressure cell. Equal amounts of protein samples of ruptured induced and uninduced cells were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis to verify the overexpression of the recombinant protein.
Purification of the fusion protein was done by a one-step affinity chromatography using amylose resin. Amylose resin (1mL of amylose resin binds 3 mg of the recombinant protein) was mixed with the crude cell extract on a shaker in the cold room for one hour and poured in a 50 mL orange capped tube and the amylose resin was washed with 15X column volumes of 1X column buffer to remove other proteins. At the final step, fusion proteins were eluted from the column by 1X column buffer containing 10 mM maltose. Purified recombinant fusion proteins were further concentrated by a passage through 100 kDa centricon columns (Amicon, Billerica, MA). The recombinant proteins were recovered from the membrane of the filter in the centricon columns. Recombinant proteins intended for antibody production were cleaved from the MBP by digestion with Factor Xa. Fifty µg of the recombinant protein was digested by 1 µg of Factor Xa enzyme in the Factor Xa digestion buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl₂ (pH 8.0) at 23°C for 4-6 hours. Purification and Factor Xa digestion of the recombinant protein was verified by performing 12% SDS-PAGE. A schematic diagram showing the outline of the cloning and purification of the recombinant MBP-CAH8 is shown in Figure 3.3.

GENERATION OF POLYCLONAL CAH7 AND CAH8 PRIMARY ANTIBODIES

The CAH7 ORF was cloned in the PQE80L vector (Qiagen, Valencia, CA) with the 6X His tag at the N-terminus using the same primers and protocol as in the cloning of CAH7 into the pMal-c2x overexpression construct. The overexpressed 6X His tagged protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose column following the manufacturer’s protocol (Qiagen, Valencia, CA). The 6X His tagged CAH7 was concentrated using a centricon column and approximately 2 mg/mL of the protein was sent to ProSci (Poway, CA) for rabbit polyclonal production of antibody by a standard 8-week protocol using two pathogen free rabbits. For the CAH8 protein, Factor Xa digested purified recombinant proteins were separated on a 12% gel by SDS-PAGE using 15 mAmp for 18-20 hours. The CAH8 protein band was
Figure 3.3. A Schematic Diagram to Show the Cloning and Amylose Column Purification of the Recombinant Fusion Protein.
excised carefully from the polyacrylamide gel. The gel pieces were shipped to Strategic Biosolutions (Ramona, CA) for production of antibody. Antibodies were raised against the CAH8 protein by a standard 70-day protocol using two pathogen free rabbits. Approximately 1.6 mg of the protein was used to raise the antibody.

**CARBONIC ANHYDRASE ASSAYS AND OTHER BIOCHEMICAL EXPERIMENTS**

CA activity was assayed electrometrically using a modification of the Wilbur-Anderson method (Wilbur and Anderson, 1948). The samples were assayed at 4°C by adding 50 to 500 µL of the test sample to 7.0 mL of 20 mM 4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid (EPPS), pH 8.0. The reaction was initiated by addition of 3.0 mL of ice cold CO2 saturated water. The time required for the pH drop from 7.7 to 6.3 was measured. The activity of the test sample was calculated using the equation: \( WAU = t_0/t - 1 \) where \( t \) is the time required for the pH change when the test sample is present and \( t_0 \) is the time required for the pH change when the buffer is substituted for the test sample. Bovine CAII (Sigma) was used as a positive control. Recombinant fusion proteins and proteins from vector only were used for activity assays.

The effect of temperature on activities of recombinant CAH7 and CAH8 proteins were studied. The temperatures used for this study are 0°C, 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. Recombinant CAH7 and CAH8 were incubated for 15 minutes at the indicated temperatures and cooled on ice. CA activity was measured at 4°C by the Wilbur-Anderson method. Activity is represented as a percentage of the activity of a sample maintained on ice throughout the experiment.

The effects of different sulfonamide inhibitors such as ethoxyzolamide and acetazolamide and anions like azide and cyanide on CA activities of recombinant CAH7 and CAH8 were studied. The \( I_{50} \) value corresponds to the concentration giving 50% inhibition. \( I_{50} \) was determined by plotting the percentage of inhibition vs the concentration of the inhibitor. Sodium
salts of azide and potassium salts of cyanide were used. To study effects of reducing agent on CA activities of recombinant CAH7 and CAH8, recombinant proteins were purified with and without the reducing agent 2-mercaptoethanol in the buffer and the activities were measured. The specific activities in the oxidized state are denoted as 100% activities (ox-CA).

**IMMUNOLOCALIZATION STUDIES USING ELECTRON MICROSCOPY**

Air adapted D66 cells were incubated in a mixture of 1% OsO₄, 2% formaldehyde and 0.5% glutaraldehyde in a 1:1 ratio for 15 minutes. The sample was then fixed for an additional 15 minutes in 1% OsO₄, 2% formaldehyde, 0.5% glutaraldehyde and 0.1 mM sodium cacodylate buffer. Materials were rinsed with distilled water and stained en block with 0.5 % uranyl acetate for 30 minutes. After this, excess stain was rinsed, dehydrated in ethyl alcohol series, infiltrated and embedded in L.R. white resin. Embedded tissues were sectioned with a Dupont Sorvall microtome and the sections were 70 µm thick.

The immunocytochemical procedure was similar to the method of Borkhsenious et al. (1998) with some modifications. Sections were pretreated with 2.5% sodium-meta periodate (Sigma, St. Louis, MA) for 10 minutes to remove residual glutaraldehyde, rinsed in distilled water and blocked twice for 20 minutes each with 2% BSA and 0.1% Tween 20 in PBS (Sigma). The sections were then incubated for 90 minutes with diluted primary antibody (1:10 dilutions of the CAH7/CAH8 primary antibody) or with the preimmune serum diluted similarly (used as a negative control). Sections were then washed with 0.5% Tween 20 six times for a total of 30 minutes and blocked again with 2% BSA for 10 minutes. The grids were transferred to 1:50 dilution of Protein A, conjugated to 20 nm colloidal gold particles. Protein A was diluted with 1% BSA and 0.1 % Tween 20 in PBS for 1 hour. Sections were washed with PBS four times; each rinse was for 5 minutes. Finally the sections were rinsed with distilled water and pictures were taken using transmission electron microscopy.
GENERATION of RNAi MUTANTS

Cloning to generate an RNAi construct

Standard molecular biological techniques were utilized to clone the genomic DNA and cDNA of target genes into the pSL72 vector to generate RNA interference (RNAi) constructs. Appendix B gives the list of primers used to generate the genomic and cDNA fragments for each of the gene whereby an RNAi construct was generated. Appendix A gives the map of the different RNAi constructs. The pSL72 vector was kindly provided by Dr. S. D. Lemaire (Univ Paris, Orsay, France). The pSL72 vector has AphVIII and Amp⁷ genes which code for an aminoglycoside phosphotransferase from *Streptomyces rimosus* and β-lactamase respectively. AphVIII and Amp⁷ confer paromomycin and ampicillin resistance respectively. Amp⁷ acts as selective marker in bacteria.

AphVIII acts as a selective marker in *C.reinhardtii* as paromomycin inhibits eukaryotic translation. The AphVIII gene has a constitutive PsaD promoter and terminator from *C. reinhardtii*. It also has an intron sequence which is the second intron of the cytochrome c₆ gene of *C. reinhardtii*. Reverse complementary sequences were cloned in two steps. In the first round of cloning, the genomic fragment was ligated before the 5’ end of the cytochrome c₆ intron in the pSL 72 vector.

Bacterial transformants containing genomic fragments were screened by PCR and verified by restriction enzyme digests. In the second cloning step the cDNA sequence complimentary to the genomic region was cloned after the 3’ end of the cytochrome c₆ intron of the vector. Transformants were screened by PCR and verified by restriction enzyme digests and DNA sequencing.

Bacterial transformation was done according to the protocol in Sambrook et al., (1989) and the ligations were done using T4 DNA ligase from NEB, following their protocol.
**Transformation of *C. reinhardtii* and Screening of RNAi Mutants**

The strain D66 (nit2-, cw15, mt+) was used for transformation. Culture conditions were similar to those used previously (Rawat and Moroney, 1991). D66 cells were first grown on 100 mL of TAP medium (Sueoka, 1960) for 2 days and then transferred to 1 L of TAP medium 24 hours before the experiment. Cells were harvested and resuspended at a density of 2 x 10^8 cells mL^-1. For electroporation, 1 µg of circular or linearized DNA was added to 300 µL of the resuspended cells in electroporation cuvettes with 0.4 cm gap width (BioRad Laboratories, Hercules, CA). These mixtures were held on ice for 15 minutes. The electroporation setting conditions were a capacitance of 25 µF and no shunt resistor. The voltage was set to 2000V cm^-1 and the pulse time was between 9.4 and 10 ms. A BioRad electroporator Gene pulser II was used. After electroporation, the cells were allowed to recover overnight in 10 mL of TAP + 60 mM sorbitol medium in minimal light. The next morning, the cells were harvested, spun 1 min and resuspended with the remaining TAP-sorbitol in the tube after pouring off the supernatant. Cells (6 x 10^7) were plated onto TAP + paromomycin (7.5 µg/mL) plates. The cells were then allowed to grow under very low-light conditions.

Transformants containing the RNAi constructs had the AphVIII gene and hence grew on TAP + paromomycin plates. The presence of the inverted repeat (IR) was checked by PCR and the decrease in the amount of message in *C. reinhardtii* cells containing the IR was quantified by real-time PCR using Sbyr Green.

Bacterial transformation was done according to the protocol in Sambrook et al. (1989) and the ligations were done using T4 DNA ligase from NEB, following their protocol.

**OTHER METHODS**

Genetic crosses and tetrad analysis were performed as previously described (Sears et al., 1980; Moroney et al., 1986; Harris, 1989). Immunoblots were performed as described earlier.
(Rawat and Moroney, 1991). Protein extracts were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with rabbit anti-*Chlamydomonas* CAH7 or CAH8 polyclonal antibodies. The specific protein bands were visualized using a secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA).

Photosynthesis rates were measured using a Clark-type O$_2$ electrode as described earlier (Pollock and Colman, 2001). The light intensity used for the photosynthesis measurements was 1000 µmol photons • m$^2$ • s$^{-1}$. Cell density values were determined by direct counting in a hemacytometer chamber. Chlorophyll concentrations were determined spectrophotometrically (Arnon, 1949) and were estimated using the equations of Holden (1976). The CO$_2$ concentration in the growth chambers was measured using an infrared gas analyzer. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.
CHAPTER 4
IDENTIFICATION AND CHARACTERIZATION OF CHLAMYDOMONAS REINHARDTII CHLOROPLAST β-CA CAH7

INTRODUCTION

Carbon dioxide (CO₂) and bicarbonate (HCO₃⁻) are substrates and products of many different metabolic reactions in the cells. The uncatalyzed interconversion of CO₂ and HCO₃⁻ is slow and thus without a catalyst these species will become limiting in the many physiological processes where they are needed. Carbonic anhydrases (carbonate dehydratase, carbonate hydrolyase; EC4.2.1.1) are zinc metalloenzymes that catalyze the interconversion of CO₂ and HCO₃⁻ (Khalifah, 1971). Thus, carbonic anhydrases (CAs) are involved in a broad range of biochemical processes that involve carboxylation or decarboxylation reactions such as photosynthesis and respiration (Moroney et al., 2001), pH homeostasis and ion transport (Tashian, 1989) and catalysis of key steps of pathways for the biosynthesis of physiologically important metabolites (Mitsuhashi et al., 2003).

Carbonic anhydrase was first purified from bovine red blood cells (Meldrum and Roughton, 1933) and now, CAs have been identified in the three domains of life: Archaea, Bacteria and Eukarya. There are clearly three distinct classes of CAs, α-, β-, and γ-CAs. Members of these different classes share very little sequence or structural similarity, yet they all perform the same function and require a zinc ion at the active site. Examples include carbonic anhydrases from mammals which all belong to the alpha class, the plant enzymes predominantly belonging to the beta class, while the enzyme from methane-producing bacteria that grow in hot springs, Methanosarcina thermophila, forms the gamma class. Thus it is apparent that these enzyme classes have evolved independently to create a similar enzyme active site. Two other classes of carbonic anhydrases, δ and ζ (Lane et al., 2005) have been reported to be present in the
marine diatom, *Thalassiosira weisflogii*. The *T. weisflogii* δ-CA similar to the other known CAs coordinates zinc (Cox et al., 2000) while the *T. weisflogii* ζ-CA coordinates cadmium (Cox et al., 2000). The presence of these two classes of CAs has so far have only been restricted in *T. weisflogii*. Whether these two CAs belong to new classes of CA is not yet well defined.

The presence of multiple CA isoforms in a single organism likely reflects their functional importance. *Arabidopsis thaliana* is reported to contain eight α-CA and six β-CA genes. Two of the α-CAs are localized to the chloroplast and are responsive to CO₂. These CAs are suggested to contribute to the transfer of CO₂ to the catalytic site of Rubisco (Fabre et al., 2007). In *C. reinhardtii*, three α-CA and six β-CA genes have been identified and six of these are already known to be active CAs.

Periplasmic CA activity contributed by α-CAs, CAH1 and CAH2 could accelerate the equilibrium between CO₂ and HCO₃⁻ and therefore can facilitate the passive and active diffusion of CO₂ across the cell membrane (Fujiwara et al., 1990; Fukuzawa et al., 1990; Rawat and Moroney, 1991; Moroney et al., 1985). The thylakoid CA, CAH3 which is required for the growth of *C. reinhardtii* at ambient levels of CO₂ has been proposed to increase generation of CO₂ from HCO₃⁻ and consequently CO₂ availability at the catalytic site of Rubisco (Funke et al., 1997; Karlsson et al., 1995). The mitochondrial CAs, CAH4 and CAH5 are highly induced under low CO₂ conditions suggestive of them being a part of the CO₂ concentrating mechanism. Their proposed functions include: (1) buffering the mitochondrial matrix by catalyzing the hydration of CO₂ whereby H⁺ will be produced that prevents alkalinization in the mitochondrial matrix which results from the uptake of H⁺ to form NH₄⁺ from NH₃ (Eriksson et al., 1998), (2) to play a role in converting the TCA and photorespiratory-generated CO₂ to HCO₃⁻ that leaves the mitochondria via a hypothetical HCO₃⁻ channel then to site of Rubisco (Raven, 2001) and (3) to supply HCO₃⁻ to PEP carboxylase for NH₄⁺ assimilation under certain conditions (Giordano et
al., 2003). The chloroplast stroma CA CAH6 possibly converts the CO₂ diffusing out back into HCO₃⁻ thereby retaining inorganic carbon within the chloroplast (Mitra et al., 2004). This chapter presents the identification and characterization of the seventh carbonic anhydrase in *C. reinhardtii*. The possible functions of this CA are proposed.

**RESULTS**

**Identification and Isolation of CAH7**

*C. reinhardtii* CAH7 was first identified from the Joint Genome Initiative (JGI) *Chlamydomonas* Database Version 2 (jgi-psf.org/chlre2/chlre2.home.html) as an annotated carbonic anhydrase. A comparison of the sequence of this new carbonic anhydrase gene indicated that it did not match any of the previously identified carbonic anhydrase genes in *C. reinhardtii*. This gene appeared to be actively transcribed as there were 13 ESTs in the database. Most of these ESTs were in the 3'UTR explaining why this gene had not been identified in the first compilation of the *Chlamydomonas* genome (Fig. 4.1). The sequence in the genome database was incomplete so complete cDNA sequence was determined.

**Gene Structure of CAH7**

The CAH7 gene consists of eleven exons (Fig. 4.2). The CAH7 genomic sequence spans approximately 5071 bases with a cDNA sequence of 2473 bp (Fig. 4.3 and 4.4). The exons range in size from 58 (exon 7) to 1342 bp (exon 11) and introns range in size from 205 (intron 2) to 368 (intron 1). The cDNA has an 84 bp 5' untranslated region in exon 1 as it contains a translation start site at nucleotide 85. Exon 11 contains the stop site which is at nucleotide 1282. CAH7 has a long 3' UTR of 1189 bp. The 3' UTR contains the common putative polyadenylation signal TGTAG, which is located 1173 bp downstream from the stop codon and 11 bp upstream of the polyadenylation site. Exons 2 through 8 encode the conserved β-CA domain.
Figure 4.1. *CAH7* in Scaffold 116 of JGI *Chlamydomonas* Genome Browser Version 2. *CAH7* in scaffold 116 showing several expressed sequence tags (ESTs) available for *CAH7*.

Figure 4.2. Exon-Intron Structure of *CAH7*. The genomic map of *CAH7* with its 11 exons. The numbers within the parentheses denote the start and stop codon positions on the map in base pairs.
Figure 4.3. The Genomic Sequence of CAH7. It is estimated to be 5071 bp. The exon sequences are in uppercase and the intron sequences are in lowercase. In gray respectively. The sequences are in uppercase and the intron sequences are in lowercase. Each exon is represented by a different color. The start and stop sequences are highlighted in gray respectively.
Figure 4.4. The cDNA Sequence of CAH7. The cDNA sequence is 2473bp. Each exon is represented by a different color. The start and stop sequences are highlighted in green and red respectively. The polyadenylation signal TGTAG is underlined.
Homology Search and the CAH7 protein

The CAH7 gene contains an open reading frame consisting of 1200 bp and its translation product consists of 399 amino acids with a calculated molecular mass of 43.0 kD and predicted isoelectric point of 7.61 (http://us.expasy.org/tools/pi_tool.html) (Fig. 4.5). There are many ORFs encoding β-CAs having significant similarities to the CAH7 amino acid sequence by BlastP searches of the non-redundant sequence database at the National Center for Biotechnology Information (NCBI).

When the deduced amino acid sequence was compared with other CAs from other organisms, the most similar CA was found to be the plastid targeted β-CA of Helicosporidium sp. ex Simulium jonesii, an obligate parasite that evolved from photosynthetic green alga (52% identity) (Fig. 4.6).

CAH7 contains the two Cys, one His coordinating Zn$^{2+}$, characteristic of β-CAs. Based on the X-ray crystal structure analysis of the red alga, Porphyridium purpureum (Mitsuhashi et al.; 2000), CAH7 contains the two Cys, His, Asp found to coordinate Zn$^{2+}$ in P. purpureum. In addition, CAH7 contains all of the 23 amino acid residues they have identified as strictly conserved among β-CAs and contains all the residues they have identified to be clustered on the concave surface of the active site clefts. Fourteen residues are clustered on the concave surface of the active site clefts as described in Mitsuhashi et al. (2000). CAH7 shares 29% and 34% identities with other C. reinhardtii β-CAs, CAH4 and CAH6 respectively (Fig. 4.7). C. reinhardtii CAH7 is most similar to C. reinhardtii CAH8 with 63% identity. Sequence alignment of CAH7 with other β-CAs in C. reinhardtii showed a C-terminal extension (Fig.4.7). TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) which is a prediction program for transmembrane region predicts a transmembrane domain at positions 269-288 close to the C-terminal domain (Fig. 4.8).
Figure 4.5. The Amino Acid Sequence of CAH7. This protein shows an open reading frame of 399 amino acids. The start and stop codons are in bold red respectively.
Figure 4.6. Amino Acid Sequence Alignment of CAH7 with other β-CAs. Highlighted are residues described to be clustered on the concave surface of the active site clefts as described by Mitsuhashi et al. (2000). Zinc ligands as determined in *P. purpureum* β-CA are marked by red asterisks.

The numbers correspond to (1) *Chlamydomonas reinhardtii* CAH7 (2) *Chlamydomonas reinhardtii* CAH8 63% (3) *Helicosporidium* sp. ex *Simulium* jonesii 55% (AY596510.1) (4) *Saccharophagus degradans* (52%) (NC_007912.1) (5) *Magnetospirillum magnetotacticum* MS-1 51% (NZ_AAAP01003796.1) (6) *Shewanella loihica* PV-4 50% (NC_009092.1) (7) *Coccomyxa* 50% (U49976.1) (8) *Aeromonas hydrophila* subsp. *hydrophila* ATCC7966 49% (NC_008570.1) (9) *Pseudomonas fluorescens* PfO-1 (NC_007492.1)

Numbers in % are corresponding sequence similarities with CAH7. Numbers in parentheses are GenBank accession numbers.
Figure 4.7. Multiple Sequence Alignment of *C. reinhardtii* CAH7 with other *C. reinhardtii* β CAs. CAH7 most closely aligns with CAH8.
SOPMA, a program for secondary structure predictions (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) was used to analyze the secondary structure of CAH7. A structural feature common to β-CAs is the domination of α-helix structure (Kannan et al., 1975; Eriksson and Liljas, 1993; Boriack-Sjodin et al., 1995; Kisker et al., 1996). An α-helix structure of 40.60% (Fig. 4.8) comprised the highest secondary structural motif based on SOPMA prediction.

Various protein prediction programs gave different results as to the localization of CAH7 (http://us.expasy.org/tools/). For instance, PSORT and Sosui, predict CAH7 is a cytosolic protein. Target P predicts CAH7 to have a chloroplast target signal. These different prediction programs assign the protein in different cellular compartments and therefore we can not rely on prediction programs in assigning a final intracellular location to CAH7. Producing an antibody to CAH7 will aid in determining the location of CAH7 and might suggest the possible role of CAH7 in *C. reinhardtii*.

**Overexpression of CAH7 in *E. coli* for Antibody Production**

The 1.5 kb fragment which corresponds to CAH7 ORF was cloned into the overexpression vector PQE80L to raise an antibody against *C. reinhardtii* CAH7. The CAH7 ORF was initially cloned into the overexpression vector pMal-c2X as a fusion to the C-terminus of the maltose-binding protein (MBP). The fusion protein was purified using an amylose affinity column and digested using Factor Xa to cleave CAH7 from the MBP. The Factor Xa digest products were first run on an SDS-PAGE to separate MBP from CAH7. SDS-PAGE was not able to separate the 42 kD MBP which migrates as a 44 kD protein from CAH7 whose theoretical MW is 43 kD. Thus, the vector PQE80L was used to overexpress CAH7 in *E. coli*. In PQE80L, CAH7 was fused with the 6X His tag. The 6X His tag is poorly immunogenic thus this recombinant protein was used as an antigen to generate antibodies for CAH7.
Figure 4.8. Secondary Structure Prediction of CAH7. Alpha helix (Hh):162 is 40.60% Extended strand (Ee):58 is 14.54% Beta turn (Tt):27 is 6.77% Random coil (Cc):152 is 38.10% (SOPMA Prediction Program). CAH7’s 20 amino acid transmembrane helix domain as predicted by TM-Pred program.is highlighted in yellow.
The presence of the insert in ampicillin resistant colonies was checked by PCR (Fig. 4.9). Several clones that have the insert were overexpressed. The bacterial clone that gave good expression and a protein fragment of the correct size was chosen and the plasmid was sequenced to ensure that an unmutated CAH7 was contained in this clone. *E. coli* cells harboring the 6XHis-CAH7 construct was induced with 1 mM IPTG for 4 hrs at 37°C to optimally overexpressed the protein. Equal amounts of proteins from the uninduced and induced cells were loaded on a 12% SDS-polyacrylamide gel. The molecular weight of the overexpressed fusion protein migrated as a 49 kD His tagged protein (Fig. 4.10). The 6XHis protein was purified using Ni-NTA affinity column to homogeneity, concentrated using as 50 kD centricon column and sent for antibody production (Fig. 4.11).

**Overexpression of CAH7 to Determine CA Activity**

CAH7 was overexpressed with an apparent MW of 84.1 kD using the vector pMal-c2x to determine if CAH7 is an active CA and to further characterize CAH7(Fig. 4.12). The 1.5 kb ORF of CAH7 was cloned at the C-terminus of the maltose binding protein. There was the need to clone CAH7 using pMal-c2x because the purification of 6XHis-CAH7 under native conditions was giving a low yield. The overexpressed recombinant MBP-CAH7 protein was purified by affinity chromatography using amylose resin following the protocol in the New England Biolabs technical catalog (Fig. 4.13). The purified recombinant CAH7 was further concentrated by using a 100-KD cut-off centricon column. The CA activity in the sample at each step of purification was assayed to check the purity of the sample (Table 4.1). The recombinant CAH7 protein has a specific activity of 3.1 WAU•mg⁻¹. This calculation of specific activity was based on the total amount of recombinant protein in the sample. CA activity was assayed by the method of Wilbur and Anderson (Wilbur and Anderson, 1948). CA activity was not detected in the extracts from uninduced cells nor *E. coli* cells containing only the pMal-c2x vector.
Figure 4.9. PCR Amplification of the 1.5 kb Insert in Ampicillin Resistant Bacterial Colonies Transformed with PQ80EL-CAH7. Lanes, (1-10) bacterial clones, (11) 0 DNA, (ST) molecular weight markers

Figure 4.10. Overexpression of CAH7 in PQ80EL. Lanes: (1) Uninduced PQ80EL-CAH7 (2) Induced PQ80EL-CAH7 (3) MW standards
Figure 4.11. 6XHis-CAH7 Fractions. Lanes: (1) crude extract, (2) 1 µL of centricon eluate and (3) 5 µL of centricon eluate.
Figure 4.12. Overexpression of CAH7 pMal-c2x. Lanes 1-9 are ampicillin resistant bacterial clones and are PCR positive for the presence of the insert. Encircled band corresponds to a clone that was used for overexpression and purification of MBP-CAH7.
Figure 4.13. Purification of MBP-CAH7. Samples from the purification steps were analyzed by SDS-PAGE. Lanes: (1) crude extract (2) crude extract unbound (3) amylose eluate (4) centicon fraction (5) MW standards.
Expression Analysis of CAH7

Reverse-trancriptase PCR showed that CAH7 was expressed under both high and low CO2 conditions (Fig. 4.14). Quantitative real time-PCR (qRT-PCR) using Sybr Green was used to quantify the expression of the various CA transcripts, including CAH7. Total RNA was isolated from wild type D66 cells grown under high CO2 and from cells acclimating to low CO2 for 12 hours. These RNA preparations were then used as templates for qRT-PCR. Similar to the results of qualitative PCR, quantitative PCR showed that CAH7 is constitutively expressed under both high and low CO2 conditions and only a 0.5 cycle or a 1.4 fold difference was observed in the amount of messages (Fig. 4.15). The comparison of critical threshold (Ct, the cycle number where the message was first detected) values of the different CAs showed that CAH7, similar to CAH6 and CAH8, is a moderately expressed CA and its expression level is lower than CAH1, CAH3 and CAH4 (Table 4.2 and Fig. 4.16).

Likewise, in order to quantify the expression of the various CA transcripts at different time points, total RNA was isolated from wild type cells grown under high CO2 or from cells acclimating to low CO2 for various times. These RNA preparations were then used as templates for qRT-PCR. The difference in the abundance of a message at a particular time point was expressed relative to the expression of the message on high CO2. CAH7 showed a 1.4-4.6 fold difference in the amount of message a range similar to CAH6 and CAH8 (Fig. 4.17). On the other hand, the genes encoding the periplasmic CA, CAH1, and the mitochondrial CAs, CAH4 and CAH5, were strongly induced under low CO2. CAH1 and CAH4/5 were the most responsive to low CO2 with a 10000 fold increase and 3500 fold increase in the amount of messages, respectively. These messages are most abundant about four hours after the shift to low CO2 (Fig. 4.18). CAH2 was the CA gene least responsive to low CO2 actually showing a decrease in expression one hour after the shift to low CO2 (Fig. 4.18).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Unit Activity (WAU/mL)</th>
<th>Specific Activity (U/mg)</th>
<th>Total Activity (WAU)</th>
<th>%Recovery</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude</td>
<td>204</td>
<td>0.44</td>
<td>0.1</td>
<td>20.4</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>amylose</td>
<td>7.6</td>
<td>4.2</td>
<td>2.2</td>
<td>16.7</td>
<td>82</td>
<td>22</td>
</tr>
<tr>
<td>centricon</td>
<td>1.8</td>
<td>21.6</td>
<td>3.1</td>
<td>5.6</td>
<td>27</td>
<td>31</td>
</tr>
</tbody>
</table>

*One WAU=(t_u/t) - 1 where t_u is the time for uncatalyzed reaction and t is the time for the enzyme catalyzed reaction. *Determined by Lowry’s protein assay (Lowry et al., 1951). *Results are averages of three replicates.
Figure 4.14. The Qualitative Expression Analysis of CAH7. CAH7 and other known C. reinhardtii carbonic anhydrases under high and low CO₂ conditions using semiquantitative RT-PCR showing the differential expression of the C. reinhardtii carbonic anhydrases.
Figure 4.15. The Quantitative Expression Analysis of CAH7. The amount CAH7 mRNA was analyzed by real-time PCR using Sybr Green and showed low CO₂ grown cells possessed 1.4 times as much message as high CO₂ cells.
Table 4.2. Comparison of the Expression Levels of the Different *C. reinhardtii* Carbonic Anhydrases Based on Ct Values\(^a\) from Real-time PCR Using Sbyr Green.

<table>
<thead>
<tr>
<th>Carbonic Anhydrase</th>
<th>High CO(_2)</th>
<th>Low CO(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAH1</td>
<td>26.3 ± 0.06</td>
<td>14.5 ± 0.02</td>
</tr>
<tr>
<td>CAH2</td>
<td>23.5 ± 0.16</td>
<td>26.4 ± 0.28</td>
</tr>
<tr>
<td>CAH3</td>
<td>18.3 ± 0.09</td>
<td>17.7 ± 0.16</td>
</tr>
<tr>
<td>CAH4</td>
<td>26.3 ± 0.07</td>
<td>14.5 ± 0.02</td>
</tr>
<tr>
<td>CAH6</td>
<td>19.3 ± 0.18</td>
<td>19.0 ± 0.28</td>
</tr>
<tr>
<td>CAH7</td>
<td>21.1 ± 0.12</td>
<td>20.6 ± 0.10</td>
</tr>
<tr>
<td>CAH8</td>
<td>18.8 ± 0.13</td>
<td>19.7 ± 0.16</td>
</tr>
</tbody>
</table>

\(^a\) Values are averages of three replicates.
Figure 4.16. The Comparison of the Expression Levels of *C. reinhardtii* Carbonic Anhydrases Based on Ct values. *CAH7* is a moderately expressed *C. reinhardtii* carbonic anhydrase.
Figure 4.17. The Relative Log Fold Difference in the Amount of Message of the Different *C. reinhardtii* Carbonic Anhydrases under Low CO\(_2\) Relative to High CO\(_2\). Results from real-time PCR showed that CAH7 does not show a dramatic response to low CO\(_2\).
Figure 4.18. Time Course Analysis of the *C. reinhardtii* Carbonic Anhydrases. There was no significant difference in the amount of CAH7 transcript under low CO₂ relative to high CO₂ at different time intervals.
**Immunolocalization of CAH7**

WT137 cells acclimated to high CO\textsubscript{2} and low CO\textsubscript{2} were used for immunolocalization of CAH7. *C. reinhardtii* cell sections were probed with either the CAH7 antibody or the preimmune serum and were observed under a transmission electron microscope (Fig. 4.19 and Fig. 4.20). Immunogold densities in different cell compartments are given in Table 4.3. Preliminary results of immunolocalization studies demonstrated that CAH7 is located in the chloroplast (Fig. 4.19).

**Characterization of CAH7 Activity**

The effects of known CA inhibitors, such as sulfonamides and anions on the CA activity of the recombinant CAH7 were determined. Table 4.4 shows the inhibition of recombinant CAH7 by acetazolamide (AZ) and ethoxyzolamide (EZ). Generally, all β-CAs are less sensitive to sulfonamide inhibition and are slightly more inhibited by the anions azide and cyanide than the α-CA bovine CAII (Johansson and Forsman, 1993). CAH7 is 10\textsuperscript{3}-fold and 10\textsuperscript{5}-fold less sensitive to sulfonamides AZ and EZ respectively compared to the α-CA, bovine CAII. CAH7 is 10-fold and 100-fold less sensitive to AZ and EZ respectively than *C. reinhardtii* CAH6. It can be observed that CAH7 exhibited a very low sensitivity to EZ compared to AZ. The I\textsubscript{50} value for AZ falls within the range of I\textsubscript{50} constants for β-CAs, 2 µM-10 µM (Johansson and Forsman, 1993) while EZ falls beyond this range. Similar to CAH6, CAH7 is more sensitive to azide than the α-CA, bovine CAII, which is typical for β-CAs. However, CAH7 unlike CAH6 is less sensitive to cyanide than α-CA, bovine CAII. CAH7 maintained 80% or better activity at temperature range of 0-40\textdegree{C}. It was stable at a temperature range of 0-50\textdegree{C} where it retained greater than 50% of its activity. Little activity (7%) was recovered when the enzyme was incubated for 15 min at 60\textdegree{C} (Fig. 4.21). CAH7 is relatively more stable than the chloroplast stroma β-CA, CAH6 which completely lost its activity at 50\textdegree{C} (Mitra et al., 2004).
<table>
<thead>
<tr>
<th>Location</th>
<th>Area (μM²)</th>
<th>Immune</th>
<th>Preimmune</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside</td>
<td>1.35 ± 0.26</td>
<td>0.00 ± 0.00</td>
<td>0.04 ± 0.04</td>
<td>-0.04 ± 0.04</td>
</tr>
<tr>
<td>Periplasm</td>
<td>0.71 ± 0.06</td>
<td>0.73 ± 0.44</td>
<td>0.85 ± 0.33</td>
<td>-0.12 ± 0.20</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>5.33 ± 0.31</td>
<td>0.17 ± 0.07</td>
<td>0.01 ± 0.01</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.86 ± 0.18</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>5.95 ± 0.28</td>
<td>0.82 ± 0.07</td>
<td>0.11 ± 0.03</td>
<td>0.72 ± 0.09</td>
</tr>
<tr>
<td>Pyrenoid</td>
<td>0.82 ± 0.31</td>
<td>0.38 ± 0.25</td>
<td>0.37 ± 0.18</td>
<td>0.01 ± 0.30</td>
</tr>
</tbody>
</table>

Table 4.3. Intracellular Localization of CAH7 Using Wild Type *C. reinhardtii*.

Immunogold densities in different cell compartments in sections were calculated by dividing the number of immunogold particles in a particular cell organelle with the area of that cell organelle. The data presented in the table is the average ± SEM of 12 cell sections. The cytoplasmic area was calculated by subtracting the total area of the chloroplast and nucleus from the cell area.
Figure 4.19. Immunogold Labeling of *C. reinhardtii* WT137 Cells Probed with CAH7 Antibody. Immunogold labeling showed localization to the chloroplast.
Figure 4.20. Immunogold Labeling of *C. reinhardtii* WT137 Cells Probed with CAH7 Pre-immune Serum.
Table 4.4. The Inhibition Constants of the Bovine CAII, *C. reinhardtii* CAH6 and CAH7.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$I_{50}$ Bovine CAII$^a$ M</th>
<th>$I_{50}$ CAH6$^a$ M</th>
<th>$I_{50}$ CAH7 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetazolamide</td>
<td>$1.4 \times 10^{-8}$</td>
<td>$2.0 \times 10^{-6}$</td>
<td>$4.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>ethoxyzolamide</td>
<td>$1.2 \times 10^{-9}$</td>
<td>$9.0 \times 10^{-6}$</td>
<td>$4.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>azide</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-5}$</td>
<td>$3.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>cyanide</td>
<td>$4.9 \times 10^{-5}$</td>
<td>$5.0 \times 10^{-6}$</td>
<td>$6.1 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

$^a$ Inhibition constants from Mitra et al., 2004
Figure 4.21. The Thermostability of CAH7 Activity. Recombinant CAH7 was incubated for 15 min at the indicated temperatures and cooled on ice and activity was determined at 4°C by the Wilbur-Anderson method. Activity is represented as a percentage of the activity of a sample maintained on ice throughout the experiment. The percentage of CAH7 activity data for each temperature treatment are the averages of three replicates.
CAH7 was more active under reduced conditions compared to oxidized conditions. CAH7 is similar to higher plant chloroplasts β-CAs which are sensitive to oxidation. On the other hand, *C. reinhardtii* CAH6 (Mitra et al., 2004) and CAH8 (Chapter 5) activities are neither decreased under oxidizing conditions nor increased under reducing conditions. CAH7 activity is increased by 69% in the presence of the reducing agent β-mercaptoethanol (Fig. 4.22).

**RNAi analysis of CAH7**

In an attempt to elucidate the function of *CAH7*, a method to suppress its expression was employed. An RNA interference (RNAi) construct was made from pSL72 vector which used the PsaD promoter and terminator to express the AphVIII paromomycin resistance gene. An inverted repeat of the genomic region containing a portion of exon 3 to portion of intron 7 and cDNA of CAH7 from portion of exon 3 to a portion of exon 8 was cloned in the vector between the AphVIII and PsaD terminator. The RNAi construct of *CAH7* is shown in Figure 4.23A. PCR was used to check the presence of the insert in the bacterial clones that were ampicillin resistant. Figure 4.23B shows the restriction enzyme digests of pSL72-IR which is diagnostic for the presence of both the genomic and cDNA inserts in pSL72 as shown by the different band patterns. Restriction enzyme digests and sequence analysis confirmed the presence of the IR in the construct that was used for the transformation of WT D66.

The strain D66 was transformed with NotI linearized pSL72-*CAH7*IR and approximately 500 transformants were selected in TAP media containing paromomycin. These transformants were screened for the presence of the inverted repeat (IR) by PCR using primer pairs that span the vector and the insert. Transformants containing the IR were then replica plated onto minimal media in high and low CO₂ conditions. No difference was observed among transformants and WT D66 grown under high and low CO₂ conditions. The transformants exhibited the same phenotype as WT D66 even under low CO₂ conditions (Fig. 4.24). Quantitative real-time PCR
**Figure 4.22. Effect of the Reducing Agent, β-mercaptoethanol on the Activity of the Recombinant MBP-CAH7.** The recombinant protein was purified in either the presence or absence of β-mercaptoethanol and CA activity was determined at 4°C by the Wilbur-Anderson method. The CAH7 specific activity data for each treatment are the averages of three replicates.
**Figure 4.23. The pSL 72-CAH7 RNAi Construct.** A. The inverted repeat was constructed by ligating a genomic fragment containing the 60 bp of exon 3 and 143 bp of intron 7 and the cDNA has the 58 bp of exon 3 and 6 bp of exon 8. B. The restriction enzyme digests of psL72-CAH7 RNAi construct. Lanes: (1-3; 7-9) bacterial clones containing the genomic and cDNA IR, (4,10) bacterial clones containing the genomic fragment only, (5,11) pSL 72 vector only and (6) MW standards.
Figure 4.24. Screening of Paromomycin Resistant Transformants Containing the pSL72-CAH7IR. No difference was observed among the transformants and WT D66 under both high and low CO$_2$ conditions.
was done to determine any difference in the amount of messages between D66 and the selected RNAi strains. A decrease in CAH7 transcript level was not observed among the transformants.

**DISCUSSION**

*C. reinhardtii* CAH7, the fourth β-CA in *C. reinhardtii*, was identified as an annotated carbonic anhydrase in Joint Genome Initiative (JGI) *Chlamydomonas* Database Version 2. The 2473 bp cDNA encoding CAH7 was identified and characterized. It has an ORF encoding a protein of 399 amino acids, that showed similarity to many β-CA protein sequences in NCBI database. The protein contains the two Cys, one His zinc-coordinating ligands characteristic of known enzymatically active β-CAs. Likewise, it has all of the 23 residues described to be strictly conserved among β-CAs (Mitsuhashi et al., 2000). It has closest sequence similarity (63%) to the *C. reinhardtii* β-CA, CAH8 which will be described in the next chapter. Among other organisms, CAH7 has 52% similarity to the parasitic green alga, *Helicosporidium* sp. ex. *Similkium jonesii*. From these comparisons it is evident that CAH7 is a β-CA. It can be observed from sequence alignment comparisons of *C. reinhardtii* β-CAs that CAH7 as well as CAH8 have C-terminal extensions. TMPred, prediction program for transmembrane regions, predicts a transmembrane domain at positions 269-288 close to the C-terminal domain. This region of CAH7 might facilitate an association for (1) oligomerization (2) interaction with other proteins or (3) with a membrane region like the chloroplast envelope. Likewise, CAH7 has a 137 amino acid C-terminal extension when aligned with other *C. reinhardtii* β-CAs. In the carboxysomal β-CA from *Synechococcus* PCC7942 and *Synechocystis* PCC6802, the 60-70 amino acid extension at the carboxyl end was proposed to be essential for both the oligomerization of the carboxysomal β-CA and in catalysis (So et al., 2002). Aside from the high degree of amino acid sequence similarity contained among β-CAs, the content of secondary structure elements was observed to be similar. For example, β-CAs from pea (Johansson and Forsman, 1993), spinach
(Rowlett et al., 1994) and *Coccomyxa* (Hiltonen et al., 1998) were described to have dominance of α-helices. Similar to these β-CAs, CAH7 was predicted by SOPMA to contain 40.60% of α-helices. The dominance of α-helices observed among β-CAs highlights one of the structural differences between the α-, β-, and γ-CAs with the α- and γ-CAs being composed mainly of β-sheet structures (Kannan et al., 1975; Eriksson and Liljas, 1993; Boriack-Sjodin et al., 1995; Kischer et al., 1996).

CAH7 was amplified by PCR using the *C. reinhardtii* core library to generate a region containing the ORF of CAH7. This fragment was cloned into the overexpression vector pMal-c2x and expressed in *E. coli* to generate the fusion protein MBP-CAH7. This fusion protein was purified using an amylose affinity column. The recombinant protein was purified 31-fold to apparent homogeneity as indicated by a single 84 kD polypeptide band after SDS-PAGE.

The purified recombinant MBP-CAH7 has a specific activity of 3.1 WAU•mg⁻¹ which is lower than the 400 WAU•mg⁻¹ from the recently reported *C. reinhardtii* β-CA, CAH6 (Mitra et al., 2004). However, CAH7 activity is significantly higher than the 0.06 WAU•mg⁻¹ specific activity determined by mass spectrophotometry reported for *R. eutropha* CA (Kusian et al., 2002). It should be emphasized that not all CAs are enzymes with high activities (Moroney et al., 2001) and activity varies even among CAs within an organism. There is at least a 1000-fold difference in specific activities among animal CAs, with CAII having the highest (2000-3000 WAU•mg⁻¹) and CAIII the lowest (1-5 WAU•mg⁻¹) (Khalifah, 1971). Although the specific activity of the recombinant protein is relatively low this may not reflect the CA activity of the endogenous CAH7, since in vivo, other factors such pH, temperature, ions and/or association with proteins could affect the catalytic activity of an enzyme.

The CAH7 activity is inhibited by generally recognized CA inhibitors and although carbonic anhydrases are inhibited by the same types of compounds, inhibition constants vary
among individual enzymes. Similar to all β-CAs, CAH7 is less sensitive to sulfonamide inhibition than the α-CA bovine CAII. On the other hand, CAH7 is as sensitive to azide as α-CA bovine CAII. Not typical of β-CAs is CAH7’s low sensitivity to cyanide as compared to α-CAs. In the human CAII, crystallographic studies have shown that inhibition by sulfonamides is brought about by the sulfonamide nitrogen ion binding to the zinc ion and thus replacing the hydroxide ion (Lindskog, 1997). Anions, such as azide and cyanide on the other hand, directly coordinate to the metal ion and displace the zinc bound water molecule (Kumar et al., 1994; Nair and Christianson, 1993).

Thus, differences in inhibition constants among the different CAs can be attributed to structural differences along the active sites among the different CAs. The more sensitive ones will have active sites more accessible to the inhibitors brought about by possibly a more exposed Zn$^{2+}$ or presence of additional residues in the active site of the enzyme that can likewise interact in the inhibitor thus bringing the inhibitor in closer contact with Zn$^{2+}$. For example, the active metal site of the β-CA enzymes is located in a shallower pocket than is seen in the α-CA family. Likewise, even among the same enzyme family, the accessibility of the zinc ion varies, as in the almost fully closed binding site cavity in Rv1284, a *Mycobacterium tuberculosis* β-CA, to the open binding site of *Methanobacterium thermoautotrophicum* β-CA (Suarez et al., 2005).

CAH7 is relatively more stable than the chloroplast β-CA CAH6. CAH7 was stable at a temperature range of 0-50°C where it has retained greater than 50% of its activity while CAH6 completely lost its activity at 50°C. Differences in thermal stability of enzymes can be attributed to differences in the strength and number of interactions that maintains the active conformation of a particular enzyme. In general, among enzymes from different organisms, the temperature where the organism thrives is usually correlated with the enzyme’s optimum temperature. For example, Cab (carbonic anhydrase β), the plant type β-carbonic anhydrase from
Methanobacterium thermoautotrophicum, the enzyme is stable up to 75°C and is the most thermostable carbonic anhydrase characterized as of this time (Smith and Ferry, 1999).

There was a significant difference between enzymatic activities of CAH7 purified under oxidizing and reducing conditions. Similar to higher plant chloroplast β-CAs, CAH7 was dependent on a reducing environment for maximum CA activity. CAs in pea and other C3 dicots are dependent on a reducing environment to retain catalytic activity (Hiltonen et al., 1998). On the other hand, CAs from E. coli (Guilloton et al., 1993), Coccomyxa sp. (Hiltonen et al., 1998), the C. reinhardtii β-CA, CAH6 (Mitra et al, 2004) and CAH8 (Chapter 5) are insensitive to oxidation.

Analysis of the amino acid sequence of CAH7 shows the presence of ten Cys residues. Except from the two conserved Cys residues that are involved in coordinating Zn$^{2+}$, none of these Cys residues are strictly conserved among the other β-CAs, thus this might imply that none of these Cys might be involved as catalytic residues. However, any of these Cys might be involved in disulfide bond formation and thus reducing conditions increased the catalytic activity of CAH7.

The expression of CAH7 transcript was observed to be approximately 1.4 -4.6 fold higher under low CO$_2$ relative to high CO$_2$ a range similar for CAH6 and CAH8. This difference can be considered insignificant if compared to the low inducible genes, CAH1 and CAH4 with 10000-fold and 3500-fold difference respectively in the amount of transcript under low CO$_2$ relative to high CO$_2$ conditions.

An attempt to elucidate the role of CAH7 using an RNA interference approach was unsuccessful. Although transformants contained the CAH7 inverted repeat as shown by PCR analysis, there was no reduction in the amount of CAH7 transcript and no difference in the phenotype between the transformants and WT D66 under high and low CO$_2$ conditions. The
inability of the RNAi mechanism to decrease the amount of CAH7 transcript may be the result of the inability of the small interfering RNAs (siRNAs) to bind to the endogenous CAH7.

The chloroplast stroma CAH6 is hypothesized to play an indirect role in the CCM by trapping CO₂ diffusing out of the pyrenoid (Mitra et al., 2004). The pyrenoid contains Rubisco in C. reinhardtii and CAH6 might play a role in the conversion of CO₂ to HCO₃⁻ thus increasing the HCO₃⁻ pool in the stroma and therefore retaining inorganic carbon within the chloroplast. The possibility that CO₂ may be elevated within the entire chloroplast rather than just the pyrenoid has been suggested (Badger, 2003). However, CO₂ can easily diffuse out of the membrane therefore in this regard it can be proposed that a rapid interconversion of CO₂ to HCO₃⁻ occurs in the entire chloroplast facilitated by CAH7 and thus CAH7 along with CAH6 supports the efficient accumulation of inorganic carbon.

A second proposed role for CAH7 is in lipid biosynthesis in the chloroplast. Biochemical (use of sulfonamides) and molecular (antisense strategy) inhibition of plastid CA reduces incorporation of lipids in cotton embryos, tobacco cell suspensions and tobacco leaves (Hoang and Chapman, 2002).

Two possible roles for chloroplast CAs have been presented by Hoang and Chapman (2002). First, CA conversion of CO₂ to HCO₃⁻ could reduce the rate of CO₂ diffusion out of plastids maintaining HCO₃⁻, the substrate for acetyl CoA carboxylase. The chloroplast CA indirectly aids in channeling carbon for lipid biosynthesis. A second role would be to modulate plastid pH. The chloroplastic spinach CA may buffer against transient pH changes in the stroma during photosynthesis (Jacobson et al., 1975), and possibly optimizing the rate of fatty acid synthesis by the fatty acid synthase complex.

The CAH7 in C. reinhardtii may play an important role in trapping HCO₃⁻ in the chloroplast for photosynthesis or other biosynthetic reactions in the chloroplast. Elucidation of
the roles of CAH7 and other CA isoforms will shed light on the physiological functions of this evolutionarily conserved enzyme.
CHAPTER 5
IDENTIFICATION AND CHARACTERIZATION
OF CHLAMYDOMONAS REINHARDTI PERIPLASMIC
β-CARBONIC ANHYDRASE CAH8

INTRODUCTION

Aquatic photosynthetic organisms such as the green alga, C. reinhardtii respond to low CO₂ conditions by inducing a carbon dioxide concentrating mechanism (CCM). The CCM is a mechanism which augments photosynthetic productivity in algal cells by increasing levels of inorganic carbon many times over the environmental concentration of carbon dioxide (Moroney, 2006). This mechanism involves active uptake of inorganic carbon into the cells mostly in the form of HCO₃⁻ and the conversion of HCO₃⁻ to CO₂ which is a substrate of Rubisco. The uncatalyzed interconversion between HCO₃⁻ and CO₂ is slow and therefore extracellular and intracellular carbonic anhydrases (CAs) are required for efficient utilization of inorganic carbon. In fact, the acclimation of C. reinhardtii to low CO₂ conditions has been correlated with increased levels of carbonic anhydrases. Carbonic anhydrases (carbonate dehydratases, carbonate hydrolyases; EC4.2.1.1) are zinc metalloenzymes that catalyze the interconversion of CO₂ and HCO₃⁻ (Khalifah, 1971).

For any photosynthetic cell, the efficient supply of inorganic species to the cell surface is the primary step in CO₂ capture. In aquatic algae, there is evidence that CA located external to the cell membrane plays a key role in providing CO₂ to the cell surface. For example in Prorocentrum micans, a dinoflagellate, the activity of a constitutive extracellular CA increases under conditions of inorganic carbon limitation (Nimer et al., 1999). In marine phytoplankton species, the development of extracellular CA activity is a response to very low concentrations of CO₂ (Nimer at al., 1997). There is no clear evidence for a role of an extracellular CA in higher
plants since CO2 is supplied to the cell surface via stomates. In addition, the cell wall environment being acidic (Badger, 2003) CO2 which can passively diffuse into the cell becomes the predominant form of inorganic carbon. The γ-CA, Cam from Methanobacterium thermophila thought to be located outside the cell increases during growth on acetate. Thus, Cam has been proposed to be required for a CH3CO2/H+ symport system or for efficient removal of cytoplasmically produced CO2 during growth on acetate (Smith and Ferry, 1999). C. reinhardtii has two periplasmic α-CAs (Fukuzawa et al., 1990; Fujiwara et al., 1990; Rawat and Moroney, 1991). These CAs are encoded by two structurally similar but differentially regulated genes, CAH1 and CAH2. CAH1 is the major periplasmic CA and its transcription is highly induced under low CO2 condition. In contrast, CAH2 is poorly expressed under low CO2 and very slightly upregulated under high CO2 (Fujiwara et al., 1990). CAH2 is only 1.4 kb bases away from the CAH1 gene (Fujiwara et al., 1990) and may be the result of a recent gene duplication and may have a poorly functioning promoter (Moroney et al., 2001).

The role of the periplasmic α-CA, CAH1 is to facilitate entry of carbon dioxide into the algal cell. At pH above 6.3, HCO3− is the predominant inorganic carbon species. This form of Ci, being an anion, cannot readily cross the plasma membrane (Gutknecht, et al., 1977). CAH1, one of the first α-CAs reported in a photosynthetic organism, converts HCO3− to CO2. Two lines of evidence have been presented for this physiological role of CAH1. First, membrane impermeant CA inhibitors have a strong inhibitory effect on photosynthetic CO2 fixation at high pHs where HCO3− predominates but a less pronounced effect at lower pHs, where most of the inorganic carbon is already in the form of carbon dioxide and activity of periplasmic CA is no longer required (Moroney et al., 1985). The above mentioned role and the observation that CAH1 is very strongly induced under limiting CO2 conditions, where CCM is operational (Fukuzawa et al., 1990) puts CAH1 as one of the few CCM components known to date.
There is a *C. reinhardtii* mutant lacking the periplasmic CAH1 that is able to grow normally (Van and Spalding, 1999) under low CO2 conditions and indicating that CAH1 is not required for optimal growth. The presence of the periplasmic CAH2 may functionally complement for the absence of CAH1 (Moroney et al., 2001). In addition to these two periplasmic CAs, this study reports the identification and characterization of another extracellular CA, CAH8. This study have shown that CAH8 is an active CA and possible functions have been presented. These proposed functions might provide a foundation for a more detailed characterization of the physiological function of CAH8. Likewise, the characterization of CAH8 can provide the basis for experimental studies of the function of this extracellular CA.

RESULTS

Identification of the *C. reinhardtii CAH8*

To find new β-CA genes, a word search using the JGI *Chlamydomonas* Database Version 2 was done to search for additional carbonic anhydrases (jgi-psf.org/chlre2/chlre2.home.html). The search indicated that scaffold 143 contained a carbonic anhydrase-like gene. Enough of the sequence of this gene was present to determine that it was likely to be a β-CA. This gene was therefore designated as *CAH8*. Only one expressed sequence tag (EST) was available for *CAH8* (Fig. 5.1) which might indicate that this CA is either not as highly expressed as the other *C. reinhardtii* CAs or it is not expressed under the conditions that the cDNA was made. Further analysis of the sole EST available for *CAH8* indicated that it is not a full length cDNA sequence and in comparison with the EST, the genewise model in scaffold 143 contains 2 more exons on the 5’ side. To establish the full length cDNA sequence of *CAH8*, forward primers were designed using exons 1 and 2 from the genewise model while reverse primers were designed from the sixth exon in the EST. PCR and sequencing analysis using the *C. reinhardtii* cDNA library have established that these putative exons were part of *CAH8*. Using a combination of
Figure 5.1. *CAH8* in Scaffold 143 of JGI *Chlamydomonas* Genome Browser Version 2. The sole expressed sequence tag (EST) available for *CAH8* is encircled.
sequencing of the cDNA library, genomic DNA and RLM-RACE, the complete sequence of
CAH8 was determined.

**Gene Structure of CAH8**

The CAH8 genomic sequence spans approximately 5570 bases with a cDNA sequence of 2649 bp (Fig. 5.2 and Fig. 5.3). The CAH8 gene consists of nine exons (Fig. 5.4). The exons range in size from 58 (exon 7) to 1483 bp (exon 9) and introns range in size from 140 (intron 5) to 702 (intron 1). The cDNA has a 255 nucleotide 5' untranslated region (UTR) in exon 1 as it contains a translation start site at nucleotide 256. Exon 9 contains the stop site which is at nucleotide 1317. CAH8 has an extremely long 3' UTR of 1392 bp. Likewise, the 3' UTR contains the most common putative polyadenylation signal TGTAA, which is located 1310 bp downstream from the stop codon and 19 bp upstream of the polyadenylation site. This polyadenylation signal is present in approximately 90% of the Chlamydomonas cDNA sequences reported to date (Silflow, 1998). Exons 2 to 7 encode the conserved β-CA domain. Comparison of the gene structures of five known C. reinhardtii β-CAs showed that the conserved Cys and His residues involved in ligand binding are usually found in exons 3 and 4.

**Homology Search and the CAH8 Protein**

The CAH8 gene contains an open reading frame consisting of 1002 bp encoding a 333 amino acid translation product. This protein has a calculated molecular mass of 35.8 kD and predicted isoelectric point of 5.71 (http://us.expasy.org/tools/pi_tool.html) (Fig. 5.5). There are 103 ORFs encoding β-CAs having significant similarities to the CAH8 amino acid sequence by BlastP searches of the non-redundant sequence database at the National Center for Biotechnology Information (NCBI). When the deduced amino acid sequence was compared with other CAs from other organisms, the most similar CA was found to be the plastid targeted β-CA of *Helicosporidium sp.* ex Simulium jonesii, an obligate parasite that evolved from photosynthetic
The genomic sequence is 5570 bp. The exon sequences are in uppercase and the intron sequences are in lower case. The start and stop sequences are highlighted in bold green and gray respectively. The polyadenylation signal GTTAA is underlined.
Figure 5.3. The Full Length cDNA Sequence of **CAH8**. The cDNA sequence is 2649 bp. The start and stop sequences are in bold green and red respectively. The putative ribosome binding site GAG is underlined. The polyadenylation signal TGTAA is underlined.

Figure 5.4. The Genomic Map of **CAH8**. The numbers within the parentheses denote the start and stop codon positions on the map in base pairs.
Figure 5.5. The Amino Acid Sequence of CAH8. This protein shows an open reading frame of 333 amino acids. The start and stop codons are in bold green and red respectively.
green alga (55% identity) (Fig. 5.6). CAH8 contains the two Cys, one His coordinating Zn$^{2+}$, characteristic of β-CAs. Based on X-ray crystal structure analysis done by Mitsuhashi et al. (2000) on the red alga, *Porphyridium purpureum*, CAH8 contains the two Cys, His, Asp found to coordinate Zn$^{2+}$ in *P. purpureum*. In addition, CAH8 contains 22 of the 23 amino acid residues they have identified as strictly conserved among β-CAs and contains all residues they have identified to be clustered on the concave surface of the active site clefts. Fourteen residues are clustered on the concave surface of the active site as described in Mitsuhashi et al. (2000). CAH8 shares 29% and 34% identities with other *C. reinhardtii* β-CAs, CAH4 and CAH6 respectively (Figure 5.7). *C. reinhardtii* CAH8 is most similar to *C. reinhardtii* CAH7 with 63% identity. Sequence alignment of CAH8 with other β-CAs in *C. reinhardtii* showed a C-terminal extension which is also observed in CAH7 (Fig. 5.7).

TMPred, a prediction program for transmembrane regions predicts a transmembrane domain (http://www.ch.embnet.org/software/TMPRED_form.html) at positions 254-276 close to the C-terminal domain (Fig. 5.8). SOPMA, a program for secondary structure predictions (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), was used to analyze the secondary structure of CAH7. A structural feature common to β-CAs is the domination of α-helix structure (Kannan et al., 1975; Eriksson and Liljas, 1993; Boriack-Sjodin et al., 1995; Kisker et al., 1996). An α-helix structure of 43.54% (Fig. 5.8) comprised the highest secondary structural motif based on SOPMA prediction.

Various protein prediction programs gave different results as to the localization of CAH8. For instance, PSORT strongly predicts CAH8 to be targeted to the chloroplast stroma and TargetP predicts it to have a chloroplast targeting signal. On the other hand Sosui (http://us.expasy.org/tools/), predicts it to be a soluble cytosolic protein. These prediction programs assign the protein in different cellular compartments and therefore we can not rely on
Figure 5.6. Amino Acid Sequence Alignment of CAH8 with other β-CAs. Highlighted are residues described to be clustered on the concave surface of the active site clefts as described by Mitsuhashi et al. (2000). Zinc ligands as determined in P. purpureum β-CA are marked by red asterisks.

The numbers correspond to (1) Chlamydomonas reinhardtii CAH7 (63%) (2) Chlamydomonas reinhardtii CAH8 (3) Helicosporidium sp. ex Simulium jonesii 55% (AY596510.1) (4) Saccharophagus degradans (52%) (NC_007912.1) (5) Magnetospirillum magnetotacticum MS-1 51% (NZ_AAP01003796.1) (6) Shewanella loihica PV-4 50% (NC_009092.1) (7) Coccomyxa 50% (U49976.1) (8) Aeromonas hydrophila subsp. hydrophila ATCC7966 49% (NC_008570.1) (9) Pseudomonas fluorescens Pf-01 (NC_007492.1)

Numbers in % are corresponding sequence similarities with CAH8. Numbers in parentheses are GenBank accession numbers.
Figure 5.7. Multiple Sequence Alignment of *C. reinhardtii* CAH8 with other *C. reinhardtii* β-CAs. CAH8 is most similar to CAH7.
Figure 5.8. Secondary Structure Prediction of CAH8. Alpha helix (Hh):145 is 43.54% Extended strand (Ee):53 is 15.92% Beta turn (Tt):24 is 7.21% Random coil (Cc):111 is 33.33% (SOPMA Prediction Program). CAH8’s 23 amino acid transmembrane helix domain as predicted by TMPred is highlighted in yellow.
prediction programs in assigning a final intracellular location to CAH8. Producing an antibody to CAH8 will aid in determining the location of CAH8 and might suggest the possible role of CAH8 in *C. reinhardtii*.

**Cloning of CAH8 in an Overexpression Vector**

The *CAH8* ORF was cloned to determine if CAH8 is an active CA, to be able to further characterize CAH8 in more detail and to raise an antibody against it. The antibody is needed to immunolocalize CAH8 in *C. reinhardtii*. The cDNA sequence corresponding to the open reading frame of CAH8 was overexpressed in *Escherichia coli* as a fusion to the C-terminus of the maltose-binding protein (MBP) using pMAL-c2x overexpression vector (Fig. 3.1 Chapter 3). *E. coli* cells harboring the recombinant construct were induced with 0.6 mM IPTG for 4 hrs at 37°C to optimally express the recombinant protein, MBP-CAH8. Equal amounts of proteins from the uninduced and induced cells were loaded in a 12% SDS-polyacrylamide gel. The overexpressed recombinant CAH8 was approximately 15% of the total *E. coli* cell protein (Fig. 5.9). The molecular mass of the overexpressed fusion protein migrated as an 80 KD fusion protein (Fig. 5.9).

**Purification and Characterization of the CAH8 Activity**

The overexpressed recombinant CAH8 protein was purified by affinity chromatography using amylose resin following the protocol in the New England Biolabs technical catalog (Fig. 5.10). Purified recombinant CAH8 was further concentrated by using a 100-KD cut-off centricon column. The CA activity in the sample at each step of purification was assayed to check the purity of the sample (Table 5.1). The recombinant CAH8 protein has a specific activity of 4.2 WAU•mg⁻¹. This calculation of specific activity was based on the total amount of recombinant protein in the sample. CA activity assays were done using the method of Wilbur and Anderson (Wilbur and Anderson, 1948). CA activity was not detected in the extracts from uninduced cells nor *E. coli* cells containing only the pMal vector.
Figure 5.9. A 12% SDS-Polyacrylamide Gel Showing Overexpression of Recombinant MBP-CAH8. Lane 1 represents prestained low molecular weight markers. Lane 2 and 3 represent 30µg of proteins from uninduced and induced E.coli cells, respectively.
Figure 5.10. Purification of MBP-CAH8. Samples from the purification steps were analyzed by SDS-PAGE. Lanes: (1) MW standards (2) crude extract (3) crude extract unbound (4) amylose eluate (5) centricon fraction
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Unit Activity (WAU/mL)</th>
<th>Specific Activity (U/mg)</th>
<th>Total Activity (WAU)</th>
<th>%Recovery</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude</td>
<td>303</td>
<td>1.8</td>
<td>0.2</td>
<td>60.6</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>amylose</td>
<td>15</td>
<td>11</td>
<td>3.6</td>
<td>54</td>
<td>89</td>
<td>18</td>
</tr>
<tr>
<td>centricon</td>
<td>5</td>
<td>86</td>
<td>4.2</td>
<td>21</td>
<td>35</td>
<td>21</td>
</tr>
</tbody>
</table>
The purified MBP-CAH8 was cleaved by the protease Factor Xa for 4 hours at 23°C to separate CAH8 from the MBP. Factor Xa cleaved the fusion protein to yield a 44 kD MBP and the 36 kD CAH8 protein. The purification and cleavage of the fusion protein was confirmed by performing SDS-PAGE (Fig. 5.11). The CAH8 protein band was cut from the gel to be used as an antigen for production of polyclonal CAH8 primary antibodies. To test the specificity of the CAH8 antibody, Factor Xa cleaved MBP-CAH8 were separated by 15% SDS-PAGE and probed with the CAH8 antibody (Fig. 5.12). The antibody did not react with the MBP from the induced *E. coli* cells containing only the pMal-c2x vector and western blot shows that the overexpressed CAH8 reacts specifically with the generated antibody.

**Expression Analysis of CAH8**

Transcript levels of CAH8 was measured under high and low CO2 conditions in order to determine if the expression of this carbonic anhydrase is affected by changes in CO2 levels. Semiquantitative RT-PCR using total RNA from wild type, D66 grown under high CO2 (5% CO2) and low CO2 (0.035%) demonstrated that CAH8 is constitutively expressed under both conditions and is upregulated at high CO2, an expression pattern similar to CAH2, a gene known to be slightly upregulated under high CO2 conditions (Fig. 5.13). Similarly, using a quantitative RT-PCR, CAH8 is shown to be 1.7 fold higher in the amount of message at high CO2 conditions. Although the difference is less than CAH2, which is 7.6 fold higher in the amount of message at high CO2 conditions (Fig. 5.14).

Western blot analysis using ammonium sulfate fractions from *C. reinhardtii* D66 crude extracts, high and low CO2 was performed to determine whether the overexpressed protein matched that of the *C. reinhardtii* endogenous enzyme and to determine if the protein is expressed in either high or low CO2 or under both conditions. Western blot analysis under denaturing conditions showed that similar to transcript expression, CAH8 protein is expressed under both high and low
Figure 5.11. SDS-PAGE Gel Showing Samples from Purification Steps and Factor Xa Digest. Protein samples were run on a 15% SDS-PAGE Gel. Lanes: (1) molecular weight markers (2) induced crude cell extract (3) amylose resin eluate (4) MBP-CAH8 cut with Factor Xa.

Figure 5.12. Western Blot Probed by the CAH8 Antibody. Lanes: (1) pMal-c2x vector induced, (2) blank, (3) and (4) 100µg purified overexpressed MBP-CAH8 cut with Factor Xa, (5) blank, (6) Pre-stained MW standards.
Figure 5.13. Qualitative Expression Analysis of CAH8.
The expression analysis of \textit{CAH8} and other known \textit{C. reinhardtii} carbonic anhydrases under high and low CO\textsubscript{2} conditions using semiquantitative RT-PCR.
Figure 5.14. Quantitative Expression Analysis of CAH8. The expression analysis under high and low CO₂ conditions using of CAH2 and CAH8, a carbonic anhydrase gene known to be upregulated under high CO₂ in C. reinhardtii. Error bars represent mean ± SE of three replicates.
CO₂ conditions (Fig. 5.15). The overexpressed CAH8 corresponded to a slightly larger sized band compared to the endogenous *C. reinhardtii* protein band. The size difference of approximately 6 kD between the apparent molecular weights of the overexpressed CAH8 and endogenous *C. reinhardtii* CAH8 can be attributed to a signal peptide possibly cleaved from CAH8.

**Immunolocalization of CAH8**

WT137 cells acclimated to high and low CO₂ conditions were used for immunolocalization of CAH8. Immunogold densities in different cell compartments are given in Table 5.2. *C. reinhardtii* cell sections were probed with either the CAH8 antibody or the preimmune serum and were observed under a transmission electron microscope (Figs. 5.16 and 5.17). Immunolocalization results demonstrated that CAH8 is located in the periplasmic space. Immunolocalization results also corroborates expression analysis results of Western blots and real-time PCR, wherein CAH8 is expressed under both high and low CO₂ conditions. It can also be observed that CAH8 is distributed at a relatively lower concentration when compared to the other periplasmic CA, the α-CA, CAH1 which is highly expressed under low CO₂ (Fig. 5.18). Likewise, it can be noted that CAH8 resides closer to the cell’s membrane compared to CAH1 which are localized farther away from the cell membrane.

**Characterization of CAH8 Activity**

The effects of known CA inhibitors, such as sulfonamides and anions on the CA activity recombinant CAH8 were determined. Table 5.3 shows the inhibition of recombinant CAH8 by ethoxyzolamide (EZ) and acetazolamide (AZ). Generally, all β-CAs are less sensitive to sulfonamide inhibition and are slightly more inhibited by the anions azide and cyanide than bovine CAII which is an α-CA (Johansson and Forsman, 1993). CAH8 is 10⁴-fold less sensitive to sulfonamide inhibition compared to the α-CA, bovine CAII and 10²-fold less sensitive than C.
Figure 5.15. Western Blotting Result for CAH8. Lanes: (1) 0-40% ammonium sulfate fraction precipitated from crude extract from *C. reinhardtii* D66 cells high CO₂, (2) 0-40% ammonium sulfate fraction precipitated from crude extract from *C. reinhardtii* D66 cells low CO₂, (3) 0-40% ammonium sulfate fraction precipitated from crude extract from *C. reinhardtii* D66 cells grown TAP as a positive control (4) 60-80% ammonium sulfate fraction precipitated from crude extract from *C. reinhardtii* D66 cells as a negative control, (5) and (6) MBP-CAH8 cut isolated from *E. coli*.
<table>
<thead>
<tr>
<th>Location</th>
<th>Area (µM²)</th>
<th>Immune</th>
<th>Preimmune</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside</td>
<td>3.09 ± 0.28</td>
<td>0.14 ± 0.14</td>
<td>0.04 ± 0.04</td>
<td>0.10 ± 0.11</td>
</tr>
<tr>
<td>Periplasm</td>
<td>1.14 ± 0.14</td>
<td>9.90 ± 1.54</td>
<td>0.63 ± 0.25</td>
<td>9.27 ± 1.09</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>8.17 ± 1.04</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.94 ± 0.10</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>7.58 ± 0.58</td>
<td>0.26 ± 0.08</td>
<td>0.05 ± 0.02</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>Pyrenoid</td>
<td>1.35 ± 0.24</td>
<td>0.94 ± 0.28</td>
<td>0.24 ± 0.20</td>
<td>0.70 ± 0.19</td>
</tr>
</tbody>
</table>

Immunogold densities in different cell compartments in sections were calculated by dividing the number of immunogold particles in a particular cell organelle with the area of that cell organelle. The data presented in the table is the average ± SEM of 12 cell sections. The cytoplasmic area was calculated by subtracting the total area of the chloroplast and nucleus from the cell area.
Figure 5.16. Immunogold Labeling of *C. reinhardtii* WT137 Cells Probed with CAH8 Antibody. CAH8 was localized to the periplasm. Western blotting has also shown that CAH8 is made under both high and low CO$_2$ conditions.
Figure 5.17. Immunogold Labeling of *C. reinhardtii* WT137 Cells Probed with CAH8 Pre-immune Serum.
Figure 5.18. Immunogold Labeling of *C. reinhardtii* WT137 Cells Probed with CAH1 Antibody. CAH1 was localized to the cell wall. CAH1 is highly expressed at low CO$_2$ conditions.
Figure 5.19. Immunogold Labeling of *C. reinhardtii* WT137 Cells Probed with CAH1 Pre-immune Serum.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$I_{50}$ Bovine CAII</th>
<th>$I_{50}$ CAH6</th>
<th>$I_{50}$ CAH8</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetazolamide</td>
<td>$1.4 \times 10^{-8}$</td>
<td>$2.0 \times 10^{-6}$</td>
<td>$1.7 \times 10^{-4}$</td>
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<tr>
<td>ethoxyzolamide</td>
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<td>$9.0 \times 10^{-6}$</td>
<td>$4.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>azide</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-5}$</td>
<td>$1.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>cyanide</td>
<td>$4.9 \times 10^{-5}$</td>
<td>$5.0 \times 10^{-6}$</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

| Inhibition constants from Mitra et al., 2004.

Table 5.3. The Inhibition Constants of the Bovine CAII, *C. reinhardtii* CAH6 and CAH8.
It can be observed that CAH8 exhibited a very low sensitivity to sulfonamides because its \( I_{50} \) value falls beyond the range of 2 µM -10 µM (Johansson and Forsman, 1993). In contrast to CAH6 which is more sensitive to azide and cyanide, 100 fold and 10 fold, respectively, CAH8 is almost as sensitive to these inhibitors as that of \( \alpha \)-CA, bovine CAII.

The optimum activity of CAH8 was at a temperature of 30°C. It was stable at a temperature range of 0-50°C where it has retained greater than 50% of its activity. Little activity (23%) was recovered when the enzyme was incubated for 15 min at 60°C (Fig. 5.20). Higher plant chloroplasts \( \beta \)-CAs are reported to be dependent on a reducing environment for maximum CA activity.

On the other hand, CAH8 activity under reducing conditions is not significantly different under oxidizing conditions (Fig. 5.21). The \( \beta \)-CA CAH6 activity is likewise not affected by either the presence or absence of a reducing agent.

**DISCUSSION**

*C. reinhardtii* genome sequence data facilitated identification of the eigth carbonic anhydrase (CA) gene in this organism. The 2649 bp cDNA encoding CAH8 was identified and characterized. It has an ORF encoding a protein of 333 amino acids, that showed similarity to at least 103 \( \beta \)-CA protein sequences in NCBI database. It contains the two Cys, one His zinc-coordinating ligands characteristic of known enzymatically active \( \beta \)-CAs.

Likewise, it has 22 of the 23 residues described to be strictly conserved among \( \beta \)-CAs (Mitsuhashi et al., 2000). It has closest sequence similarity (63%) to the *C. reinhardtii* \( \beta \)-CA, CAH7, which was described in the earlier chapter. Among other organisms, CAH8 has 55% similarity to the parasitic green alga, *Helicosporidium sp.* ex Similium jonesii \( \beta \)-CA. From these comparisons it is evident that CAH8 is a \( \beta \)-CA.
Figure 5.20. The Thermostability of CAH8 Activity. Recombinant CAH8 was incubated for 15 min at the indicated temperatures and cooled on ice and activity was determined at 4°C by the Wilbur-Anderson method. Activity is represented as a percentage of the activity of a sample maintained on ice throughout the experiment (4.16 WAU/mg). The percentage of CAH8 activity data for each temperature treatment are the averages of three replicates.
Figure 5.21. Effect of the Reducing Agent, β-mercaptoethanol on the Activity of the Recombinant MBP-CAH8. The recombinant protein was purified in either the presence or absence of β-mercaptoethanol and CA activity was determined at 4°C by the Wilbur-Anderson method. The CAH8 specific activity data for each treatment are the averages of three replicates.
On the basis of the observed coordination of the catalytic zinc ion, the structures of the β class of carbonic anhydrases fall into two distinct subclasses. The first subclass of β-CAs that coordinate Zn$^{2+}$ tetrahedrally with four protein-derived ligands is represented by the structures of orthologues from *P. purpureum*, *E. coli* and *M. tuberculosis* Rv3588c and *H. influenza*. The second subclass represented by *P. sativum*, *M. thermoautotrophicum*, *M. tuberculosis* Rv1284, the active site zinc ion is coordinated by one histidine and two cys residues with a fourth coordination site occupied by water or a substrate analogue (Cronk et al, 2006). In CAH8, the conserved aspartate may not necessarily belong to the first subclass since for example in the pea enzyme this aspartate was likewise conserved but is involved instead in a salt link with nearby arginine (Suarez et al., 2005). Since the second group of β-CAs is characterized by little or no CO$_2$ hydration activity at pH values less than 7.0 compared to the first group (Suarez et al., 2005) the determination of CAH8 activity at these pH values will then put CAH8 in either of these two subclasses of β-CAs.

It can be observed from sequence alignment comparisons of *C. reinhardtii* β-CAs that CAH8 as well as CAH7 have C-terminal extensions. TMPred, prediction program for transmembrane regions predicts a transmembrane domain at positions 254-276 which is located at C-terminal extension. This region of CAH8 might facilitate an association for (1) oligomerization (2) interaction with other proteins or (3) with a membrane region like the cell membrane.

Aside from the high degree of amino acid sequence similarity among β-CAs, the content of secondary structure elements was observed to be similar. For example, β-CAs from pea (Johansson and Forsman, 1993), spinach (Rowlett et al., 1994) and *Coccomyxa* (Hiltonen et al., 1998) were described to have dominance of α-helices. Similar to these β-CAs, CAH8 was predicted by SOPMA, a program for secondary structure prediction to contain 43.54% of α-
helices. The dominance of α-helices observed among β-CAs highlights one of the structural differences between the α-, β-, and γ-CAs with the α- and γ-CAs being composed mainly of β-sheet structures (Kannan et al., 1975, Eriksson et al., 1998, Eriksson and Liljas, 1993; Boriack-Sjodin et al., 1995; Kisker et al., 1996).

CAH8 was PCR amplified using a C. reinhardtii core library to generate a region containing the ORF of CAH8. This fragment was cloned into the overexpression vector pMal-c2x and expressed in E. coli to generate the fusion protein MBP-CAH8. This fusion protein was purified using an amylose affinity column. The recombinant protein was purified 21-fold to apparent homogeneity as indicated by an 80 kD single polypeptide band after SDS-PAGE.

The purified recombinant MBP-CAH8 has a specific activity of 4.2 WAU•mg\(^{-1}\) which is lower than the 400 WAU•mg\(^{-1}\) from the recently reported C. reinhardtii β-CA, CAH6 (Mitra et al, 2004). However, CAH8 activity is significantly higher than the 0.06 WAU•mg\(^{-1}\) specific activity determined by mass spectrophotometry reported for R. eutropha (Kusian et al, 2002). It should be emphasized that not all CAs are enzymes with high activities (Moroney et al., 2001) and activities were observed to vary even among CAs within an organism. There is at least a 1000 fold difference in specific activities between animal CAs, with CAII having the highest (2000-3000 WAU•mg\(^{-1}\)) and CAIII the lowest (1-5 WAU•mg\(^{-1}\)) (Khalifah,1971). Although the specific activity of the recombinant protein is relatively low this could not reflect the CA activity of the endogenous CAH8, since in vivo, other factors such pH, temperature, ions and/or association with proteins could affect the catalytic activity of an enzyme. There was no difference observed between the CA activities of the Factor Xa cleaved MBP-CAH8 fusion protein and the uncut fusion protein.

The CAH8 activity is inhibited by generally recognized CA inhibitors and although carbonic anhydrases are inhibited by the same types of compounds, inhibition constants vary
among individual enzymes. Similar to all β-CAs, CAH8 is less sensitive to sulfonamide inhibition than the α-CA bovine CAII. On the other hand, CAH8 is as sensitive to azide and cyanide as α-CA bovine CAII. In the human CAII, crystallographic studies have shown that inhibition by sulfonamides is brought about by the sulfonamide nitrogen ion binding to the zinc ion and thus replacing the hydroxide ion (Lindskog, 1997). Anions on the other hand such as azide and cyanide directly coordinate to the metal ion and displace the zinc bound water molecule (Kumar et al., 1994; Nair and Christianson, 1993). Thus, differences in inhibition constants among the different CAs can be attributed to structural differences along the active sites among the different CAs. The more sensitive ones will have active sites more accessible to the inhibitors brought about by possibly a more exposed Zn$^{2+}$ or presence of additional residues in the active site of the enzyme that can likewise interact in the inhibitor thus bringing the inhibitor in closer contact with Zn$^{2+}$.

CAH8 is relatively more stable than the chloroplast β-CA CAH6. It was stable at a temperature range of 0-50°C where it has retained greater than 50% of its activity while CAH6 completely lost its activity at 50°C. Differences in thermal stability of enzymes can be attributed to differences in the strength and number of interactions that maintains the active conformation of a particular enzyme. Likewise, among enzymes from different organisms, the temperature where the organism thrives is usually correlated with the enzyme’s optimum temperature. For example, in Methanobacterium thermoautotrophicum, the plant type β-carbonic anhydrase (Cab) is stable up to 75°C and is the most thermostable carbonic anhydrase characterized as of this time (Smith and Ferry, 1999).

There was no significant difference between enzymatic activities of CAH8 purified under oxidizing or reducing conditions. Similarly insensitive to oxidation are CAs from prokaryotes E. coli (Guilloton et al., 1993), Synechococcus sp. PCC 7942 (Price et al., 1993), Coccomyxa sp.
(Hiltonen et al., 1998), the *C. reinhardtii* β-CA, CAH6 (Mitra et al, 2004), whereas CAs in pea and other C3 dicots are dependent on a reducing environment to retain catalytic activity (Hiltonen et al., 1998). Analysis of the amino acid sequence of CAH8 shows the presence of ten Cys residues. Except for the two conserved Cys residues that are involved in coordinating Zn$^{2+}$, none of these Cys residues are strictly conserved among the other β-CAs, thus this might imply that none of these Cys might be involved as catalytic residues. In addition, these Cys might not be involved in disulfide bond formation and therefore neither oxidizing nor reducing conditions had an effect on the catalytic activity of CAH8.

The difference in CAH8 transcript level expression under high and low CO$_2$ conditions, was observed to be 1.7 fold higher under high CO$_2$ compared to low CO$_2$ conditions. This difference is low to be considered as significant. Likewise, this transcript level difference was not apparent at the protein level. However, similar to transcript expression, Western blot analysis under denaturing conditions showed that CAH8 is expressed under both high and low CO$_2$ conditions. The overexpressed CAH8 corresponded to a slightly lower sized band compared to the endogenous *C. reinhardtii* protein band which can be attributed to a signal peptide cleaved from the endogenous *C. reinhardtii* CAH8.

Immunolocalization has shown that CAH8 is located in the periplasmic space more towards the cell membrane when compared with the other periplasmic CA, CAH1. CAH1 is strongly induced at low CO$_2$ conditions and it has therefore been proposed as part of the CCM. The role of this CA is thought to accelerate the equilibrium between HCO$_3^-$ and CO$_2$ so that the latter can easily diffuse across the plasma membrane. Through the action of the periplasmic CA, the external CO$_2$ pool is replenished from HCO$_3^-$ as CO$_2$ enters the cell, a role that was thought to be especially important in high pH aquatic environments where HCO$_3^-$ predominates (Moroney, et al., 2001). The utilization of the impermeant CA inhibitor, acetazolamide (AZ)
showed a strong inhibition of photosynthesis in *C. reinhardtii* at high external pH, but did not inhibit photosynthesis at acidic external pH. In contrast, the membrane permeable CA inhibitor EZ inhibited photosynthesis at both low and high pH (Moroney et al., 1985). Their work clearly demonstrated that extracellular CAs are required for photosynthesis specifically for the conversion of $\text{HCO}_3^{-}$ to $\text{CO}_2$. With these, the extracellular $\beta$-CA, CAH8 might perform the same role as CAH1 and can be thought of as a means to provide for a more efficient $\text{CO}_2$ diffusion towards the cytosol since CAH8 is located closer to the membrane as compared to CAH1. On the other hand, owing to its location CAH8 might be needed for recycling any $\text{CO}_2$ leaking out of the cytosol.

The second possible role of CAH8 is the maintenance of a localized $\text{CO}_2$ gradient. CAH8 can be envisioned as a CA in the vicinity of a $\text{CO}_2$ uptake system. CAH8 will maintain a localized high $\text{CO}_2$ concentration on the extracellular side of the uptake system thus facilitating the movement of $\text{CO}_2$ molecules towards the cytosol where a localized low concentration of $\text{CO}_2$ is maintained via the action of a cytosolic CA that rapidly converts $\text{CO}_2$ from the extracellular side to $\text{HCO}_3^{-}$ inside the cytosol. This proposed role of CAH8 can be analogous to the role of the bicarbonate transport metabolon in human cells. A metabolon is a complex of proteins that allows metabolites to move rapidly from one active site to the other (Srere, 1987), such as the interaction between peripheral enzymes and membrane transport proteins that move the product of the enzyme. In the human body, bicarbonate transport proteins are closely associated functionally with carbonic anhydrases (Sterling et al., 2002). Cytosolic CA, CAII associates with a Cl$^-$/HCO$_3^-$ anion exchanger (AE1). CAII localizes HCO$_3^-$ production at the site close to the exchanger. This concentrates production of HCO$_3^-$ on one side of the membrane, a “push” effect. On the extracellular side of the membrane is CAIV that mediates the conversion of the transported HCO$_3^-$ to $\text{CO}_2$, providing the “pull” effect by minimization of the HCO$_3^-$ at the
transport side. This push-pull mechanism, established by having CA activities on both sides of the membrane, accelerates the bicarbonate transport through the anion exchanger (Sterling et al., 2002). Likewise, the same “push-pull” mechanism was reported for the extracellular CAIV-sodium/bicarbonate co-transporter (NBC1)-cytosolic CAII metabolon complex (Alvarez et al., 2003). Together with the previous findings with AE1, it was suggested that the interaction of bicarbonate transporters with intracellular and extracellular carbonic anhydrases to be a universal component of bicarbonate transport physiology, at least in animal system (Alvarez et al., 2003).

*C. reinhardtii* CAH8 possibly functions to increase the cell’s efficient uptake of CO₂ both under high and low CO₂ conditions.
CHAPTER 6

ISOLATION AND CHARACTERIZATION OF AN INSERTIONAL MUTANT OF CHLAMYDOMONAS REINHARDTII

INTRODUCTION

Aquatic photosynthetic organisms account for 50% of world’s photosynthesis (Falkowski, et al., 1998; Field et al., 1998). They grow in an environment that is often limiting for CO₂ (Raven, 1997b; Kaplan and Reinhold, 1999; Colman et al., 2002). In order to compensate for this nutrient limitation algae have developed a means of concentrating CO₂ at the site of Rubisco thereby increasing their photosynthetic capabilities (Badger et al., 1980). This process is known as the CO₂ concentrating mechanism (CCM). The CCM is a biological adaptation to low carbon dioxide concentrations in the environment. It is a variation of photosynthesis in which organisms raise the level of carbon dioxide in the cell many times over the environmental concentration of carbon dioxide (Moroney, 2006).

The green alga C. reinhardtii has an active CCM and this mechanism is operational only when the alga is grown under limiting CO₂ conditions. While a number of candidate proteins have been identified in C. reinhardtii only a few these of these proteins have been conclusively proven to be an essential part of the CCM. Thus it is clear that many more genes need to be discovered in order to fully understand how the CCM operates in eukaryotic algae.

One approach shown to be useful in identifying genes important to the acclimation to low CO₂ is tagged insertional mutagenesis. Previous insertional mutagenesis approaches involved transforming ammonia- or arginine-requiring mutants with the corresponding endogenous gene that complements the mutation. For example, the genes for the master regulatory element CCM1 (CIA5) was cloned through the use of the Nit1 insertional mutant, C16 (Fukuzawa et al., 1998; Fukuzawa et al., 2001) while the phosphoglycolate dehydrogenase mutant HCR89 was generated
by complementation of an *arg*-mutant (Van et al., 2001; Nakamura et al., 2005). However, the use of an endogenous gene for mutagenesis have made it more difficult to determine the location of the insertion.

Two useful tools have been exploited to make insertional mutagenesis a more powerful tool in identifying new genes that might be involved in acclimation to low CO$_2$ conditions (Colombo, et al., 2002). One is the use of a chimeric construct engineered by Saul Purton and colleagues. It has an antibiotic resistance gene (*Ble*R) using the coding region from *Streptoalloteichus hindustanus*. The Ble$^R$ protein binds to Zeocin and inhibits its DNA strand cleavage activity (www.invitrogen.com/content/sfs/manuals/zeocin_man.pdf). Zeocin is a glycopeptide antibiotic of the bleomycin family. In addition, the chimeric construct has the 5’ introns and 3’ regulatory regions from the *C. reinhardtii* RbcS2 gene (Stevens et al., 1996; Lumbreras et al, 1998). This construct expresses well in *C. reinhardtii* and provides an insert with a unique tag into the genome. The second tool was the electroporation method described by Shimogawara et al. (1998) that allows researchers to generate large number of primary transformants. The *Ble*R construct used along with this electroporation method presented a practical methodology to help identify genes required for growth under low CO$_2$ conditions. This approach has been used to demonstrate that cells defective in Rubisco activase grow poorly under low CO$_2$ conditions (Pollock, et al., 2003). Likewise, a *C. reinhardtii* insertional mutant with a *Ble*R insert in a novel gene, *HDH1* that encodes a protein with similarity to halo-acid dehalogenase-like hydrolases has been described (Adams et al., 2005).

This chapter describes another insertional mutant, *slc211*, generated by the above described insertional mutagenesis approach. This mutant grows normally under high CO$_2$ conditions but grows poorly in a low CO$_2$ environment, thus the gene disrupted by the insert has been designated as *CIA7*. This gene encodes a protein of unknown function which has high
similarity to conserved bacterial proteins. Although the specific function of this protein has not been determined this chapter presents data which suggests that the protein encoded by CIA7 facilitates growth of C. reinhardtii under low CO₂ conditions.

RESULTS

Screening of Ble<sup>R</sup> Insertional Mutants

In the insertional mutagenesis screen, C. reinhardtii strain D66 was transformed with linearized pSP124s DNA, which contains the modified Ble<sup>R</sup> gene (Lumbreras et al., 1998). Approximately 42,000 Ble<sup>R</sup> insertional mutants were generated and screened for a high CO₂ requiring phenotype (Colombo et al., 2002). Some of the selected transformants required high CO₂ for optimal growth and grew slowly in a low CO₂ atmosphere (Pollock et al., 2003). For this type of insertional mutagenesis to be successful studies the Ble<sup>R</sup> must be linked to the high CO₂ requiring phenotype. To test the mutant strain crossed with WT CC124<sup>−</sup>, and for linkage to be established all of the Zeocin resistant progenies of a cross should also require elevated CO₂ for growth.

Genetic crosses were performed to determine if the resistance to Zeocin was genetically linked to the mutation in several of these mutants. Previous studies have reported that approximately 30% (8 of 25) of the transformants demonstrated linkage (Pollock, 2003). Table 6.1 presents results of linkage analysis done on several insertional mutants. In this study, 4 out of 15 mutants tested (27%) showed a high CO₂ requiring phenotype along with Zeocin resistance.

Identifying the Genomic DNA Disrupted by the Insert

Inverse-PCR (i-PCR), adaptor-PCR and thermal asymmetric interlaced PCR (TAIL-PCR) have been suggested to be useful methods of obtaining flanking sequence information from the insertional mutants (Colombo et al., 2002). Table 6.2 lists several insertional mutants with their
<table>
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<td>Insertional Mutant</td>
<td>PCR method used to Determine Flanking Sequence</td>
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identified flanking sequences. The method of i-PCR was proven useful in identifying 3 flanking
sequences. The method of i-PCR (Gasch, et al., 1992) can take advantage of the unique
sequences provided by the $Ble^R$ gene which is originally from *Streptoalloteichus hindustanus*.
The multiple cloning site from the plasmid was retained in the plasmid used for transformation to
leave a number of restriction sites to use for i-PCR. The strategy was to digest genomic DNA
from the mutant with restriction enzymes that cut within the multiple cloning site but not in the
$Ble^R$. The hypothesis is that the next restriction site will lie outside of the insert of the flanking
DNA. The PCR primers were designed to anneal in the opposite directions in the original $Ble^R$
but after ligation, the primers will face each other and the flanking *C. reinhardtii* DNA will be
amplified (Fig. 6.1) (Colombo et al., 2002). When the method works the fragments amplify and
a nested PCR shows a difference in size consistent with the difference in location of the primers
used (Fig. 6.2).

The second method used to obtain flanking DNA is TAIL-PCR (Liu et al., 1995). However, in this study no flanking sequences were obtained using this method. The third method useful in obtaining flanking sequences is the adaptor-mediated PCR. In this method
 genomic DNA was digested with a blunt-end restriction enzyme followed by ligation with a
blunt-ended adaptor. PCR was performed using primers in the pSP124s vector and in the
adaptor. Two flanking sequences were obtained using the adaptor-mediated PCR (Table 6.2).
This method was useful to obtain flanking sequences for mutants that might contain
concatameric insertions of the vector.

**Identification of the slc211 Mutant**

One of the insertional mutants that showed linkage was *slc211* (*sick on low CO$_2$). The
mutant *slc211* belongs to the class of insertional mutants designated as Class II mutants. These
are mutants that can survive but have a reduced growth than the parent D66 at 30 ppm CO$_2$
Figure 6.1. Schematic Diagram of i-PCR. (Adapted from Colombo et al., 2002)

Figure 6.2. Part of pSP124s Map Used to Generate the Insertional Mutants. The map shows positions of primers used for i-PCR.
level. On the other hand mutants designated as Class I mutants could not grow at 30 ppm CO₂ levels. Figure 6.3 shows the growth characteristics of slc211 under different growth conditions. The mutant grew well on minimal media and elevated CO₂ (5% [v/v]) but did not grow as well as the wild type parent D66 under low CO₂ (30 ppm) conditions. Like the CCM deficient mutant, cia5 and the carbonic anhydrase (CAH3) mutant, cia3, slc211 showed a reduction in photoautotrophic growth rates under low CO₂ conditions compared to the parent strain D66 (Fig. 6.3). Thus we designated the gene as CIA7. This mutant also showed resistance to the antibiotic Zeocin, indicating that it had a BleR insert.

Genetic crosses were performed to determine if the resistance to Zeocin was genetically linked to the mutation in slc211. The progeny that resulted from at least 16 crosses between slc211 and CC124, which grows normally on low CO₂ concentrations, showed a 1:1 ratio in all tetrads examined and in all cases the Ble resistant cells exhibited a sick on low CO₂ phenotype (Fig. 6.4).

Likewise, using random spore analysis all of the 85 Ble resistant segregants exhibited the growth phenotype of slc211 which further supports that the pSP124’s insertion is the cause of the high CO₂ requiring phenotype. The genetic linkage means that the functional BleR insert was at or extremely close to the disrupted gene that leads to the poor growth phenotype.

**Identification of the CIA7 Gene by iPCR**

Genomic DNA from slc211 was digested with SacI. The DNA flanking the BleR insert in slc211 was cloned by using inverse PCR (i-PCR). The 2° and 3° i-PCR products were approximately 1.4 and 1.2 kb, respectively, the difference in the size of the nested PCR products being consistent with the distance between the nested primers used for i-PCR (Fig. 6.5). The resulting sequence from the i-PCR product from the 3’ side of the insert was used in a BLAST search against the *C. reinhardtii* genome, and the insertion site was shown to be located on
Figure 6.3. Growth Characteristics of *slc211* Under High and Low CO$_2$. *slc211* grows well under high CO$_2$ conditions but grows poorly under low CO$_2$ conditions. D66 is the parental wild type strain of *slc211*, *cia5* and *cia3* are CCM deficient mutants of *C. reinhardtii*.

Figure 6.4. Linkage Analysis of the Mutant *slc 211* Crossed with WT CC124$^+$. In the cross between *slc211* and CC124$^+$, which grows normally on low CO$_2$ concentration, the segregation of *Ble$^R$* resistance showed a ratio of 1:1 in all tetrads examined and in all cases the *Ble$^R$* cells showed a reduced growth under low CO$_2$ conditions. A. Growth on TAP + Zeocin plates  B. Growth on minimal media low CO$_2$  C. Growth on minimal media on high CO$_2$
Figure 6.5. i-PCR Results Using Ble$^R$ Specific Primers. SacI digested \textit{slc211} genomic DNA was ligated and used as template for iPCR. Lanes:
(1) 1$^0$ reaction using Ble 5-1 and 3-1; (2) 2$^0$ reaction using Ble 5-2 and 3-2; (3) 3$^0$ reaction using Ble 5-3 and 3-3; (4-6) 0 DNA 1$^0$, 2$^0$ and 3$^0$ reactions respectively.
scaffold 40 of the genome draft (version 3.0 of the C. reinhardtii genome, http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). However, there were no ESTs in this region of the scaffold suggesting that they are either genes with low expression or are genes expressed under conditions different for those used for EST identification and from those inducing expression of CIA7.

Eukaryotic Genemark was used to predict the exon-intron structure of CIA7 and to design primers for PCR and sequence analysis by using the 1000 bp on the left side and 1000 bp on the right side of the flanking sequence. The predicted exon-intron structure was helpful in elucidating the actual gene structure of CIA7. Eukaryotic Genemark predicted five exons while PCR amplification of cDNA showed that CIA7 actually has 4 exons (Figs. 6.6A and 6.6B). The BleR insert was in the 3’UTR in exon 4 of CIA7 (Fig. 6.6C). The opposite end of the insert was amplified using primers designed to complement the sequences of the BleR insert and CIA7. PCR analysis showed that there is at least no more than 15 base pair deletion upon insertion of the linearized pSP124s (Fig. 6.7).

**Sequence Analysis of CIA7**

The genomic sequence of CIA7 is approximately 2010 bp (Fig. 6.8), which is 60% GC rich. The C. reinhardtii genome is characterized to be GC-rich. The CIA7 coding sequence consists of three introns flanked by consensus spliced junctions. The 5’ end of CIA7 was established by 5’RLM-RACE while the 3’ end was established by PCR analysis of the C. reinhardtii core cDNA library and D66 genomic DNA. The CIA7 cDNA is approximately 1.2 kb. The sequence does not contain a canonical polyadenylation site, TGTAA, commonly found in C. reinhardtii nor TGTAG or TGTTA found in few of the C. reinhardtii polyadenylation sites.

The putative ORF consisting of 315 bp encodes a polypeptide of 104 amino acids (Fig. 6.9), with a calculated molecular weight of 11290 and theoretical pI of 10.20 using ProtParam.
A. Predicted *CIA7*

Exon 1                                          Exon 2               Exon 3                          Exon 4                                                   Exon 5

B. Wild-type

Exon 1                                          Exon 2               Exon 3                          Exon 4                                                   Exon 5

C. *cia7* in *slc211* showing position of the Ble\textsuperscript{R} insert

Exon 1                                          Exon 2               Exon 3                          Exon 4a                                                  Exon 4b

Ble insert

**Figure 6.6.** Exon-Intron Structures of *CIA7*. 
Figure 6.7. Deletion Check in slc211. A. Map of CIA7 with the Ble\textsuperscript{R} insert showing location of primers used for deletion check. B. PCR results showing that insertion of pSP124s did not cause a big deletion. Lanes:(1) slc211, (2) D66, (3) 0 DNA A: Rf1-Rr2, B: Rf1- Rr1, C: Lf1-Lr1, D: Lf2-Lr1, E: Ble3-3-Lr1
Figure 6.8. The Genomic Sequence of CIA7. The genomic sequence is 2010 bp. The exon sequences are in uppercase and the intron sequences are in lower case. The start and stop sequences are highlighted.
Figure 6.9. Predicted Amino Acid Sequence of CIA7. The (M) in bold text indicates potential translation start site and the stop codon is also indicated in bold.
Table 6.3 presents the amino acid composition of CIA7. CIA7 consists of a relatively high percentage of charged amino acids, 28.8%; arginine for example is 11.5%.

Using protein-protein BLAST and domain searches showed the presence of a conserved domain found in 91 hypothetical proteins from bacteria, and one from the marine algae, *Ostreococcus tauri* (http://www.ncbi.nlm.nih.gov/BLAST/). Figure 6.10 shows the sequence alignment of CIA7 with other bacterial protein sequences. Three are defined as zinc finger proteins and another homologous protein found in *Idiomarina loihensis*, with 78% identity to CIA7 was described to be an uncharacterized conserved metal-binding protein. Figure 6.11 shows the multiple sequence alignment of CIA7 with the proteins defined to have metal binding domains.

The similarity of CIA7 to the other conserved proteins starts after 49 amino acids (Fig. 6.11). The N-terminal extension present in CIA7 and in the unicellular marine alga *Ostreococcus tauri*, is possible to be a chloroplast targeting signal (Fig. 6.12). In addition, the prediction program ChloroP v1.1 predicts the first 35 amino acids of CIA7 to be a chloroplast leader sequence (http://www.expasy.org/tools/). Thus these suggest that CIA7 could be targeted to the chloroplast.

**Expression Analysis of CIA7**

To determine whether the insertion of *Ble*<sup>R</sup> resulted in the absence or a decrease in the CIA7 message, a qualitative PCR using primers designed in the coding region of the CIA7 was performed and results showed that there was no difference in the amount of the message between the WT D66 and the insertional mutant *slc211*. However, using primers designed to span the insert, no message was amplified in *slc211* which indicates that a longer abnormal message, thus unamplifiable by regular qualitative PCR is present in *slc211* (Fig. 6.13). Likewise, RT was
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Figure 6.10. Multiple Sequence Alignment of CIA7 with Bacterial Proteins.
A. Using CDART: Conserved Domain Architecture Retrieval Tool showed the presence of a conserved domain in CIA7 which is also present in other bacterial proteins. B. ClustalW alignment of CIA7 with bacterial proteins showing how well conserved the domain is in the protein sequence.
**Figure 6.11.** ClustalW Alignment of CIA7 with Bacterial Proteins Defined to have Metal Binding Domains.

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<tr>
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</tr>
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**Figure 6.12.** ClustalW Alignment of CIA7 with *Ostreococcus tauri* Protein Product.

The N-terminal extension highlighted in green present in CIA7 aligning with the N-terminal sequence of unicellular marine alga *Ostreococcus tauri* protein sequence is possible to be a chloroplast targeting signal.
Figure 6.13. Expression Analysis of CIA7 in WT D66 and slc211. A. RT-PCR to show difference between the message in WT D66 and the mutant slc211 using primers designed to span the insert in slc211. Lanes: (1) D66 High CO2, (2) D66 Low CO2, (3) slc211 High CO2, (4) slc211 Low CO2, (5) cia5, (6) 0 RNA (ST) Molecular weight standards. B. RT-PCR using primers that span the BleR insert and CIA7. Lanes: (1) D66 (2) slc211 (3) 0 DNA (a) using CIA7 gene specific primer and Ble 3-2 (b) using CIA7 gene specific primer and Ble 3-2 C. RT-PCR using primers designed in the coding region of CIA7. Lanes (1) D66 (2) slc211
done in cia5 to determine whether CIA7 is under the control of C. reinhardtii master regulator CIA5. The results showed that CIA7 is being expressed in cia5.

To further assess any quantitative difference between the amount of message in the mutant and WT which can not be determined by a qualitative RT-PCR, a real-time quantitative PCR was done. Results has shown that there was no significant difference between the amount of CIA7 message between slc211 and WT D66 (data not shown). In addition, in order to determine if CIA7 is expressed at a particular time after switch from high CO2 to low CO2 a time course analysis of the expression level of CIA7 at different time points was done. Results showed that CIA7 is constitutively expressed at high and low CO2 and at different time intervals tested (Fig. 6.14). Likewise, critical threshold (Ct) values show that CIA7 is a moderately expressed gene.

**Complementation of slc211**

To establish the role of CIA7 as a gene that facilitates growth on low CO2 an attempt to complement the sick on low CO2 phenotype of slc211 was done. Transforming the mutant with the wild-type gene should produce transformants whose phenotype will be almost similar to the wild-type. An attempt to complement slc211 with a cosmid was not performed because screening of the cosmid library did not amplify the 5’ end of CIA7, thus regulatory regions that might be required for the expression of CIA7 will be absent. Thus pSL18 vector that has been previously used that partially complemented the insertional mutant rca1 (Pollock et al., 2003) was used to clone the ORF plus the 3’UTR of CIA7 into the vector. The pSL18 vector has the constitutive PsaD promoter and terminator linked to a paromomycin resistance cassette (Fig. 6.15) (Depege et al., 2003). Cells of slc211 were transformed with the pSL18 vector containing the wild-type gene of CIA7. Paromomycin resistant colonies were screened for growth on low
Figure 6.14. Expression Level of CIA7 under High and Low CO₂ at Different Time Intervals. CIA7 is constitutively expressed. Controls using the constitutive gene CBLP were run to determine equal loading.

Figure 6.15. pSL18-CIA7 Construct. Linearized pSL18 containing the wild-type gene of CIA7 carrying the paromomycin resistance gene AphVIII used to transform slc211.
CO₂. Only few colonies, 2 out of approximately 1,000 colonies screened showed improved growth compared to slc211 (Fig. 6.16). Although only two transformants were obtained from the transformation an oxygen evolution was done to determine whether transformant designated G will have a higher photosynthesis rate compared to slc211. Results in Figure 6.17 showed that transformant G has an increased photosynthesis rate. Due to the very low number of transformants which showed improved growth under low CO₂, a second construct was made. In the second construct a PCR product was amplified from the genomic DNA of D66 in order to include CIA7’s intronic region. The aim of this experiment was to improve the expression of CIA7 and possibly increase the number of transformants which will show improved growth compared to slc211. Restriction enzyme digests and sequencing analysis of the two constructs were done to ensure that no mutation was introduced during PCR and in the process of cloning into the bacterium and thus only contracts that will generate the full length CIA7 protein were used to transform slc211. Several colonies, 1.2% (six out of 485) showed slight improved growth compared to slc211 under 35-40 ppm CO₂ in air (Fig. 6.18).

The colonies were further characterized by PCR amplification with primers that spans the BleR insert. The fact that the diagnostic WT 396 bp fragment that spans the region of the insert was restored in slc211 transformants demonstrated that these paromomycin resistant transformants contained the WT copy of CIA7. This region can not be amplified in slc211 because of the interruption of the BleR insert (Fig. 6.19).

**CIA7 RNAi Strains Have a Sick on Low CO₂ Phenotype**

To provide further evidence for the role of CIA7 as a gene that facilitates growth under low CO₂, the method of RNA interference (RNAi) was used. RNAi has been utilized to silence the expression of several genes in *C. reinhardtii* (Sineshchekov et al., 2002; Koblenz et al., 2003, Pollock et al., 2003; Rohr et al., 2004). Figure 6.20 shows the RNAi construct for CIA7. The
Figure 6.16. Growth Characteristics of \textit{slc211} Transformants Containing WT cDNA copy of \textit{CIA7}. Growth characteristics of D66, \textit{slc211}, \textit{slc211p} (progeny of a cross between CC124 and \textit{slc211}), CCM mutants \textit{cia3} and \textit{cia5} and two paromomycin resistant colonies that grew better than \textit{slc211}. A. Growth on TAP plus paromomycin  B. Growth on Minimal media low CO$_2$

Figure 6.17. Rates of Photosynthesis as a Function of the DIC Concentration for Air Grown D66, \textit{slc211} and Paromomycin Resistant Transformant G
Figure 6.18. Growth Characteristics of slc211 Transformants Containing the WT Genomic Cia7. Growth characteristics of D66, slc211, CCM mutant cia5 and paromomycin resistant colonies (T4-T6) that grew better in 35-40 ppm CO₂ in air than slc211 but not as well as the WT D66.

Figure 6.19. PCR Showing the Presence of the WT Gene in slc 211 Transformants. Lanes: (1-8) paromomycin resistant colonies T1-T8,(9) slc211, (10) D66, and (11) 0 DNA
Figure 6.20. **pSL72-CIA7IR Construct.** The genomic sequence of CIA7 and its complementary cDNA were cloned in pSL72. The vector pSL72 contained the paromomycin resistant gene *AphVIII.*
construct was made from pSL72 vector which used the PsaD promoter and terminator to express the *AphVIII* paromomycin resistance gene. An inverted repeat of the genomic region and cDNA of *CIA7* containing exons 1 to portion of exon 4 was cloned in the vector between the *AphVIII* and PsaD terminator.

PCR was done to check the presence of the insert in the bacterial clones that were ampicillin resistant. Figure 6.21 shows the restriction enzyme digests of pSL72-IR which is diagnostic for the presence of both the genomic and cDNA inserts in pSL72. Restriction enzyme digests and sequence analysis confirmed the presence of the IR in construct that was used for the transformation of WT D66.

The strain D66 was transformed with NotI linearized pSL72-*CIA7*IR and 204 transformants were selected in TAP media containing paromomycin. These transformants were then replica plated onto minimal media in high and low CO₂ conditions. Figure 6.22 shows the growth characteristics of selected paromomycin resistant transformants. Forty-three (21%) of the paromomycin resistant colonies grew slower than the parent strain in a low CO₂ environment but grew normally in high CO₂ environment. Similarly, it has been observed that the high CO₂ growth requirement became less over a period of months (Pollock, et al., 2003).

*CIA7* RNAi strains have decreased levels of *CIA7* mRNA

Several transformants that grew slower in low CO₂ were selected for further analysis. These transformants were first screened by PCR for the presence of the inverted repeat using primers that will amplify the region that spans the vector and *CIA7*-IR (Fig. 6.23). Among the strains showing the presence of the inverted repeat (Fig. 6.24), RNAi strains 3, 5 and 10 showed a sick on low CO₂ phenotype (Fig. 6.22), and also showed a decrease in the message using qualitative RT-PCR (Fig.6.25), on the other hand RNAi strain 4, although has the presence of the inverted repeat was similar to WT on low CO₂ and did not show a decrease in the *CIA7* message
Figure 6.21. Restriction Enzyme digests of psL72 with Genomic and cDNA, pSL72 with Genomic and pSL72 without an Insert.
A. Sma cut constructs and B. Fsp cut constructs
Figure 6.22. Growth Characteristics of RNAi Transformants. A. Minimal media low CO₂ (35-40 ppm), B. Minimal media air (360 ppm) and C. TAP plus paromomycin
Figure 6.23. Map Showing Primers Used to Check Presence of IR.

Figure 6.24. PCR Results to Check Presence of CI47-IR Among Paromomycin Resistant Transformants. Using A. primers psL72-f and 211RNAi-GR, B. primers 211RNAi-GF2 and RNAiR1 and C. primers RNAiF1 and psL18r.
Figure 6.25. Qualitative RT-PCR to Determine Expression Levels of CIA7 in WT D66 and RNAi strains. Total RNA isolated from WT D66, slc211 and RNAi strains grown in minimal media under high CO₂ and switched to low CO₂ for 12 hrs was used as template for Qualitative RT-PCR using A. CIA7 gene specific primers B. CBLP primers as loading control.
Quantitative real-time PCR was done to determine the difference in the amount of messages between D66, the insertional mutant slc211 and the selected RNAi strains. The difference between WT D66 and the insertional mutant slc211 was not significantly different. However, with the RNAi mutants there was a 2.6 fold difference in the amount of messages between D66 and 3-RNAi, 4.3 fold difference between D66 and 5-RNAi and a 1.5 fold difference between D66 and 10-RNAi (Fig. 6.26).

When grown on low CO₂ levels, growth curve analysis showed a significant difference in the growth rates among WT D66, slc211 and the RNAi transformants shown to have a decreased in CIA7 mRNA (Fig. 6.27). The RNAi-5 strain with the greatest fold difference in the amount of CIA7 message relative to the parent strain D66 also showed the slowest growth rate. A difference in growth rates between WT D66 and cia7 mutants was evident after 8 hrs on low CO₂ and greatest difference in growth among the strains was observed after 48 hrs on low CO₂.

DISCUSSION

This chapter characterizes the C. reinhardtii insertional mutant, slc211. While tetrad analysis of 15 other insertional mutants showed a low level of linkage of 27%, the sick on low CO₂ phenotype of slc211 was established to be linked to the Zeocin resistance. As of this time, the reason for this low level of linkage which was also observed using T-DNA tagging approaches in higher plants (Dent et al., 2001) is still not known.

Using i-PCR, the DNA flanking the pSP124s insert in slc211 was cloned and sequenced. The BLAST search done against the Chlamydomonas JGI Database ver3 gave a match to scaffold 40 and the gene disrupted by the insert was designated as CIA7. PCR and sequencing analysis of genomic DNA and core library cDNA have established the 2.0 kb genomic sequence and 1.2 kb cDNA sequence of CIA7. This gene consists of 4 exons and the BleRX insert in slc211 was in exon 4, at the 3’UTR.
Figure 6.26. Quantitative Expression Analysis of *CLA7* in WT D66, insertional mutant *slc211* and RNAi transformants RNAi-3, RNAi-5 and RNAi-10 using Quantitative Real-time PCR. Results are averages of three replicates. Controls using the constitutive gene *cblp* were run to determine equal loading.
Figure 6.27. Growth Curves of WT D66 and CIA7 Mutants Grown on Air. Cell count of RNAi transformants grown on liquid minimal media bubbling with air showed slower growth compared to WT D66.
Recent reports have viewed the 3′ UTR as a regulatory region that is essential for the appropriate expression of many genes (Shen et al., 2001). The $Ble^R$ insertion may affect mRNA transcription rate and/or stability. In the study, by Shen et al. (2001) using phosphorothioate-modified triplex forming oligonucleotides (TFO) targeted to the 3′UTR of the bcl-2-gene showed a significant down regulation of the expression of bcl-2-gene in Hela cells as demonstrated by Western blotting. TFO targeting to the 3′UTR resulted into a truncated mRNA lacking polyA tail which is very unstable and rapidly degraded in cells (Shen et al., 2001). In Fuyama type congenital muscular dystrophy (FCMD) a retro-transposal 3 Kb insertion of tandemly repeated sequences within the 3′UTR of the gene results in almost undetectable level of transcripts encoding the protein fukutin (Kobayashi et al., 1998). However, in slc211 qualitative and quantitative PCR has shown no significant difference in the amount of $CIA7$ message between the mutant and WT which implies that the $Ble^R$ insertion did not affect mRNA transcription rate and stability. Therefore the insertion of the $Ble^R$ did not affect $CIA7$ at the transcriptional level.

Another possible effect of the $Ble^R$ insertion is at the post-transcriptional level. The 3′UTR of mRNA is strongly implicated in post-transcriptional regulation of the gene expression in humans. It has been suggested that diseases arise from anomalies in this region (Conne, et al., 2000). The 3′UTR has been reported to possess cis-acting elements that bind trans-acting transport or regulatory proteins. The region of the 3′UTR disrupted by pSP124s in slc211, might play a role in the recruitment of these proteins. Thus, the $CIA7$ transcript in slc211 might have been retained in the nucleus and was not transported to the cytoplasm for protein translation. For example, mutated transcripts as in cyclic AMP-dependent protein kinase gene (DMPK) in myotonic dystrophy cells (DM), are retained within the nucleus possibly due to lack of recognition by an RNA binding protein which regulates splicing and transport. Mutated DMPK
transcripts caused by expanded number of trinucleotide (CTG) repeats in the 3’UTR of DMPK are defective in nucleocytoplasmic export of mutant DMPK transcripts (Korade-Mirnics, et al., 1998; Timchenko, et al., 1999).

Other possibilities of the effect of insertion in slc211 are (1) the inability of the region disrupted by the insert to recruit proteins either for translational activation or inactivation or (2) the region disrupted by the insert is by itself required for translational activation or inactivation. Regulatory nucleotide sequences in the 3’UTR of mRNAs which act as protein binding sites are very common. However, the molecular mechanisms by which they affect translational efficiency of mRNAs is poorly understood. There are trans-acting factors that bind to the 3’UTR which might regulate translation. The mRNA encoding the enzyme lipoxygenase (LOX) present in erythroid precursor cells are translationally silent. Translational silencing is due to the CU-rich repeated sequences in the 3’UTR (DICE, differentiation control element) that bind hnRNP K and E1 proteins (Ostareck et al., 1997). These proteins inhibit joining of the 60S ribosomal sub-unit to 40S initiation complexes (Ostareck, et al., 2001). Translational activation on the other hand is brought about by phosphorylating hnRNP K by a protein kinase (Ostareck-Lederer et al., 2002). In another example, in vitro experiments have shown that the 3’UTR of p53 mRNA contains an Alu-like element that can repress translation of synthetic p53 mRNA and heterologous transcripts in cell-free extracts (Fu et al., 1996). Barley yellow dwarf virus mRNA carries in its 3’UTR a translational enhancer which upregulates translation 30-100 fold (Wang, et al., 1997). Conversely, in ceruloplasmin (Cp) mRNA, a ribosomal protein L13a once phosphorylated and released from ribosomes bind to a so-called GAIT element in the 3’UTR of CpmRNA and inhibits translation (Mazumder et al., 2003). The insert in CIA7 might have resulted in translational silencing of CIA7 which can be directly involved as a protein that facilitates growth under low CO₂ conditions or be indirectly involved as an activator of a protein that facilitates
growth under low CO$_2$. On the contrary, the insert in $CIA7$ might have resulted in translational activation of $CIA7$ which can be an inhibitor of a protein that facilitates growth under low CO$_2$. The detection of the presence or absence of $CIA7$ in WT D66 and the mutant $slc211$ can answer which between the two possibilities is true for the effect of the $Ble^R$ insert in $CIA7$.

Although the complementation analysis showed that $slc211$ transformants contain the WT locus of $CIA7$, the low number of transformants and the slight improvement in growth of these transformants under low CO$_2$ conditions did not provide a strong evidence that the mutants were rescued by the introduction of the WT copy of $CIA7$. A possible explanation for these observations is positional effect. The chromosomal position of the gene affects its expression. In the case of $CIA7$, its expression might be regulated by the sequences flanking $CIA7$ or the recruitment of proteins by these sequences that enhances the expression of $CIA7$. Likewise even sequences thousands of bases away from $CIA7$ might serve as enhancer elements for gene expression. This is why transformation using the cosmid library will be helpful. However, the cosmid library available to us did not contain an intact $CIA7$ gene.

The use of an RNAi construct was effective in decreasing $CIA7$ message which resulted in RNAi strains, with the sick on low CO$_2$ phenotype similar to the insertional mutant $slc211$. The inverted repeat of $CIA7$ cloned into the vector was specific only for $CIA7$. Likewise, a relatively high percentage of transformants that were sick on low CO$_2$ were generated which makes it unlikely that a random insertional mutagenesis cause the decreased amount of $CIA7$ and the sick on low CO$_2$ phenotype. RNAi analysis suggest that $CIA7$ is a gene that facilitates growth under low CO$_2$ conditions.

Qualitative and quantitative RT-PCR show that in WT D66, $CIA7$ is constitutively expressed at both high and low CO$_2$ conditions. Likewise Ct values from quantitative PCR shows that $CIA7$ is a moderately expressed gene. $CIA7$ encodes a protein of 104 amino acids.
with a conserved domain found in 91 bacterial sequences and in the marine unicellular alga, *Ostreococcus tauri*. The majority (66 of 91) of these hypothetical proteins are small soluble proteins with 45-55 amino acids. The conserved domain has 4 conserved cysteine residues and contain sequences of the form Cys-X$_2$-Cys-X$_{19}$-Cys-X$_3$-Cys. Cysteine as well as histidine are residues identified to bind metal ions. Examples of short Cys-containing sequences that provide two ligands to single metal ions include: Cys-X$_2$-Cys: liver alcohol dehydrogenase (Zn), aspartate carbamoyltransferase (Zn), metallothionein (Zn,Cd), rubredoxin (Fe); Cys-X$_3$-Cys: metallothionein (Cd); Cys-X$_4$-Cys: aspartate carbamoyltransferase (Zn), ferredoxin (Fe). It is unlikely that CIA7 is a metal transporter as it is a small hydrophilic protein with no transmembrane domain. However it is still possible for CIA7 to function as a transporter if it is part of a complex with a protein that possesses a transmembrane domain.

CIA7 can be a candidate metallochaperone. Metallochaperones function in the trafficking and delivery of essential, yet potentially toxic, metal ions to distinct locations and particular proteins in eukaryotic cells (Rosenzweig, 2001). Most studied are the small, soluble cytoplasmic Cu chaperones in yeast. Cu in the form of Cu(I) is transported across the plasma membrane by the Cu transporter, Ctr1 and then transferred to the three currently known Cu chaperones--Atx1, Lys7, and Cox17. Each feeds Cu to a specific protein; Cox17 guides Cu to the mitochondria for insertion into cytochrome c oxidase (CCO), the terminal oxidase of the respiratory chain (Glerum et al., 1996); Lys7 targets Cu to CuZnSOD, a primary antioxidant enzyme in the cytosol (Culotta et al., 1997); and Atx1 directs Cu to a post-Golgi compartment, by way of Ccc2 a P-type adenosine triphosphatase (ATPase) transmembrane Cu transporter, for final insertion into Fet3, a multicopper oxidase essential for high-affinity iron uptake (Pufahl et al.,1997; Yuan et al., 1997). In the cyanobacteria, *Synechocystis* PC 6803, it has been established that copper is supplied to thylakoid proteins, such as plastocyanin and cytochrome c oxidase.
which is required for the electron transport system via two copper transporting ATPases and a metallochaperone intermediary. Although, the metal binding motif for yeast copper metallochaperones has been characterized as Met-X-Cys-X-Cys (X,any residue) (Pufahl et al., 1997) and CIA7 does not have this motif, CIA7 motif can be different. The difference can be attributed to CIA7 transporting a different metal ion and/or being from a different organism. CIA7 as well as a number of bacterial protein conserved domains, has the sequence motif Cys-X$_2$-Cys-X$_{10}$-Cys-X$_3$-Cys, has the presence of four conserved cysteines and its arrangement is suggestive of metal binding

This work provides evidence that CIA7 is a gene that facilitates growth under low CO$_2$ conditions. The protein encoded by CIA7 may play a potential role as either a domain of a metal transporter or as a metallochaperone. The deficiency of essential metal ions might have been aggravated under low CO$_2$ conditions and thus resulted in the inability of the mutants to grow optimally. The possibility of the conserved domain in CIA7 being a metal binding domain is of interest. Experiments which include determination of the metal content, the metal ion it binds, its sensitivity to chelating agents and site directed mutagenesis might answer whether it has a metal binding domain. Its localization might provide a clue as to its function. This protein is found in quite a number of organisms and the domain is highly conserved which implies that this protein performs essential function.
CHAPTER 7

CONCLUSIONS

As of this time *C. reinhardtii* has nine carbonic anhydrases, three α-CAs and six β-CAs. A list of known *C. reinhardtii* CAs to date are presented in Table 7.1. This dissertation has identified and characterized two new carbonic anhydrases, designated CAH7 and CAH8. CAH7 and CAH8 are presented in Chapters 4 and 5, respectively. The genes, *CAH7* and *CAH8* were identified using the *Chlamydomonas* JGI Database. Using molecular biology techniques the full length cDNA sequence for these genes were established.

The ORF of *CAH7* and *CAH8* was each cloned in the overexpression vector pMal-c2x as a fusion to the C-terminus of the maltose-binding protein (MBP). Both the recombinant CAH7 and recombinant CAH8 are active CAs. Using the Wilbur-Anderson activity assay, it was found that recombinant CAH7 has a specific activity of 3.1 WAU•mg⁻¹ while recombinant CAH8 has a specific activity of 4.2 WAU•mg⁻¹. Although these two CAs shared 63% amino acid sequence identity, they have different biochemical properties. Comparative analyses of characteristic properties of CAH7 and CAH8 activities are shown in Table 7.2.

The *CAH7* transcript is constitutively expressed under high and low CO₂ conditions. Preliminary results of immunolocalization studies have shown that CAH7 is localized in the chloroplast. Likewise, CAH7 is constitutively expressed under high and low CO₂ conditions at the protein level.

The next interesting yet challenging step is the determination of the role that CAH7 plays in *C. reinhardtii*. CAH7 might play an important role in trapping HCO₃⁻ in the chloroplast for efficient inorganic carbon assimilation. It might play a role in photosynthesis, by facilitating a rapid interconversion of CO₂ to HCO₃⁻ which occurs in the entire chloroplast and thus CAH7 along with CAH6 supports the efficient accumulation of inorganic carbon in the
Table 7.1. A Summary of Carbonic Anhydrases Known to Date in *C. reinhardtii*.

<table>
<thead>
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<th>Carbonic Anhydrases</th>
<th>Gene Family</th>
<th>Location</th>
<th>References</th>
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<tbody>
<tr>
<td>CAH1</td>
<td>α</td>
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<td>α</td>
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<td>α</td>
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<td>Karlsson et al., 1995</td>
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<td>β</td>
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<td>Eriksson et al, 1996</td>
</tr>
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<td>β</td>
<td>Chloroplast stroma</td>
<td>Mitra et al., 2004</td>
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<td>Chloroplast</td>
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<td>Chapter 5</td>
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<tr>
<td>CAH9</td>
<td>B</td>
<td>(possibly cytosolic)</td>
<td>Deb and Moroney*</td>
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*unpublished observation
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<th>Biochemical traits</th>
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<td>Specific activity</td>
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<td>4.2 WAU/mg</td>
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<tr>
<td>pI</td>
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<td>5.71</td>
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<td>Thermostability</td>
<td>Loses 50% of CA activity at 51.5 °C</td>
<td>Loses 50% of CA activity at 53.2 °C</td>
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<tr>
<td>Effect of SH-reducing</td>
<td>Increases CA activity by 69%</td>
<td>No effect on CA activity</td>
</tr>
<tr>
<td>activity</td>
<td></td>
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<tr>
<td>Sulfonamide inhibition</td>
<td>AZ $I_{50}$ 4.4 ± 0.2 x 10^{-5}M</td>
<td>AZ $I_{50}$ 1.7 ± 0.2 x 10^{-4}M</td>
</tr>
<tr>
<td></td>
<td>EZ $I_{50}$ 4.4 ± 0.6 x 10^{-4}M</td>
<td>EZ $I_{50}$ 4.1 ± 0.1 x 10^{-4}M</td>
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<tr>
<td>Azide inhibition</td>
<td>$I_{50}$ 3.2 ± 0.3 x 10^{-5}M</td>
<td>$I_{50}$ 1.9 ± 0.0 x 10^{-3}M</td>
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<tr>
<td>Cyanide inhibition</td>
<td>$I_{50}$ 6.1 ± 0.9 x 10^{-4}M</td>
<td>$I_{50}$ 1.5 ± 0.7 x 10^{-5}M</td>
</tr>
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</table>
chloroplast. On the other hand, CAH7 might be involved in other biosynthetic reactions in the chloroplast such as lipid biosynthesis.

The \textit{CAH8} transcript is also constitutively expressed under high and low CO$_2$ conditions. Likewise, CAH8 is constitutively expressed under high and low CO$_2$ conditions at the protein level. Results of immunolocalization studies have shown that CAH8 is localized in the periplasm. CAH8 might perform the same role as CAH1 and can be thought of as a means to provide for a more efficient CO$_2$ diffusion towards the cytosol since CAH8 is located closer to the membrane as compared to CAH1 as shown in Figures 5.16 and 5.18. It would be interesting to determine if there is any upregulation of \textit{CAH8} in the \textit{cah1} mutant.

A more interesting possible role of CAH8 is the maintainance of a localized CO$_2$ gradient. CAH8 can be envisioned as a CA in the vicinity of a CO$_2$ uptake system and along with CAH9, a possible cytosolic CA, they provide a “push- pull” mechanism to increase CO$_2$ transport efficiency. CAH8 will maintain a localized high CO$_2$ concentration on the extracellular side of the uptake system thus facilitating the movement of CO$_2$ molecules towards the cytosol where a localized low concentration of CO$_2$ is maintained via the action of a cytosolic CA that rapidly converts CO$_2$ from the extracellular side to HCO$_3^-$ inside the cytosol. At least in animal systems, it was suggested that the interaction of bicarbonate transporters with intracellular and extracellular carbonic anhydrases might be a universal component of bicarbonate transport physiology, (Alvarez et al., 2003). A protein-protein interaction experiment using either FRET (Fluorescence Resonance Energy Transfer) or yeast-two-hybrid assay can be useful to test this proposed role, an initial experiment could be to determine whether CAH8 interacts with LCI1, a candidate transporter which is likely to be targeted to the plasma membrane.

On the basis of the results of this dissertation, it was clearly established that \textit{C. reinhardtii} CAH7 and CAH8 are active $\beta$-carbonic anhydrases. However whether these CAs
directly play a role in the CCM still needs further investigation. Even though the attempt to determine the functional role of CAH7 by RNAi analysis using the pSL72 vector was unsuccessful, RNAi still seems to be the appropriate tool to elucidate the roles of these two CAs. The Maa vector presented in Appendix A might be able to provide a more direct screening of RNAi transformants since CAH6 RNAi transformants were generated using this vector. However, since CAs have a high turnover number and are known to be catalytically very efficient enzymes, there might be a need to underexpress a CA gene to a level of 99-100% in any RNAi-CA mutant to see a visible phenotypic difference between the wild type and mutant cells. Previous studies have reported that plants with even the lowest CA levels (2% of wild type levels) were not morphologically different from the wild type plants (Price et al., 1994).

Chapter 6 presented the isolation and characterization of a novel \textit{C. reinhardtii} mutant that requires high CO$_2$ to grow. Interestingly, the gene disrupted in the insertional mutant \textit{slc211} encodes a protein with high homology to bacterial proteins of unknown function. This gene which was designated \textit{CIA7} consists of 4 exons and the \textit{Ble$^R$} insert was in exon 4, in the 3’UTR. \textit{CIA7} is constitutively expressed at both high and low CO$_2$. \textit{CIA7} encodes a protein of 104 amino acids. The conserved domain has 4 conserved cysteine residues and its arrangement is suggestive of metal binding. The protein encoded by \textit{CIA7} may play a potential role as either a domain of a metal transporter or as a metallochaperone.

The use of an RNAi construct was effective in decreasing \textit{CIA7} message which resulted in RNAi strains, with the sick on low CO$_2$ phenotype similar to the insertional mutant \textit{slc211}. RNAi analysis suggests that \textit{CIA7} is a gene that facilitates growth under low CO$_2$ conditions. The deficiency of essential metal ions might have been aggravated under low CO$_2$ conditions and thus resulted in the inability of the mutants to grow optimally. The possibility of the conserved domain in \textit{CIA7} being a metal binding domain is of interest. Experiments which include
determination of the metal content, the metal ion it binds, its sensitivity to chelating agents and site directed mutagenesis might answer whether it has a metal binding domain.

The challenge for *C. reinhardtii* researchers remains to be the identification of CCM components since as of this time only CAH1 and CIA5 are the clearly identified essential components of the CCM.
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thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂.

**EMBO J.** 17:1208-1216.


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APPENDIX A

CAH 3 AND CAH6 RNAI CONSTRUCTS

In an attempt to elucidate the functions of the different *C. reinhardtii* β-CAs, a method to suppress the expression of the individual carbonic anhydrases was employed. An RNAi construct that was developed for direct selection of effective transgenic RNAi lines was adapted to be able to screen for RNAi lines showing a down regulation in the expression of a target carbonic anhydrase. The approach was the production of double stranded RNAs (dsRNA) by transcription from transgene that will generate a transcription unit consisting of two inverted repeats (IRs) separated by a spacer. This approach will generate a hairpin-loop shaped RNA (Rohr, et al., 2004).

RNAi constructs (pSL72-CA IR) specific for *CAH3* and *CAH6* using the pSL72 vector were generated (Figs. A.1 and A.2). The IR repeat has the genomic region and the its cDNA oriented in a reverse orientation. Several studies have reported that IR constructs containing introns appears to improve its effectiveness. RNAi transformants whose phenotypic defect is unknown need to be molecularly characterized. It is only after the reduction of the transcript levels of the gene interest will they be considered true RNAi transformants and thus be screened for the phenotype of interest. The MAA vector which contains the IR repeat of tryptophan synthase β-subunit (TSB) was expected to generate mutants defective in TSB.

In tryptophan producing organisms, TSB converts the indole analog 5-fluoroindole (5-FI) into toxic tryptophan analog 5-fluorotryptophan. Therefore, mutants defective in TSB will be resistant to 5-FI. The use of the MAA vector will allow for the direct selection of transgenic strains showing an RNAi-induced phenotype. The construct containing the MAA-CA tandem repeat was done by restriction enzyme digest of the CA-IR from the previous pSL72-CA IR
followed by ligation of the cut CA-IR into the MAA vector forming a tandem repeat of MAA and CA in the construct (Figs. A.4 and A.5). Preliminary results of the RNAi analysis for CAH3 and CAH6 are presented (Figs. A.6, A.7 and A.8).

**Figure A.1** Map of the Portion of psL72-CAH3IR Construct. Portion of Exon 4 to portion of Exon 6 genomic (1045 bp) was cloned in the vector using primers CAH3RNAi EcoRV-F and CAH3RNAi EcoRI-R (625 bp) followed by cloning of the reverse complement of the cDNA sequence of the genomic using primers CAH3RNAi MluI-F and CAH3RNAi Xba-R.

**Figure A.2.** Map of the Portion of psL72-CAH6IR Construct. Portion of Exon 1 to portion of Exon 3 genomic (746 bp) was cloned in the vector using primers CAH6RNAi EcoRV-F and CAH6RNAi EcoRI-R followed by cloning of the reverse complement of the cDNA sequence of the genomic using primers CAH6RNAi MluI-F and CAH6RNAi Xba-R (501 bp).
Figure A.3. Restriction Enzyme Digests to Determine the Right Clone Containing the Genomic and cDNA Inserts. Digests were performed with two different restriction enzymes on A (genomic + cDNA + vector), B (genomic + vector) and C (vector only).
Figure A.4. Map of the Portion of the MAA Vector. The MAA vector contains the inverted repeat of β-trptophan synthase’s 3’UTR, the inability of transformants to convert fluoroindole (FI) to the toxic form fluorotryptophan confers resistance of RNAi transformants to FI.

Figure A.5. Map of the Portion of MAA-CAH3 RNAi Vector. The CAH3 IR was cut from the pSL72-IR construct and was ligated to the MAA vector. A similar construct was also made using the CAH6 IR.
Figure A.6. Transformants Screened on TAP Media with 50 µM Fluoroindole (FI). CC124 was transformed with the linearized plasmid containing the inverted repeat (IR) of MAA (β tryptophan synthase) 3’UTR and CAH3 or CAH6 IR. Possible RNAi transformants confer resistance to FI.
Figure A.7. Quantitative Real-time PCR to Determine the Decrease in the Amount of Messages of *MAA* and *CAH6* in the Transformants. The cells were grown on minimal liquid media on high CO$_2$ and then switched to low CO$_2$, the cells were harvested 8 hrs after the switch.
Figure A.8. Growth Characteristics of *MAA-CAH6* RNAi Transformants. The transformants were grown in minimal media under low CO\textsubscript{2} (70-80 ppm) conditions. Encircled are transformants which showed a decrease in the amount of *MAA* and *CAH6* transcript levels. D66 is WT, *cia3* and *cia5* are CCM mutants.
## APPENDIX B

### LIST OF PRIMERS

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VITA

Ruby A. Ynalvez is married to Marcus Antonius Ynalvez, and they have two children—Ma. Leslie Ammabel and Marcus Amiel. Ruby, together with her family, came to the United States in August 2001 to pursue her doctoral degree at Louisiana State University.

She received her Bachelor of Science degree in Food Science and Technology, and her Master of Science degree in Biochemistry from the University of the Philippines at Los Baños. A Department of Science and Technology, Engineering Science and Education Program scholarship funded her masteral studies. Ruby was awarded Best Master of Science Thesis in 1997 by the University of the Philippines Los Baños Chapter of the Gamma Sigma Honor Society in Agriculture.

Prior to her graduate studies in the U.S., she was assistant professor at the University of the Philippines Los Baños, Institute of Chemistry. In 2004, she received a Louisiana Economic Development Fund Award, a Best Graduate Student Oral Presentation from the American Society of Plant Biology-Southern Section Meeting and a Daisy B. and William J. Luke Outstanding Teaching Assistant Award given by the Department of Biological Sciences, Louisiana State University. Ruby has accepted an assistant professor of cell biology position at Texas A&M International University in Laredo.