Molecular, genetic and physiological characterization of a Chlamydomonas reinhardtii insertional mutant

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MOLECULAR, GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF A CHLAMYDOMONAS REINHARDTII INSERTIONAL MUTANT

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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A.A., Las Positas Junior College 1989
B.S., California State University, Stanislaus, 1996
May, 2004
ACKNOWLEDGMENTS

I am grateful to Dr. James Moroney for his patience, faith, and guidance over my graduate career. I would also like to thank my committee: Dr. John Larkin, Dr. Thomas Moore and Dr. Sue Bartlett for their useful input and guidance.

I would like to thank Catherine Mason for her technical assistance and support, and camaraderie; Pat Arbour-Reily for her comments; Dr. Kevin Carman and Soraya Silva for their help with HPLC; Tracey Lavezzi for help with TLC; Baran Tural for his hard work on RT-PCR and northern blots; Ruby Ynalvez for her help with 5’ RACE; Dr. Steve Pollock, Dr. Aravind Somanchi, and Dr. Sergio Colombo for their input; and the Moroney lab’s excellent undergraduate workers: Elizabeth Streva, Ashley Godfrey, Kristen Lebleu, Scott Lato, Lacey Howard, Michael Mc Cormick for their hard work and patience in assisting me in my research.

I would like to thank my wife, Randi Adams, my parents, James and Marlys Adams, as well as my grandmother, Elenore Thor, for their unconditional love and support over the last few years.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Antheraxanthin</td>
</tr>
<tr>
<td>APX</td>
<td>Ascorbate Peroxidase</td>
</tr>
<tr>
<td>Asc</td>
<td>Ascorbate</td>
</tr>
<tr>
<td>CAB</td>
<td>Chlorophyll a/b binding protein</td>
</tr>
<tr>
<td>CAM</td>
<td>Crassulacean acid metabolism</td>
</tr>
<tr>
<td>CCM</td>
<td>Carbon Concentrating Mechanism</td>
</tr>
<tr>
<td>Chl a</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>Chl b</td>
<td>Chlorophyll b</td>
</tr>
<tr>
<td>C(_i)</td>
<td>Inorganic Carbon</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper Zinc Superoxide Dismutase</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>EB</td>
<td>Elution Buffer</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>iPCR</td>
<td>Inverse Polymerase Chain Reaction</td>
</tr>
<tr>
<td>LHC</td>
<td>Light harvesting complex</td>
</tr>
<tr>
<td>mAu</td>
<td>Maximum absorbance units</td>
</tr>
<tr>
<td>MDA</td>
<td>Monodehydroascorbate</td>
</tr>
<tr>
<td>MGDG</td>
<td>Monogalactosyldiacylglycerol</td>
</tr>
<tr>
<td>MIN</td>
<td>Minimum Media</td>
</tr>
<tr>
<td>NPQ</td>
<td>Non-Photochemical Quenching</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcribed PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>TAP</td>
<td>Tris-Acetate-Phosphate Media</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>V</td>
<td>Violaxanthin</td>
</tr>
<tr>
<td>VDE</td>
<td>Violaxanthin De-Epoxidase</td>
</tr>
<tr>
<td>YA</td>
<td>Yeast-Acetate Media</td>
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ABSTRACT

Photosynthetic microorganisms must acclimate to environmental conditions that may lead to photo-oxidative stress, such as low CO₂ environments or high light intensities. *Chlamydomonas reinhardtii*, a unicellular, green alga with a Carbon Concentrating Mechanism (CCM), effectively accumulates inorganic carbon (Cᵢ) to levels higher than external concentrations. The CCM concentrates inorganic carbon around Rubisco to increase the CO₂ fixation efficiency of *C. reinhardtii*. Photorespiration, the water-water cycle, the xanthophyll cycle, and the CCM are adaptations that also prevent the over-reduction of photosystems and thus photoinhibition by dissipating the energy from the absorption of excess photons. In an effort to dissect elements of the CCM, insertional mutants of *C. reinhardtii* were transformed using the $Ble^R$ cassette, selected for Zeocin resistance, and then screened for a “sick on low CO₂” phenotype. One insertional mutant selected was slc-230. This dissertation describes the molecular and the physiological characterization of slc-230.

slc-230 was shown to have a $Ble^R$ insert in the first exon of *Hdh1*, a novel, single copy gene that seems to be slightly upregulated under low CO₂ and whose predicted gene product has homology with open reading frames in archaebacteria. The *Hdh1* gene product has similarity to general phosphatases. Proteins in this family include phosphatases and epoxide hydrolases. In addition, *Hdh1* is predicted to be localized to the chloroplast or mitochondria in *C. reinhardtii*. It was found that a genomic copy of *Hdh1* can complement slc-230.

Physiological studies were conducted to determine the effects of the altered expression of *Hdh1* in slc-230. slc-230 exhibits a lower affinity for inorganic carbon
(slightly elevated $K_{0.5}$), a decreasing photosynthetic rate ($V_{max}$) over time, and a lower content of chlorophylls and quenching xanthophylls than wild-type. Some possible roles of $Hdh1$ are discussed.
CHAPTER 1
INTRODUCTION

Introduction

In an effort to identify elements of the carbon concentrating mechanism, 42,000 insertional mutants were generated using the BleR cassette and screened on low CO₂ for a Sick on Low CO₂ (slc) phenotype. In this context, “sick” or “sickly” meant that the insertional mutant grew more slowly and had a more bleached or yellowish appearance than the parental wild-type strain, D66, when grown on low CO₂ (100 ppm). A BleR insertional mutant, slc-230, was originally screened and selected as a slc mutant and found to have an insert in Hdh1, a novel, single copy gene. After linkage analysis, it was found that the slc-230 phenotype appeared sickly under high and low CO₂ indicative of a defect in photosynthesis or dealing with photooxidative stressors. One question that needed to be answered was; What do photooxidative stress and acclimation to low CO₂ have in common. To this end, and to introduce the systems possibly affected by the interruption of Hdh1, the following subjects are discussed in this introduction:

- Photosynthesis, specifically, electron transport in the light dependent reactions
- Photorespiration
- The water-water cycle.
- The xanthophyll cycle
- The Carbon Concentrating Mechanism or CCM

The detrimental effects of excess light are parallel to the effects of low CO₂ on the photosynthetic apparatus in that they both lead to over reduction. Excess light causes the
over reduction of photosystem II directly. When the light intensity exceeds the capacity of the photosynthetic apparatus, over reduction occurs due to the generation of singlet oxygen from oxygen reacting with triplet state chlorophylls. During acclimation from high CO$_2$ to low CO$_2$, the light independent reactions transiently cease using the ATP and NADPH produced by the light dependent reactions. This causes a “back-up” of reductive potential causing over reduction of the electron transport chain leading to the over reduction of the photosystems, and subsequently leading to triplet state chlorophylls that generate singlet oxygen.

**Photosynthesis**

Photosynthesis is responsible for the conversion of light energy to chemical energy and generally considered to be the source of energy for most life on earth. Briefly, light quanta are used to generate a three carbon sugar from inorganic carbon (C$_i$). The reactions responsible for forming the three carbon sugar are separated into the light-dependent reactions and the light-independent reactions. The light-dependent reactions use light energy to generate ATP and NADPH. The light-independent reactions generate the three carbon sugar from C$_i$ using the ATP and NADPH made during the light-dependent reactions.

**Electron Transfer in Light Dependent Reactions**

The light-dependent reactions occur in the thylakoid membrane. These reactions are powered by quanta absorbed by chlorophylls held together with CAB’s (chlorophyll a and b binding proteins) in antenna complexes. Once quanta are absorbed, an electron in the pigment is elevated to an excited state. Pigments other than the chlorophylls, referred to as “accessory pigments”, also participate in the harvesting of quanta at other wavelengths. One class of accessory pigments is the carotenoids such as $\alpha$-carotene, $\beta$-
carotene, lutein, and violaxanthin. Discrete protein complexes, reaction centers, accept the quanta from the antennae pigments and transfer their energy to an electron acceptor. The D1 and D2 subunits and the P$_{680}$ reaction center (containing pheophytin and quinone) located in Photosystem II (PSII) (Fig. 1.1) accept quanta and the P$_{680}$ goes into an excited state. Pheophytins are subsequently reduced by the P$_{680}$. The electron then reduces two plastiquinone molecules designated Q$_a$ and Q$_b$. Once the P$_{680}$ has reduced the pheophytin, the P$_{680}$ accepts electrons from a tyrosine residue (Y$_z$) in the D1 subunit. This electron is garnered from the splitting of two water molecules into four protons and a diatomic oxygen molecule at the oxygen evolving complex on the luminal side of the thylakoidal membrane. Reduced quinones then reduce the cytochrome b$_{6f}$ complex. Cytochrome b$_{6f}$ in turn reduces plastocyanin. Photosystem I, containing P$_{700}$ also absorbs light energy exciting chlorophylls. Excited electrons from the P$_{700}$ center reduce ferredoxin; which in turn generates NADPH from NADP$^+$ and H$^+$ on the stromal side of the thylakoidal membrane (Fig. 1.1). The reduced plastocyanin reduces the oxidized P$_{700}$. While both of these successive reductions are occurring, the gradient of protons generated from the original splitting of water at the manganese binding protein and by the Q cycle is being harnessed to generate ATP from ADP and P$_i$ by ATP synthases located in the thylakoid membrane (Buchanan et al., 2000).

**Light Independent Reactions**

The light-independent reactions take place in the stroma of the chloroplast and consist of thirteen enzymatic reactions that use nine ATP generated by the ATP synthase and six
**Figure 1.1** A scheme of electron and proton flow in photosynthesis. Mn, manganese binding proteins; PSII, photosystem II; D1, subunit D1; D2, subunit D2; PQ, plastiquinone; PQH2, plastiquinol; b$_{6}$f, Cytochrome b$_{6}$f; PC, plastocyanin; PSI, photosystem I; Fd, ferredoxin; Fnr, ferredoxin-NADP$^{+}$ reductase.
NADPH created from the reductive potential of the photosystems to reduce 3 CO₂ molecules to the level of carbohydrate. The initial fixation of CO₂ results in the generation of 3-phosphoglycerate from C₁ and is catalyzed by ribulose 1, 5 bisphosphate carboxylase/ oxygenase (Rubisco). This conversion of CO₂ to carbohydrate is called the C3 cycle (Buchanan et al., 2000). Consistent with the importance of its role and its slow turnover rate (approximately three times per second), Rubisco is the most abundant protein in the chloroplast and considered to be the most abundant protein on the planet. Rubisco is composed of eight large, catalytic subunits (56 kDa) coded by the chloroplastic genome and eight small, regulatory subunits (14 kDa) coded by the nuclear genome.

**Prevention of Photodamage**

Although light is essential for photosynthesis, photosynthetic organisms sometimes absorb photons in excess of what the photosystems can handle. In this way, light is potentially deleterious in that it can damage the photosynthetic apparatus by photoinhibition. When the light intensity exceeds the photosynthetic apparatus’ capacity, over reduction of PSII occurs due to the generation of singlet oxygen from oxygen reacting with triplet state chlorophylls. Over reduction of PS II causes damage to the D1 subunit so that the D1 subunit must be degraded and PSII must be reassembled (Buchanan et al., 2000). Likewise, the antenna protein (Lhcb) can be damaged (Zolla and Rinalducci, 2002). Singlet oxygen can damage the photosystems and can also generate peroxidated lipids in the membranes of the chloroplast. Additionally, diatomic oxygen can generate hydrogen peroxide.

Photosynthetic organisms have adapted to high light conditions by evolving various mechanisms for dissipating the excess light energy that is experienced in natural environments. In nature, about 80% of absorbed photon energy is used for CO₂ fixation at a light intensity of 100 mmol m⁻² s⁻¹; however, at full sunlight (2000 mmol m⁻² s⁻¹), it is
thought that only 10% of the energy absorbed is actually used for CO₂ fixation (Long et al., 1994) (Fig. 1.2). The remaining photon energy is dissipated through photorespiration (Asada, 1981), the water-water cycle, and/or down regulation of PSII triggered by the xanthophylls cycle (Horton et al., 1996). The relative roles of each of these mechanisms are presented in figure 1.2. Photorespiration, the water-water cycle, the xanthophyll cycle, and the carbon concentrating mechanism (CCM) are adaptations that prevent the over-reduction of photosystems and thus photoinhibition by dissipating the energy from the absorption of excess photons.

**Photorespiration**

As the name indicates, Rubisco can also function as an oxygenase. If oxygen is reacted with ribulose 1, 5 bisphosphate instead of carbon dioxide, one molecule of phosphoglycolate and one 3-phosphoglycerate are formed instead of two molecules of 3-phosphoglycerate. Phosphoglycolate goes through a process called photorespiration or the C2 cycle. To recycle the two carbon phosphoglycolate, it first is dephosphorylated and then shuttled out of the chloroplast as glycolate to the peroxisome where it is converted to glycine. Glycine is moved to the mitochondria where two molecules of glycine are converted to one serine generating ammonia and CO₂. Serine is moved back to the peroxisome and converted into hydroxypyruvate. Finally, the hydroxypyruvate is reduced to glycerate that is shuttled back to the chloroplast and phosphorylated to 3-phosphoglycerate to once again become part of the C3 cycle. The cost to the photosynthetic cell for recouping the oxygenated product is one carbon, lost as CO₂, and 2 ATP. Although this process seems wasteful to the point of detriment to the organism in the cost of fixed carbon and energy, one must realize that Rubisco evolved when there
Figure 1.2 The fate of photon energy. These pie graphs show the relative proportions of how photons are used in chloroplasts under increasing photon intensity. As photon intensity increases, a decreasing proportion of photons are used for CO₂ assimilation and increasing proportions of photons are dissipated through the water-water cycle, downregulation of PSII by the xanthophyll cycle, and photorespiration. These plots have been generated from K. Asada (1999).
was a relatively low concentration of oxygen and a relatively high concentration of carbon dioxide in the atmosphere. The C2 cycle helps prevent damage from oxygen by firstly forming glycolate and indirectly by preventing over-reduction of the photosystems through use of ATP and NADPH. Besides photorespiration, oxygen species are the key players in photooxidation and photoinhibition.

**The Water-Water Cycle**

The water-water cycle starts with the splitting of H₂O to protons and oxygen at PSII in the thylakoid lumen, singlet oxygen (O^•) is formed then combined with protons to form H₂O₂, then H₂O₂ is detoxified to water again. The foremost physiological role of the water-water cycle is the rapid, immediate scavenging of photoproduced O₂⁻ and H₂O₂ (Asada, 1999). The photoreducing site of O₂ was indicated to be PSI; and, in 1974, its primary product was identified to be super oxide anion radical (O₂⁻) (Asada et al., 1974). Therefore, H₂O₂ is photoproduced in thylakoids via the spontaneous disproportionation of O₂⁻, but not directly through the two electron reduction of O₂ (Asada, 1999). To deal with the O₂⁻ produced, superoxide dismutases (SOD) catalyze the disproportionation of superoxides by combining two superoxides with diatomic hydrogen to form hydrogen peroxide (Fig. 1.3) (Asada, 1999). This hydrogen peroxide is then scavenged by ascorbate peroxidases that use two ascorbates as electron donors to form two molecules of water and two molecules of monodehydroascorbate (MDA) (Fig. 1.3) (Asada, 1999). The MDA is then regenerated to ascorbate by MDA reductase (Fig. 1.3) (Asada, 1999).

Immunogold labeling of the chloroplastic CuZn-SOD indicates that at least 70% of the enzyme is located within 5 nm of the stroma thylakoids where the PSI complex is located (Ogawa et al., 1995). Simulations show that if the CuZn-SOD
Figure 1.3 Elimination of superoxide radicals (O$_2^•$). Superoxide dismutase (SOD) forms hydrogen peroxide from protons and superoxide radicals. Hydrogen peroxide is converted to water by ascorbate peroxidase (APX) that uses ascorbate (Asc) as electron donors to form 2 water and two monodehydroascorbate (MDA) which is regenerated to ascorbate by monodehydroascorbate reductase (MDAR). Hydrogen peroxide can also be broken down to water and diatomic oxygen by catalase (CAT) (adapted from Buchannan et al., 2000)
was uniformly distributed in the stroma, superoxide could diffuse up to 100 nm away from the point where it was produced, possibly damaging the chloroplast (Ogawa et al., 1995). By attachment to the thylakoid, the superoxide is scavenged within 5 nm of the surface. This location of the CuZn-SOD prevents the superoxide from interacting with Calvin-cycle enzymes and prevents its diffusion to the stroma (Asada, 1999). Tobacco transformed with anti-sense CuZn-SOD showed photobleaching and PSI inactivation under bright light (Ogawa et al., 1997).

The ascorbate-specific peroxidases (APX), are in chloroplasts at a content of 1 molecule per P$_{700}$ in two isoforms: thylakoid bound APX (tAPX) and a soluble stromal APX (sAPX) (Miyake et al., 1993). The tAPX, like the CuZn-SOD, also binds to the stroma thylakoids where the PSI complexes are located (Miyake and Asada, 1992).

**The Xanthophyll Cycle**

The mediated dissipation of excess light energy as heat in the light harvesting complexes of PSII by zeaxanthin and antheraxanthin is generally accepted as a system that dynamically modulates the energy transfer of PSII, thereby protecting it, and is highly conserved in higher plants (Baroli et al., 2003). The first relevant observation was that the concentration of violaxanthin could be reversibly decreased by light-dark treatments (Sapozhnikov et al., 1957). It was later demonstrated that the changes were due to cyclical, stoichiometric conversions among the species violaxanthin, antheraxanthin, and zeaxanthin in the xanthophyll pool (Yamamoto et al., 1962). The xanthophyll cycle is critical in the prevention of triplet chlorophyll species.

Violaxanthin de-epoxidase (VDE) is one of the key enzymes in the xanthophyll cycle that is localized to the thylakoid lumen and converts violaxanthin (V) to antheraxanthin (A) and then to the fully de-epoxidated zeaxanthin (Z) (Figure 1.4) in the
presence of an acidic lumen and ascorbate. The accumulation of antheraxanthin and zeaxanthin in addition to the transthylakoid pH gradient mediates non-radiative dissipation of light energy (non-photochemical quenching or NPQ) in the antennae. The ability to dissipate excess energy as heat prevents the chlorophylls and the D1 subunits in PSII from getting over-reduced and singlet oxygen from being formed. Purification of VDE from *Lettuca sativa* L. showed that the 43kDA polypeptide VDE had an affinity for the lipid monogalactosyl-diacylglyceride (MGDG), a major lipid found in thylakoid membranes (Rockholm and Yamamoto, 1996). Although VDE precipitation by MGDG appears to be fairly specific, low activity was recovered by the use of other lipids as well (Rockholm and Yamamoto, 1996). Also, VDE is sensitive to dithiothreitol indicating that it has at least one di-sulfide bond. (Rockholm and Yamamoto, 1996). It was found that purified violaxanthin made a poor substrate for VDE unless suspended with MGDG (Yamamoto *et al.*, 1974). VDE has also been cloned and expressed in *E. coli* and shown to have activity (Bugos and Yamamoto, 1996). It was also hypothesized that VDE might be developmentally expressed and this was shown to be so by growing tobacco in growth chamber conditions (300 μmol photons m⁻² s⁻¹) and field grown with variable light conditions with a maximum PFD of 2400 μmol photons m⁻² s⁻¹. Results suggested that the younger leaves had a larger xanthophyll pool and lower VDE levels whereas older leaves had a smaller xanthophyll pool and higher VDE levels. The size of the xanthophyll pool on a per chlorophyll basis in the full-grown (high light) plants remained higher than the growth chamber grown plants throughout the growth experiment (Bugos *et al.*, 1999).
Figure 1.4  Diagram of carotenoid biosynthesis from lycopene with highlighted xanthophyll cycle (in blue). ZE, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase
A mutant of VDE in *C. reinhardtii* was originally identified by video imaging of chlorophyll fluorescence quenching by Niyogi *et al.*, (1997). However, the actual gene for VDE has as yet to be identified as insertional mutagenesis using ARG7 was employed to cause the lesion. The mutant npq1 completely lacks both antheraxanthin and zeaxanthin implying that there was a defect in VDE and that this defect was not fatal in excess light (Niyogi *et al.*, 1997). The lor1 mutation prevents constitutive accumulation of lutein and its derivative loraxanthin which does not affect the xanthophyll cycle, but does make the mutant partially defective in non-photochemical quenching and is non-fatal (Baroli and Niyogi, 2000). However, recently, the double mutant npq1-lor1 (which lacks zeaxanthin and lutein) in *C. reinhardtii* was found to be fatal, presumably due to build up of reactive oxygen species (ROS) (Baroli *et al.*, 2004). The mutant npq2 was presumed to have a defect in zeaxanthin epoxidase (ZE); although this mutant does not have a completely functional xanthophyll cycle, it is still protected against photooxidative stress due to the presence of zeaxanthin (Baroli *et al.*, 2003). In January of 2003 a sequence for the *C. reinhardtii* ZE was published. However, even with the advent of a searchable *C. reinhardtii* genome and EST databases, the sequence for an algal VDE has as yet to be identified either because it has as yet to be posted on the genome database (which is in its second draft) or it has a sequence that is not homologous to the VDE’s of higher plants.

**The Xanthophyll Cycle and Nitrogen Starvation**

In higher plants, the xanthophyll cycle is also in operation under nitrogen starvation. Under nitrogen starvation the amount of excess light that a plant absorbs can increase due to the resultant decrease in the capacity of photosynthesis. Numerous studies have shown that nitrogen starved plants exhibit decreases in photosynthetic
capacity and quantum yield of photosynthesis when compared to nitrogen replete controls (Terashima and Evans, 1988). Terashima and Evans found that nitrogen deficiency does not alter the properties of spinach thylakoid membranes, but the amounts of thylakoid proteins per chloroplast are decreased (Terashima and Evans, 1988). Studies also showed that under nitrogen starvation there are decreases in chlorophyll and Rubisco content, and the ratio of chlorophyll to Rubisco decreases (Evans and Terashima, 1987). The key effect of nitrogen starvation is a lowering of the capacity for carbon assimilation due to the limitation of synthesis of the Calvin cycle enzymes (Terashima and Evans, 1988). This lowered capacity for carbon assimilation means that the plant under nitrogen starvation is more sensitive to photodamage.

Verhoeven, et al., (1997) showed that in nitrogen starved spinach (Spinacia oleracea L. cv Nobel) the photosynthetic rate, as measured indirectly by oxygen evolution, was approximately half of that of the nitrogen replete controls at 2000 μmol photons m⁻² s⁻¹ photo flux density (PFD). Also, an increase in quenching xanthophylls at 500 and 900 μmol photons m⁻² s⁻¹ PFD was found in nitrogen starved spinach. At 500 PFD, there was approximately two times the amount of quenching xanthophylls as the nitrogen replete controls; and, at 900 PFD, there was approximately three times the amount of quenching xanthophylls as the nitrogen replete controls. The amount of quenching xanthophylls in nitrogen replete spinach was not statistically different (Verhoeven et al., 1997). It was found in nitrogen replete controls under 900 PFD, μmol m⁻² s⁻¹ the percent of light used was 53% for photosynthesis; and, 36.1% of the light energy was dissipated while nitrogen starved spinach used 23.1% for photosynthesis and dissipated 63.7%. Under the same conditions, the xanthophyll pool contained 18% quenching xanthophylls in nitrogen replete spinach while in nitrogen starved spinach the
quenching xanthophylls comprised 65% of the xanthophyll pool. Lastly, they showed that there was 66% as much chlorophyll per leaf area in nitrogen starved spinach at 500 μmol photons m⁻² s⁻¹, and about one twelfth at 900 μmol photons m⁻² s⁻¹ as their nitrogen replete controls (Verhoeven et al., 1997). Verhoeven et al., (1997) showed, that under nitrogen starvation, there are more quenching xanthophylls in the xanthophylls pool and more light is dissipated as heat.

Likewise, algal productivity is often limited by nitrogen (Dugdale, 1967). When growth of algae is limited by nitrogen availability, photosynthetic light harvesting, energy transduction, and C fixation are compromised (Herzig and Falkowski, 1989). The interactions between photosynthesis and nitrogen metabolism are particularly evident in nitrogen stressed algae where nitrogen enrichment has been observed to induce a transient suppression of photosynthetic ¹⁴C fixation and oxygen evolution rates (Turpin et al., 1997). Nitrogen starvation arises when algal cells are deprived of all external nitrogen sources. Under nitrogen starvation, an unbalanced, continuously increasing, nitrogen stress results (Young and Beardall, 2003). It was found in Dunaliella tertiolecta (Butcher) batch cultures that cellular Chl a quickly declined during starvation from 1.25 pg cell⁻¹ to 0.5 pg cell⁻¹ in 30 hours and that after 20 hours of nitrogen deprivation there was a sharp increase in the affinity for Cᵢ (uptake rate expressed as nmol hr⁻¹ (10⁵ cells)⁻¹). Young and Beardall (2003) showed that after starvation, addition of nitrate (NO₃⁻) quickly caused a drop in fluorescence emission that was mediated by non-photochemical quenching. This drop in fluorescence emission was not seen with the control (distilled water). Additionally, oxygen evolution rates initially increased after the addition of nitrate to nitrogen starved cells (Young and Beardall, 2003). They also had a
higher affinity for nitrate (0.5 mM than for ammonium (NH$_4^+$) 10 mM by k$_{0.5}$ determined by mM nitrogen source vs fluorescence (Young and Beardall, 2003).

**Carbon Concentrating Mechanisms (CCM’s)**

Because Rubisco is a relatively slow enzyme which turns over only a few times per second (compared to carbonic anhydrase which turns over a thousand times per second) and will react with either CO$_2$ or O$_2$. As a result, higher plants, algae, and cyanobacteria have evolved means of making Rubisco more productive in response to the increase of O$_2$ and decrease of CO$_2$ in the atmosphere. These adaptations are used to concentrate CO$_2$ for Rubisco. This favors the carboxylase reaction and decreases the detrimental oxygenase reaction. CCM’s prevent loss of energy and carbon caused by photorespiration and can indirectly prevent the photosystems from being over-reduced.

**CCM’s in Higher Plants**

Higher plants have separately evolved systems that increase CO$_2$ fixation to reduce the loss of energy and carbon caused by photorespiration and still prevent the photosystems from getting over-reduced. Because CO$_2$ is a non-polar molecule it can pass easily through membrane enclosed compartments. C4 plants, such as sugarcane and maize, separate the two sets of reactions spatially, by converting CO$_2$ to HCO$_3^-$. And then PEP carboxylase condenses the bicarbonate with phosphoenolpyruvate to form oxaloacetate (Buchanan et al., 2000). Oxaloacetate is then turned into malate by NADP$^+$-malate dehydrogenase or can be reacted with glutamate to form aspartate by aspartate aminotransferase. Malate and aspartate are transported as charged molecules, which will not pass easily through compartmental membranes, through the plasmodesmata to the bundle sheath cells, which have suberized membranes to aid in keeping CO$_2$ from diffusing out. Here the malate is decarboxylated by NADP$^+$-malic enzyme. Or, in the
case of aspartate, converted back to oxaloacetate and decarboxylated. CO₂ is formed at the site of Rubisco thus locally raising the concentration of CO₂ around Rubisco making it more efficient.

Plants such as Ananas sativus (pineapple), Crassula and Agave have CAM (Crassulacean Acid Metabolism) photosynthesis. Here, the CO₂ is fixed into oxaloacetate by PEP carboxylase then reduced by malate dehydrogenase to form malate at night and stored in the vacuole (Buchanan et al., 2000). The Calvin cycle occurs normally during the day with the stored malate decarboxylated at the site of Rubisco. This type of photosynthesis has the added benefit of limiting transpiration as the plants’ stomata are closed during the day.

**The CCM in Chlamydomonas reinhardtii**

Aquatic microalgae not only have the detriment of an inefficient system to fix CO₂ they additionally need to overcome the potential limitation caused by the slow diffusion of CO₂. CO₂ diffusion is ten thousand times slower into an aqueous environment versus simple diffusion through a gaseous medium. Most aquatic microalgae have carbon concentrating mechanisms (CCM’s) to acclimate to low CO₂ concentrations (Moroney and Somanchi, 1999). The requirements of CCM’s in microalgae include a mechanism to transport inorganic carbon species against a concentration gradient and the rapid inter-conversion of CO₂ and HCO₃⁻ inside and outside of the cell catalyzed by carbonic anhydrases (Badger, 1987).

As Chlamydomonas reinhardtii is easy to culture and maintain, will grow on an inorganic or organic carbon source, has means for nuclear and chlorplastic transformation, has a CCM, and, more recently, has a sequenced genome
Figure 1.5 Diagram of the induced *C. reinhardtii* CCM. Bicarbonate (HCO$_3^-$) is moved to Rubisco then converted to CO$_2$. ctCA- chloroplast carbonic anhydrase. From Moroney and Somanchi, 1999.
(http://genome.jgi-psf.org/chlre2/chlre2.home.html), it is a choice model organism to study CCM’s (Van Winkle-Swift, 1992; Lefebvre and Silflow, 1999). Figure 1.5 shows the current model of the CCM in *C. reinhardtii* cells grown in a low CO₂ environment (Moroney and Somanchi, 1999). When *C. reinhardtii* is grown in a high CO₂ environment its ability to actively accumulate Cᵢ is repressed; however, when cells are switched to a low CO₂ environment they induce their CCM. By increasing the abundance of several proteins, they attain a high affinity for Cᵢ.

**Components of the *C. reinhardtii* CCM**

The most studied component of the CCM in *C. reinhardtii* is the extracellular carbonic anhydrase Cah1, an α-carbonic anhydrase that is encoded by the gene Cah1 (Fukuzawa et al., 1990). Cah1 catalyzes the reversible dehydration of HCO₃⁻ to CO₂. In an alkaline environment, CO₂ comprises a small proportion of the total Cᵢ. Thus, Cah1 quickly equilibrates the HCO₃⁻ and CO₂ to supply the cell with a continuous source of CO₂ in an alkaline environment.

*Cah3* encodes a thylakoid-bound α-carbonic anhydrase that is required for growth in a low CO₂ environment (Karlsson et al., 1998). The cia3 mutant (Moroney et al., 1986) does not grow at air levels of CO₂ and requires acetate or high CO₂ for optimal growth. cia3 retains the ability to concentrate Cᵢ intracellularly, it actually over-accumulates Cᵢ, but it is not able to effectively concentrate CO₂ in the vicinity of Rubisco (Moroney et al., 1986). It is thought that Cah3 is the carbonic anhydrase that helps convert built up HCO₃⁻ to CO₂ for use by Rubisco.

*C. reinhardtii* cells that are defective for Cia5 do not acclimate to a low CO₂ environment (Moroney et al., 1989). The cia5 mutant lacks the induction of Cᵢ transport,
induction of *Cah1, Mcal* and *Mca2, Ccp1* and *Ccp2, Lci1*, or any of the unidentified low CO$_2$ induced polypeptides. Additionally, the upregulation of phosphoglycolate phosphatase and glycolate dehydrogenase, and down-regulation of Rubisco biosynthesis fail to occur in the *cia5* mutant (Moroney *et al.*, 1989; Spalding *et al.*, 1991; Marek and Spalding, 1991; Burow *et al.*, 1996). Since the generation of *cia5*, the gene responsible for the mutant phenotype has been cloned (Fukuzawa *et al.*, 2001; Xiang *et al.*, 2001).

*Cia5* encodes a protein of 76 KDa that contains a putative zinc-finger motif that is crucial to its regulatory function and this motif suggests a role as a transcription factor (Xiang *et al.*, 2001). *Cia5* appears to be constitutively expressed (Xiang *et al.*, 2001). When mutants lacking *Cia5* are transformed with a truncated *Cia5* gene the transformed cells constitutively expresses the CCM (Xiang *et al.*, 2001). Thus, *Cia5* is a component of the signal transduction cascade that senses, and either represses or de-represses, the response to low CO$_2$.

**Other Elements Implicated in the CCM of *C. reinhardtii***

Differential screening of *C. reinhardtii* grown in high and low CO$_2$ environments has been employed to identify mRNAs that are up-regulated in cells grown under low CO$_2$. Many of these mRNAs encode enzymes of the photorespiration pathway and another set of low CO$_2$ inducible mRNAs is comprised of messages from the two light-harvesting complex families, LHCA and LHCB. Conditions of low CO$_2$ and of high light have been shown to increase the abundance of the LHCs (Somanchi *et al.*, 1998a). Other mRNA’s identified by differential screening have as yet to have their roles elucidated (Spalding *et al.*, 2002).

*Ccp1* and *Ccp2* (Chen *et al.*, 1997) are two low CO$_2$ inducible transcripts that give rise to proteins with a molecular mass of 36 kDa that have been purified from
membrane fractions of *C. reinhardtii* (Spalding and Jeffrey, 1989) and are immunologically distinct from the Cah1 peptide which has a similar molecular weight of 37 KDa (Geraghty *et al.*, 1990). LIP-36 was localized to intact chloroplast envelopes using $^{35}$S labelling of *C. reinhardtii* proteins, (Ramazanov *et al.*, 1993). The proteins are predicted to have 6 transmembrane domains and have a very high homology to proteins of the mitochondrial carrier protein super family. The proteins are closest to carnitine acyl carriers (CAC’s) which are proteins are involved in the transport of lipid-carnitine molecules formed in the cytosol across mitochondrial membranes for energy.

*Mcal* and *Mca2* are two low CO$_2$ induced mRNAs encoding a β-carbonic anhydrase (Eriksson *et al.*, 1996). The β-CA's are localized to the mitochondria and have a molecular weight of 20.7 kDa and are proposed to act as pH stabilizers and facilitate the diffusion of CO$_2$ out of the mitochondrial matrix under low CO$_2$ conditions when CO$_2$ is produced by glycine decarboxylation during photorespiration (Eriksson *et al.*, 1996).

The mutant, hf-9 is defective in omega-6-desaturase (ω6 desaturase). The mutant hf-9 was originally identified as a high fluorescence mutant with a lower content of unsaturated fatty acids, lower Chl a: Chl b ratios (2.23 *versus* 2.51 in CC-137c (wild-type)), and lower oxygen evolution (29 [Mol mg Chl$^{-1}$ h$^{-1}$ *versus* 185 [Mol mg Chl$^{-1}$ h$^{-1}$ in CC-137c (wild-type)) (Sato *et al.*, 1995) and subsequently cloned (Sato, *et al.*, 1997). ω6 desaturase catalyzes the desaturation of monoenoic to dienoic acids in chloroplasts. The fluorescence was postulated to be due to the fact that hf-9 may be damaged in the desaturase itself or an electron transport system for the desaturation (Sato *et al.*, 1995) as chloroplast ω6 desaturation in spinach was shown to require NADPH and ferredoxin as electron donors (Schmidt and Heinz, 1990). Transformation of hf-9 with des6 ( the
Genomic transcript of w6 desaturase was found to complement the desaturation mutation, but not the photosynthetic activity (Sato et al., 1997). It was found that the complemented hf-9 had lower PSII activity at 45°C (Sato et al., 1997). It was found independently that in differential screening of cDNA libraries of high CO₂ and low CO₂ grown cells of C. reinhardtii that the transcript for w6 desaturase was found under low CO₂ conditions (Somanchi, et al., 1998b). w6 desaturase may be transiently expressed under high temperature, high light, and low CO₂ to acclimate the lipid composition of the thylakoid membrane resulting in inhibition of PSII activity thus preventing over reduction.

Additionally, there are four low CO₂ up-regulated genes that encode novel proteins known as low CO₂ inducible (Lci1, Lci2, Lci3, and Lci5) that have no significant homology to existing proteins (Burow et al., 1996; Somanchi et al., 1998a; Lavigne et al., 2001). The roles of these proteins in acclimation to low CO₂ or the CCM remain to be established as little research has been devoted to the regulation of specific CCM components in microalgae (Spalding et al., 2002).

In Summary

In summary, photorespiration, the water-water cycle, the xanthophylls cycle, and CCM’s all have a parallel function, which is to prevent over-reduction of PSII, which prevents photoinhibition due to inactivation of the D1 subunit. In an effort to learn more about components of the C. reinhardtii CCM and possibly describe the effects of mutants in genes already implicated in the CCM by differential screening of cDNA libraries, insertional mutagenesis utilizing the Ble<sup>R</sup> gene was employed. Ble<sup>R</sup> confers a stable, selectable marker (Zeocin resistance) and incorporates a unique sequence with a lower GC bias than is native to C. reinhardtii. However, some mutants were found to have
defects in photosynthesis or defects in dealing with photooxidative stress and have been additionally selected as these systems are not exclusive. The mutant, slc-230 was generated using \( \text{Ble}^r \) insertional mutagenesis and originally selected as a “sick on low \( \text{CO}_2 \)” mutant, but after linkage analysis had a phenotype more consistent with a defect in photosynthesis or dealing with photooxidative stress. In the following chapters, the generation of slc-230 and other \( \text{Ble}^r \) insertional mutants will be explained (Chapter 3), the subsequent molecular biology showing that \( Hdhl \) is disrupted by the \( \text{Ble}^r \) insertion (Chapter 4), and the physiological symptoms of the disruption of \( Hdhl \) (Chapter 5). Lastly, although the function of \( Hdhl \) has as yet to be fully elucidated, the plausibility of it’s role in photorespiration, the water-water cycle, the xanthophylls cycle, and the CCM will be discussed.
CHAPTER 2
MATERIALS AND METHODS

Generation, Screening, and Maintenance of Insertional Mutants in *Chlamydomonas reinhardtii*

*Ble*\(^R\) mutants were generated by the electroporation of *Chlamydomonas reinhardtii* strain D66 (nit2', cw-15, mt') (Schnell and LeFebvre, 1993) with the construct pSL124 conferring Zeocin resistance (Stevens *et al.*, 1996; Lumbreras *et al.*, 1998) (Fig. 2.1) according to the method of Shimogawara, *et al.*, (1998). After transformation cells were allowed to grow up under the dark or low light (25 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) conditions on TAP (tris-acetate phosphate) plates containing 7.5\(\mu\)g mL\(^{-1}\) Zeocin (Invitrogen Carlsbad, CA). Cells showing resistance to Zeocin were then replica plated on to TAP + Zeocin plates and allowed to grow. In the secondary screen, Zeocin resistant transformants were streaked onto two plates containing MIN (minimal) media and placed under low CO\(_2\) conditions (28-50 ppm CO\(_2\)) and high CO\(_2\) conditions (5% or approximately 50,000 ppm CO\(_2\)) along with appropriate controls (Fig. 2.2). The control strains were cia-3 (a mutant in the gene encoding the carbonic anhydrase cah3, which dies on air levels of CO\(_2\) (approximately 400-600 ppm CO\(_2\)), cia-5 (which will die on low CO\(_2\) levels due to a defective regulatory element), and D66 (the parental wild-type strain which will grow under low CO\(_2\) levels as its carbon concentrating mechanism is not compromised). Mutants that grew poorly on low CO\(_2\) and grew normally on high CO\(_2\) were dubbed “slc” (Sick on Low CO\(_2\)) mutants, which meant that they grew more slowly and had a more bleached appearance than wild-type (which when originally
Figure 2.1 Map of pSP124 with restriction sites and primer locations. This construct was used to confer bleomycin resistance to *C. reinhardtii* strain D66 to generate insertional, tagged mutants.
D-66
• 250 ml of cells

Electroporate
• Capacitance = 25 mF
• Voltage = 2000 V/cm
• Pulse time = 10 to 11 msec
• Temperature = ice cold

Ble®
• 2.5 µg plasmid
• Linearized with Kpn I

Several Days
Ble® colonies come up

Library Plate
High CO₂ Screen
Low CO₂ screen

**Figure 2.2** Diagram of steps in generating and screening insertional tagged mutants. Using pSP124 *C. reinhardtii* was transformed then subsequently screened to select mutants in the carbon concentrating mechanism.
selected, slc-230 fit), and were screened for their affinity for C\textsubscript{i} indirectly by photosynthetic oxygen evolution. Insertional mutants that did poorly under both low CO\textsubscript{2} and high CO\textsubscript{2} conditions were additionally screened by photosynthesis assays and examination under long-wave UV light. Mutants that fluoresced under long-wave UV light (280 nm) and had lower than wild-type V\textsubscript{max}’s were classified as having “hf” or high-flourescence phenotypes, which will not be discussed again in this dissertation.

**Purification of Plasmids and Cosmids from *E. coli***

*E. coli* strain DH5\textsuperscript{a} (Invitrogen, Carlsbad, CA), containing the plasmid to be purified, was grown in 2-5 mL of Luria-Bertani media (LB) with 50 µg mL\textsuperscript{-1} ampicillin (Sigma, St. Louis, MO) overnight at 37° C in 13x100 mm borosilicate test tubes. Purification of plasmid DNA was done with Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) columns as outlined by Qiagen. Likewise, cosmids from Saul Purton’s genomic cosmid library (Purton and Rochaix, 1994) in *E. coli* strain DH5\textsuperscript{a} were grown overnight in 500 mL of LB media with 50 µg mL\textsuperscript{-1} ampicillin overnight at 37° C in 1 L flasks. Purification of cosmid DNA was done with Qiagen-tip 100 by the procedure outlined by Qiagen. Quantification of resulting cosmid DNA was done on a 0.8% agarose gel.

**Purification of Total Genomic DNA from *C. reinhardtii***

100 mL of TAP grown *C. reinhardtii* cells grown at log phase were harvested at 1430xg for 5 min in sterile, 50 mL polypropylene tubes (Corning, Corning NY) in a Beckman JS-13 rotor using a Beckman J2-HS centrifuge (Beckman, Fullerton, CA). Cells were resuspended in 500 mL of TEN buffer and centrifuged again at 1430xg for 5 min. The harvested pellet was then resuspended in 150 µL of H\textsubscript{2}O and 300 µL of SDS + EB (2% sodium dodecyl sulfate, 400 mM NaCl, 40 mM EDTA, 100 mM Tris-HCl, pH
8.0) and the pellet was briefly vortexed to resuspend the cells. 350 μL of PCI (Phenol (pH 4.3): chloroform isoamyl alcohol 25:24:1) was added and vortexed for 2-5 min. The PCI + cell lysate was centrifuged for 5 min at 1430xg to separate the organic phase from the aqueous phase. The aqueous phase was removed and transferred to a new tube and extracted with chloroform: isoamyl alcohol (24:1), vortexed for 5 min and centrifuged for 5 min at 1430xg. The sample was then transferred to a new 1.5 mL tube where the DNA was precipitated with 2 volumes of ethanol, put on ice for 30 min and centrifuged for 10 min. The pellet was washed with 200 μl of ethanol, allowed to air dry and resuspended in 200 μL of sterile H₂O or EB buffer.

**Colony PCR Protocol for E. coli**

This protocol was adapted from Gussow and Clackson (1989) and used as described by Chuck Wimpeee at the University of Wisconsin - Milwaukee (http://garnet.acns.fsu.edu/~bgs4765/ColonyPCR.html). Individual E. coli colonies were selected with a sterile toothpick and transferred into a 250 μL microfuge tube containing 50 μL of 10 mM EDTA. The tube was then placed in a thermocycler (DNA Thermocycler 48C (Perkin Elmer, Emeryville, CA)) set at a 94° C for 5 min. The lysate was vortexed briefly and 1-2 μL of the lysate was used per PCR reaction. Samples of lysate were either used immediately or stored at –20° C.

**Mini Genomic PCR Protocol for C. reinhardtii Used for Rapid Screening of Putative Transformants**

This protocol was adapted from the previous colony PCR protocol for E. coli (Gussow and Clackson, 1989) where stabs of different C. reinhardtii colonies were selected with a sterile toothpick and a “toothpick full” was transferred to a 500 μL microfuge tube containing 20 μL of 10 mM EDTA (Fig. 2.3). The volume of 10 mM
EDTA was decreased as compared to that of the *E. coli* protocol to concentrate genomic DNA per sample. The tube was then placed in a thermocycler (DNA Thermocycler 48C, Perkin Elmer, Emeryville, CA) set at a 94° C soak file for 8 min. The longer denaturation time was used to compensate for the cell walls, high GC content, and size of the *C. reinhardtii* DNA molecules. The lysate was vortexed briefly and 5-10 μL of the lysate was used per PCR reaction. An increased amount of template was used to insure that there was enough product to visualize on an agarose gel.

**Purification of Total RNA from *C. reinhardtii***

Cells of *C. reinhardtii* were grown to early to mid log phase (1x10⁶ to 5x10⁶ cells mL⁻¹) in 100 mL of TAP in 250 mL flasks. Cells were then pelleted at 1430xg in a swinging bucket rotor (Beckman, sw13.1) in a J2-HS centrifuge (Beckman, Fullerton, CA) at 10° C in 50 mL polypropylene tubes (Corning, Corning, NY) for 5 min. The supernatant was decanted and cells were resuspended by brief agitation or vortexing. Ten mL of lysis buffer (20 mM Tris (pH 8.0), 20 mM EDTA, 5% SDS, 100 mg mL⁻¹ Proteinase K (Sigma, St. Louis, MO), and diethyl pyrocarbonate (DEPC; Sigma, St. Louis, MO) treated H₂O was added to the pellet and the mixture was allowed to incubate without stirring at room temperature (ca. 22° C) for 4 hours. One mL of 3 M sodium acetate was added and mixed. Fifteen mL of PCI [(phenol: (pH 8.0 as RNA is extracted by a basic pH and DNA is extracted by an acidic pH) chloroform: isoamyl alcohol (Fisher, Fairlawn, NJ) 50:49:1 (v/v/v)] was added, vortexed, and centrifuged at 1430xg for 15 min then a second PCI extraction was performed on the aqueous phase. The aqueous layer was again transferred to a clean 50 mL polypropylene tube and an equal volume of isopropyl alcohol was added and allowed to incubate for 15 min at room temperature to precipitate the RNA. The RNA was pelleted by centrifuging for 10 min at
• Sterile Toothpick-full of *Chlamydomonas*.

• Twirl culture into 20 µl of 10 mM EDTA.

• Denature in thermo-cycler set to soak at 94°C for 5min to 8min.

• Vortex briefly

• Use 5 µl to 10 µl in a 25 µl PCR reaction.

**Figure 2.3** Diagram of small-scale preparation of genomic DNA from *C. reinhardtii*
1430xg. The resulting pellet was washed with 80% ethanol (AARP, Shelbyville, KY) in DEPC treated H₂O and inverted to dry. Each pellet was resuspended in DEPC treated, sterile dH₂O (100 μl - 500 μl) according to visual estimation of amount of RNA. RNA samples were usually quantified by both absorbance at 260 nm and by gel electrophoresis through a denaturing, formaldehyde gel immediately after resuspension of the pellet. Equal loading was done by spectrophotometric and visual quantitation of total RNA preps using the 28s, 18s, and 5s ribosomal bands as markers on a formaldehyde gel.

**Southern and Northern Blots**

DNA (2 μg in each lane) was loaded and separated on a 0.8% agarose gel and blotted onto a nylon (S&S) as described in Sambrook *et al.*, (1989). ³²P-dCTP labeled probes were prepared using a random primer procedure (Sambrook *et al.*, 1989). The restriction endonucleases digests obtained with NcoI, NotI, PstI, and SacI were consistently of high quality and totally digested genomic DNA for the Southern blot experiments. Southern analyses were performed by standard procedures (Sambrook *et al.*, 1989).

For northern blots, formaldehyde gel was loaded with 10μg to 15μg of total RNA, ran, then blotted onto a nylon as described in Sambrook *et al.*, 1989. ³²P-dCTP labeled probes were prepared using a random primer procedure (Sambrook *et al.*, 1989).

**Polymerase Chain Reaction (PCR)**

PCRs (Polymerase Chain Reactions) were performed using reagents from Epicentre Technologies (Madison, WI). Each 25 μl reaction contained 3x Masteramp™ 10x PCR enhancer (containing Betaine), 2.5 mM MgCl₂, .15 mM dNTP’s (NEB Beverly, MA), 1x Masteramp™ Tfl 20x PCR buffer, 2.5 mM each of the forward and backward
primer, 0.5 U Masteramp™ Tfl DNA polymerase, and water to a volume of 25 µL. Denaturation was 95° C. The annealing temperature used was dependent on the Tm of the primers used. Extension was 72° C.

**Inverse PCR**

Inverse PCR (iPCR) was performed by digesting 40 to 80 ng of the slc mutant’s genomic DNA with a battery of enzymes supplied by NEB (Beverly, MA). The restriction endonucleases BamHI, EcoR1, PstI, and SacI were used as they did not cut in the sequence of the BleR insert, but did cut in the multiple cloning site of pSP124. The resulting digests were precipitated by adding ethanol to 70%, washed, and ligated using T4 ligase (NEB, Beverly, MA) overnight at 16° C. The ligation was then precipitated using ethanol, centrifuged, and allowed to dry followed by resuspension in 200 µl dH2O. The fragments were then used as a template for PCR using the Ble3b and BleDR primers (IDT, Coralville, IA).

**RT-PCR**

Reverse Transcriptase PCR (RT-PCR) was done on 2 µg total genomic RNA from *C. reinhardtii* prepared using Qiagen One-step RT-PCR System (Qiagen, Valencia, CA). PCR primers were designed to straddle exons so that amplified fragments from contaminating genomic DNA would be larger than amplified products from the mRNA.

**5’ RACE (Rapid Amplification of cDNA Ends)**

5’ Race was performed by using the First Choice RLM-RACE kit (Ambion, Austin, TX) as instructed by Ambion. The adaptor primer (5’-GCU GAU GGC GAU GAA UGA ACA CUG CGU UUG CUG GCC UUG AUG AAA-3’) supplied by Ambion was used for the 5’ primer.
Sequencing

DNA (cosmids, plasmids, and PCR products) was sequenced using the ABI Big Dye terminator Kit or the dGTP Big Dye Kit. For cosmids, 500 ng to 1μg was used as template; for plasmids, 250 ng to 500 ng was used as template; for PCR products, 100 ng was used as template. Each sequencing reaction was performed in a volume of 20 μL. 3.2 pMol of primer and 2 μL of Big Dye were used per sequencing reaction. PCR was carried out in 30 cycles: 96° C for 30 s (denaturation), the annealing temperature of the chosen primer for 30 s, and 60° C for 4 min (extension). The resulting product was precipitated in 95% ethanol, washed in 70% ethanol then dried at 90° C for 1 min to drive off any residual ethanol. All sequencing was done at the LSU Museum of Natural Science DNA Sequencing Laboratory, Baton Rouge, LA.

Identification of Loci and Homologies Using BLAST

Homology searches of nucleotide sequences were performed against the full NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), the C. reinhardtii EST database (http://www.biology.duke.edu/chlamy_genomeblast/blast_form.html), and the C. reinhardtii genome (http://aluminum.jgi-psf.org/prod/bin/runBlast.pl?db=chlre1). All alignments used the BLAST program (Altshul et al., 1997). Exon/ intron splice sites and open reading frames were identified manually as well as by using greenGenie (http://www.cse.ucsc.edu/~dkulp/cgi-bin/greenGenie ) (Kulp et al., 1996).

Genetic Analysis of Linkage

Mutant strains (mt+) and CC-124 (mt-) cells were first grown for several days on TAP medium plates and transferred to TAP medium plates containing 10% the normal level of nitrogen for two days. They were then transferred to liquid TAP medium without nitrogen or trace elements and incubated in high light overnight (Moroney et al., 1986).
Tetrad dissections were then carried out as previously described (Levine and Ebersold, 1958; Harris, 1989). Colonies derived from meiotic products were then transferred and grown on TAP medium. These colonies were then screened on TAP medium containing Zeocin (7.5 µg mL⁻¹) to check for the presence of the Ble™ insert. Colonies were also replica-plated on minimal media and grown in high and low CO₂ to determine which progeny required high CO₂ levels for normal growth. Linkage was determined by associating Ble™ with those progeny that were sick on low CO₂ levels.

**Screening of the Cosmid Library**

To obtain wild-type genomic clones of disrupted genes, a PCR-based screen of an indexed cosmid library was used. The indexed cosmid library was constructed using a cosmid library from Saul Purton, University of London (Purton and Rochaix, 1994). A cosmid is a strand of genomic DNA (up to 45 kb in length) spliced into φ phage DNA and packaged into a φ virion which is used to infect *E. coli* which acts as a carrier for the genomic strand in the cos vector (Stryer 1995). Briefly, 7680 different *E. coli* cells carrying single cosmids were grown in LB media on 80 different 96-well microtiter plates. 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 single cosmids) that were suitable for PCR. Using the sequence information obtained from the iPCR or TAIL PCR fragments, sets of primers 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, at least one unique cosmid, containing the desired gene can be isolated assuming that the cosmid is present in the library.

**Nuclear Transformation of C. reinhardtii by the Glass Bead Method**

Nuclear transformation of *C. reinhardtii* was done by the glass bead method as described by Kindle (1990). Cells were started in 100 mL TAP medium and grown to mid-log-phase. Starter cultures were harvested in 50 mL Corning tubes at 1430xg for 5 min. Then cells were incubated in 4 mL of crude autolysin preparation for 2 hr at room temperature (ca. 22° C) if there was the presence of a full or partial cell wall. Cells were then resuspended in 10 mL MIN media and re-harvested to wash excess acetate off cells. The final pellet was then resuspended in 1 mL MIN and added to 1 L MIN media and grown to a density of 1-3x10⁶ cells mL⁻¹. The density of the culture was then determined by adding a small drop of tincture of iodine to approximately 2 mL of culture and counting the cells using a hemocytometer. Cells were then harvested in 500 mL centrifuge tubes (Nalgene, Rochester, NY) at 1590xg for 5 min in a Beckman JA-10 rotor. The resulting pellet was resuspended to a density of 2x10⁸ cells mL⁻¹ in MIN + 5% polyethylene glycol 8000 (Sigma, St. Louis, MO). One mL of resuspended culture was transferred to sterile screwtop test tubes containing 300 mg of acid washed beads (Sigma, St. Louis, MO). Transformation was performed by adding 1 mg of transforming DNA to a 1 mL aliquot of culture and vortexing at full speed for 2 rounds of 30 s with a 5 s pause between each round. 200 μL of cells (4x10⁷ cells) were then plated on MIN plates with 1% agar and put under low CO₂ conditions for screening of complementation.

**Crude Autolysin Preparations**

Autolysin was prepared as described in Harris (1989), except that the cells were grown in MIN media and the preparation was not concentrated. Instead the preparation
was filter sterilized using a 13 mm nylon membrane syringe filter with a pore size of 0.20 μm (Titan Filtration Systems) on a 5mL syringe (Becton-Dickinson) into a sterile 10 mL Corning tube and stored at –80° C.

**Nuclear Transformation of *C. reinhardtii* by Electroporation**

Using an electroporation technique modified from Shimogawara *et al.* (1998), D66 cells were started in 100 mL of TAP medium (Sueoka 1960) for 2 days then transferred to 1 L TAP medium and allowed to grow to mid-log phase. Cells were harvested by centrifugation at 1430xg and resuspended to 2x10^8 cells mL⁻¹. 300 μL of cells were allowed to sit in an electroporation cuvette (0.4 cm gap width; Biorad, Hercules, CA), on ice with 1 μg of pSP124S that had been linearized with KpnI (NEB, Beverly, MA). The electroporation settings on the Biorad electroporator Gene Pulser II (Hercules, CA) were as follows: capacitance of 25 mF and no shunt resistance, voltage set to .84 V cm⁻¹, and the pulse time was between 9.4 and 10 ms. After electroporation, the cells were allowed to recover overnight in 10 mL TAP + 60 mM sucrose medium. Cells were then harvested, resuspended in 4 mL TAP, and 4x10⁷ cells were plated onto media containing Zeocin. Likewise, complementation of slc mutants that had been backcrossed to CC-124 mt⁻ was performed by using 1 μg of purified cosmid containing the wild-type locus and a setting of .96 V cm⁻¹ (Somanchi, 2003, personal communication). The transformants were then allowed to recover overnight in MIN + 60 mM sucrose, washed in liquid MIN and plated onto MIN plates.

**Oxygen Evolution Assays**

Cultures were started heterotrophically in 100 mL of media containing acetate (TAP) and grown for 48 hours then transferred to media without a carbon source (MIN) and bubbled with 5% CO₂ in air (final [C₅] = 2mM) for 24 hr. Cultures were then
acclimated to low CO\textsubscript{2} by bubbling with air (final [C\textsubscript{i}] = 4\mu M) for 4 to 96 hours. All conditions were carried out under continuous light and shaking. Photosynthesis was determined indirectly by oxygen evolution as done by Rawat and Moroney (1995), with a few modifications. Algae were centrifuged at 994xg for 5 min, the pelleted algae were resuspended at 25 \mu g Chl mL\textsuperscript{-1} in 0.5 mL of 25 mM Hepes-KOH (pH 7.3). Chlorophyll concentrations were determined spectrophotometrically (Arnon, 1949). The photosynthetic rate of algal cells was measured with an oxygen electrode (Rank Brothers, Cambridge, UK). Cells were transferred to the electrode chamber, where they were allowed to exhaust the C\textsubscript{i} of the buffer and until no net O\textsubscript{2} exchange was observed which varied with each transformant. Sodium bicarbonate at 25\mu M, 50\mu M, 100\mu M, 250\mu M, 500\mu M, 1mM, 2mM, and 4mM was added and the rate of O\textsubscript{2} evolution was measured for the next 30 s to 5 min The K\textsubscript{0.5} (CO\textsubscript{2}) value is the CO\textsubscript{2} concentration required to give half-maximal rates of O\textsubscript{2} evolution.

**n-propanol Lipid Extraction**

Lipid extraction was performed as modified from Bligh and Dyer (1959) in Moore (1990). 100 mL of mid-log-phase cells were pelleted in 50 mL polypropylene tubes (Corning, Corning, NY) at 1430xg in a swinging bucket rotor (Beckman, sw13.1) in a J2-HS centrifuge (Beckman, Fullerton, CA) for 5 min. The pellet was vortexed briefly so that it could be taken up in a micropipette. Samples were prepared for extraction as in Christie (1982) by adding 4 mL n-propanol that had been heated to 70° C. Tubes were incubated in a heater block for 30 min at 70° C. Samples were cooled to room temperature, 2 mL chloroform was added followed by 3 mL 1 M KCl. Lipids were extracted into chloroform and the aqueous layer discarded. Samples were washed by adding 1.5 mL 1 M KCl, vortexing until completely mixed then centrifuging at 1430xg
for 3 min and discarding the aqueous layer 3 times. 1.5 mL H$_2$O was added, vortexed, centrifuged and removed. The organic layer was evaporated in low light under nitrogen gas at 50° C. The resulting pellet was resuspended in chloroform and the amount of chlorophyll was determined spectrophotometrically in each sample.

**Thin Layer Chromatography (TLC)**

To separate polar and non-polar lipids with different head groups TLC (Thin layer Chromatography) was performed. Lipid extractions equivalent to 20 mg were spotted onto a 20 x 20 cm silica gel GHL Thin Layer Chromatograph plate using a 10 mL syringe. Lipids were separated using a two-dimensional system involving chloroform: methanol: H$_2$O (65:25:4 v:v:v) in the first dimension and chloroform: methanol: isopropylamine: concentrated NH$_4$OH (65:35:0.5:5 v:v:v:v) in the second dimension (Vogel and Eichenberger, 1992). Lipids were visualized in an iodine vapor chamber, and selected spots were circled to generate a template. The marked TLC plate was scanned. Plates from different algal growth conditions (bright light and low light) and of different strains (D66 and slc-230) were compared for any change in quantity in the major membrane lipids.

**Exposure of C. reinhardtii to Bright Light**

Cultures of *C. reinhardtii* were started in 100 mL TAP and grown for approximately 24 hr to mid-log phase, cultures were harvested, washed, and resuspended in MIN media followed by dark acclimation for 24 hr. Afterward, the cells were put under an illumination apparatus (Fig. 2.4). An aliquot of cells was taken at time 0 then cells were exposed to 500 μmol photons m$^{-2}$ s$^{-1}$ for 90 min with 10 mL aliquots taken at 30 min, 60 min, and 90 min. A thermometer was used to monitor temperature of the
cultures (ca. 25° C) and a LiCor light meter was used to determine photon flux density (PFD) of the flasks.

**Extraction of Photosynthetic Pigments for HPLC Analysis**

For extraction of pigments for HPLC, cells were harvested in 50 mL polypropylene tubes (Corning, Corning, NY) at 1430xg in a swinging bucket rotor (Beckman, sw13.1) using a J2-HS centrifuge (Beckman, Fullerton, CA) at 10° C for five minutes. In the dark, the supernatant was then removed and tubes were inverted for a minute for the pellet to dry. One to four mL of HPLC grade acetone (Fisher, Fairlawn, NJ) (the volume depends on the initial concentration of the cells) were used to extract pigments for 1 hr on ice. The extraction was then filtered twice using a 13 mm nylon membrane syringe filter with a pore size of 0.20 mm (Titan Filtration Systems, Sun-Sri, Wilmington, NC) on a 5mL syringe (Becton-Dickinson, Franklin Lakes, NJ) into an amber HPLC 1.5 mL sample vial (Sun-Sri, Wilmington, NC), topped off with nitrogen gas and stored at –80° C if not used immediately.

**Analysis of Pigments Using HPLC**

High Performance Liquid Chromatography (HPLC) was performed as described by Buffan-Dubau and Carman (2000). HPLC was performed with a Hewlett-Packard1100 liquid chromatograph comprised of a 100 mL loop autosampler, a quaternary solvent delivery system coupled to a diode array spectrophotometer, and a Hewlett Packard 1046A fluorescence detector. The diode array detector had a setting of 436 nm for detection of carotenoid and chlorophyll pigments. Chlorophylls were detected without interference from carotenoids using the fluorescence detector set at 407 nm (excitation)/ 672 nm (emission) and at 430 nm (excitation)/ 665 nm (emission) (Jeffrey et al., 1997).
**Figure 2.4** Setup for high light regimen. Light from Sylvania floodlights passes through a vertical lucite tank filled with water that acts as a heat sink by removing infrared radiation. The lamp illuminates cultures in 250 mL flasks with 100 mL cultures sitting on stirrers.
Separation of pigments was performed by reverse-phase liquid chromatography using an Adsorbosphere C18, 5 μm column (250 mm x 4.6 mm i. d.; All Tech, Deerfield, IL) coupled to a guard column (C18, 5 μm; All Tech, Deerfield, IL). Data analysis was performed using Hewlett Packard HP Chemstation software. The mobile phase was prepared and programmed according to the analytical gradient protocol described by Wright et al., (1991). All organic solvents and water were HPLC grade (Mallinckrodt Phillipsburg, NJ). *C. reinhardtii* pigments were identified by comparing retention times and absorption spectra with those of authentic standards (Chl a, Chl b, and β-carotene were from Sigma, St. Louis, MO; antheraxanthin and violaxanthin were from DHI, Hoersholm, Denmark) or standards prepared from algal cultures. Zeaxanthin and lutein from *Oscillatoria sp.* were provided by Dr. Thomas Bianchi (Tulane University, LA, USA). Retention times of extracted pigments from *Dunaliella tertiolecta* (Appendix III) were used to determine pigment peaks in HPLC done from *C. reinhardtii* pigments (Appendix IV).
CHAPTER 3

USE OF THE BLEOMYCIN RESISTANCE GENE TO GENERATE TAGGED INSERTIONAL MUTANTS OF CHLAMYDOMONAS REINHARDTII THAT REQUIRE ELEVATED CO\textsubscript{2} FOR OPTIMAL GROWTH

Introduction

Tagged insertional mutagenesis has been used to identify the role of novel genes in a number of organisms including \textit{C. reinhardtii} (Nelson and Lefebvre, 1995; Tam and Lefebvre, 1995; Davies \textit{et al.}, 1996; Pazour \textit{et al.}, 1998). In \textit{C. reinhardtii}, transforming ammonia-requiring or arginine-requiring mutants with the endogenous gene that complements the mutation has regularly been used to perform insertional mutagenesis. The \textit{Nit1} (Kindle \textit{et al.}, 1989; Tam and Lefebvre, 1995) and the \textit{Arg7} (Davies \textit{et al.}, 1996; Wykoff \textit{et al.}, 1999) genes have been the most commonly used. Fukuzawa and his colleagues used \textit{Nit1} insertional mutagenesis to investigate the CCM of \textit{Chlamydomonas} (Fukuzawa \textit{et al.}, 1998). They provided evidence that one of their mutants, C16, had a \textit{Nit1} insert in the Ccm1/Cia5 gene (Fukuzawa \textit{et al.}, 2001). This work demonstrated the power of the insertional mutagenesis approach; however, the use of an endogenous gene for the mutagenesis made it more difficult to determine the location of the insertion. In the work described in this chapter, a foreign gene has been employed to circumvent some of the problems with determining the location of the insert.

Two recent advances have made insertional mutagenesis with \textit{C. reinhardtii} a more powerful tool. First, Saul Purton and his colleagues constructed an antibiotic resistance gene (\textit{Ble}\textsuperscript{R}) using the coding region from \textit{Streptoallotrichus hindustanus} and the 5’, introns and 3’ regulatory regions from the \textit{C. reinhardtii} \textit{RbcS2} gene (Stevens \textit{et al.}, 1996; Lumbreras \textit{et al.}, 1998). This chimeric gene expresses well in \textit{C. reinhardtii} and allows one to insert a tag into the genome with a unique sequence. The second
advance, described by Shimogawara et al., (1998), was the development of highly efficient nuclear transformation utilizing electroporation in C. reinhardtii allowing generation of large numbers of primary transformants. The Ble\textsuperscript{R} construct and a modified electroporation method have been employed to develop an improved insertional mutagenesis strategy. Also, two PCR based methodologies have been used to identify the locations of the Ble\textsuperscript{R} inserts in these transformants. Using this transformation method, 42,000 independent transformants were generated and screened, resulting in 120 mutants that were unable to grow well on low CO\textsubscript{2}.

Results

Production and Screening of Insertional Mutants

42,000 Ble\textsuperscript{R} insertional mutants were produced and screened. The primary goal of the screen was to identify mutants with visible defects in the CO\textsubscript{2} concentrating mechanism (CCM) although other photosynthesis mutants were also selected in the initial screen. Any transformant (about 2,000) that appeared to grow poorly on low CO\textsubscript{2} was kept and rescreened. In the second screen, cells were grown on both high CO\textsubscript{2} and low CO\textsubscript{2}. Cell lines that grew well on high CO\textsubscript{2} but grew poorly, if at all on low CO\textsubscript{2}, were kept as potential CCM mutants (Fig. 3.1). About 120 mutants had the phenotype of good growth on elevated CO\textsubscript{2} but very poor growth on low CO\textsubscript{2}. In addition to those transformants with a CCM-like phenotype, 110 mutant strains could not grow photosynthetically at all (Fig. 3.1 and Table 3.1). Also, 38 of the transformants exhibited high fluorescence properties (Table 3.1).
**Figure 3.1** Growth of insertional mutants. A, Growth of mutants on MIN media and elevated CO₂; B, Growth of mutants on MIN media and 30 ppm CO₂; C, Growth of mutants on TAP media; D, Growth of mutants on TAP + 7.5 μg mL⁻¹ Zeocin. D-66 is the wild-type parental strain for the slc insertional mutants and cia-5 is a high CO₂ requiring strain previously derived by UV mutagenesis.
Those transformants that grew well on elevated CO₂ but poorly on low CO₂ were further characterized. First, these strains were grown on plates on elevated CO₂ and in a chamber with extremely low CO₂ (30 ppm). Many of the transformants could not grow at all at 30 ppm and these transformants were designated as Class I mutants. The rest of the mutants, which could still survive at this extremely low CO₂ concentration were called Class II mutants. Class II mutants still grew more slowly and appeared more bleached than the parental strain at these low CO₂ concentrations (Fig. 3.1). The affinity of each mutant for external inorganic carbon (Cᵢ) was then estimated by measuring net O₂ evolution at different Cᵢ concentrations and determining the concentration resulting in one-half the maximal rate of O₂ evolution [K₀.₅(Cᵢ)]. The mutants exhibited a wide range of affinities for Cᵢ with some having a K₀.₅(Cᵢ) similar to wild-type cells (20 M) while others had very high K₀.₅(Cᵢ) values of greater than 300 M (Fig. 3.2A and 3.2B). The range of growth and Cᵢ affinities observed suggests that a number of different genes had been disrupted by the insertional events.

For insertional mutagenesis to be useful, transformation conditions needed to be developed which introduce only a limited number of insertions into the target cell. The original transformation protocol of Shimogawara et al., (1998) was modified in two ways. The carrier DNA was eliminated and the plates were not overlaid with agar containing cornstarch. These modifications dropped the transformation efficiency by about 50 fold; however, the rate of transformation (1 in 10⁵ cells) was high enough to provide enough transformants for this study.
Table 3.1 Results from the large-scale screen of insertional mutants using the Blc<sup>e</sup> gene. Out of 42,000 primary transformants 246 exhibited poor growth on low CO<sub>2</sub> and were retained. Out of that 246, 120 were able to grow well on elevated CO<sub>2</sub> but poorly on low CO<sub>2</sub>. 110 of the selected mutants were unable to grow on either high or low CO<sub>2</sub>, consistent with a photosynthesis mutation.

<table>
<thead>
<tr>
<th>Transformant phenotype</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeocin resistant</td>
<td>~42,000</td>
</tr>
<tr>
<td>Exhibiting poor growth on 100 ppm CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>246</td>
</tr>
<tr>
<td>Able to grow on elevated CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>120/246</td>
</tr>
<tr>
<td>Unable to grow on high or low CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>110/246</td>
</tr>
<tr>
<td>High fluorescence phenotype</td>
<td>38/246</td>
</tr>
</tbody>
</table>
Figure 3.2 The apparent affinity of the transformants for inorganic carbon and subclassification of mutants based on $K_{0.5}(C_i)$.  

A, Rate of photosynthesis vs. inorganic carbon concentration for wild-type D66 cells, and cells transformed by the insertion of the $Ble^R$ gene (slc-15, slc-18, and slc-21). Cells were allowed to acclimate to low CO$_2$ for 16 h. Photosynthesis was measured as O$_2$ evolution.  
B, Number of class I (unable to survive at 30 ppm CO$_2$) and Class II (able to survive at 30 ppm CO$_2$) mutants sorted by their relative affinity for $C_i$. Relative affinity for $C_i$ was judged by $K_{0.5}(C_i)$, and was estimated as described in “Materials and Methods”. A $K_{0.5}(C_i)$ of between 20 and 39 $\mu$M was considered normal, a $K_{0.5}(C_i)$ of between 40 and 135 $\mu$M was considered elevated and a $K_{0.5}(C_i)$ greater than 134 $\mu$M was considered highly elevated.
Southern blots were performed on a number of primary transformants and most transformants had a single copy of the $\text{Ble}^R$ gene insertion (Fig. 3.3). The probe used in these experiments was a PCR product that included the coding regions of $\text{Ble}^R$ plus one of the introns of the endogenous $\text{RbcS}2$ gene. The intron of the $\text{RbcS}2$ gene is required for a high expression of $\text{Ble}^R$ in $\text{C. reinhardtii}$ (Lumbreras et al., 1998) and also provides an internal control for the Southern blots (Fig. 3.3). This intron hybridizes only weakly with DNA isolated from D66 but this weak hybridization does indicate whether the DNA digestion is complete (Fig. 3.3).

Another requirement for insertional mutagenesis studies is that the inserted $\text{Ble}^R$ gene be linked to the high CO$_2$-requiring phenotype. The insert was determined to be linked by backcrossing the primary transformant with wild-type cell lines. For linkage to be established, all of the Zeocin resistant progeny of a cross should also require elevated CO$_2$ for optimal growth. At this point, it has been found that about 30% (8 of 25) of the transformants backcrossed demonstrate the required linkage. Transformants that show linkage are then subjected to the PCR protocols to identify the gene disrupted by the insertion.

**Identifying the Genomic DNA Disrupted by the Insert**

Two methods have been successfully used to identify DNA sequences flanking the $\text{Ble}^R$ insertion (Table 3.2). The first method is to amplify the flanking DNA using inverse PCR (Gasch et al., 1992). This method takes advantage of the fact that the $\text{Ble}^R$ gene is originally from *Streptoallotrichus hindustanus* and contains unique sequences. We purposely left the multiple cloning site from the plasmid in our transforming DNA.
Figure 3.3 Southern blots indicating the number of $Ble^R$ inserts. D66 (1) and slc-23 (2) genomic DNA digested with the indicated restriction enzymes and probed with part of the $Ble^R$ construct. The bands that hybridized strongly indicate the $Ble^R$ insertion, while the bands with weak hybridization indicate the endogenous $RbcS2$ intron.
Genomic DNA from the transformant is digested with restriction enzymes that cut within the multiple cloning site but not in Ble\(^8\) (Fig. 3.4A). The hypothesis is that the next restriction site will lie outside of the insert in the flanking DNA. The PCR primers used face away from each other in the original Ble\(^8\), but after ligation these primers will face each other and a piece of the flanking *C. reinhardtii* DNA will be amplified. When a DNA fragment is amplified (Fig. 3.4B, lanes 8-11, Fig. 3.4C, lanes 1 and 3) the fidelity of the fragment can be checked by a nested PCR (Fig. 3.4C, lanes 2 and 4). The difference in size observed is consistent with the difference in location of the primers used. Alternatively, the PCR fragment can be rapidly checked by digestion with XhoI (or HindIII). This digestion yields a diagnostic three DNA fragment pattern if all of the XhoI sites are present (Fig. 3.4D, lanes 1 and 2). Two of the bands obtained with this digestion correspond to the remaining Ble\(^8\) portions (from the primer used to the Xho I site) and the third corresponds to the flanking region. In some cases, the insertion occurs with a deletion of the multiple cloning site on the 3’UTR of pSP124S. The Ble\(^8\) is still functional but the digestion generates only 2 bands (Fig. 3.4D, lanes 3 and 4).

**Obtaining the Wild-Type Gene**

As soon as a flanking DNA sequence was obtained, that sequence was compared to sequences deposited in databases, particularly those contained in the *C. reinhardtii* EST database and the DOE Joint Genome (Altschul *et al.*, 1997). This part of the research has become easier since the *Chlamydomonas* genome sequence has been completed (http://www.jgi.doe.gov/index.html). From our studies, insertions in different genes including rubisco activase, a putative protein kinase, a dioxygenase and a histone have been identified (Table 3.2). Once enough sequence data is obtained from an
**Table 3.2** Genes disrupted by \( Ble^R \) random insertional mutagenesis. For each slc mutant the gene disrupted is identified where known. The linkage between the insert and the “sick on low CO\(_2\)” phenotype is indicated. N.D. indicates that linkage has yet to be determined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Method utilized</th>
<th>Homology</th>
<th>Linked</th>
</tr>
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<tbody>
<tr>
<td>SLC18</td>
<td>iPCR/TAIL PCR</td>
<td>Dioxygenase-FeS protein</td>
<td>No</td>
</tr>
<tr>
<td>SLC25</td>
<td>iPCR/TAIL PCR</td>
<td>Rubisco Activase</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC51</td>
<td>iPCR</td>
<td>No Homology</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC67</td>
<td>TAIL PCR</td>
<td>Histone</td>
<td>No</td>
</tr>
<tr>
<td>SLC144</td>
<td>TAIL PCR</td>
<td>5’ of Rbcs2</td>
<td>No</td>
</tr>
<tr>
<td>SLC225</td>
<td>TAIL PCR</td>
<td>No Homology</td>
<td>N.D.</td>
</tr>
<tr>
<td>SLC280</td>
<td>iPCR</td>
<td>SNF-Protein Kinase</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 3.4 iPCR of Ble<sup>R</sup> mutants. (A) Schematic representation of iPCR. RE, restriction endonuclease; pBS, pBluescript. (B) iPCR results for slc-280. Lane 1, DNA markers; lane 2, negative control (Ble5-Ble3); lane 3, negative control (Ble5a-Ble3a); lane 4, positive control (original Ble<sup>R</sup> cassette without intron, Ble5a-Ble3a); lane 5, positive control (slc-280 genomic DNA, Ble5a-Ble3a); lane 6, negative control (iPCR on D66 genomic DNA digested with Bam HI, Ble5-Ble3); lane 7, negative control (on D66 genomic DNA, Ble5-Ble3); lane 8, slc-280 iPCR (Sac I digest, Ble5-Ble3); lane 9, slc-280 iPCR (Eco RV digest, Ble5-Ble3); lane 10, slc-280 iPCR (Eco RI digest, Ble5-Ble3); lane 11, slc-230 iPCR (Bam HI digest, Ble5-Ble3). (C) iPCR of slc-163 and slc-51 using Pst I. Lanes 1 and 3, iPCR performed using Ble5-Ble3; lanes 2 and 4, nested PCR using BleC-BleD. The difference in size of the PCR products (lanes 1 and 2, or, lanes 3 and 4) indicates the difference in the position of the primers used. M1, DNA markers. (D) Diagnostic restriction digests. A simple digest of an iPCR product with Xho I (or Hind III) creates three bands (lanes 1 and 22, mutant slc-23 digest with Xho I and Hind III respectively). In some cases, the insertion occurred with the deletion of the multiple cloning site at the 3' UTR of the BleR cassette, and only two bands were identified (mutant slc-25 lane 3, nested iPCR BleC-BleD, digested with Xho I; lane 4, iPCR Ble3-Ble5, digested with Xho I). M2, DNA markers.
insertional mutant, a wild-type copy of the genomic region can be obtained from an indexed cosmid library using a PCR-based methodology. Primers have been used on an indexed cosmid genomic library (Purton and Rochaix, 1994, Funke et al., 1997) that has about 90 cosmid pools each containing 96 individual cosmids. This set of cosmids covers the C. reinhardtii genome about 3 times. Also, “superpools” of cosmids with each pool containing cosmids from 10 microtiter plates have been made. The primers are first used on the superpools, each of which contains 960 individual cosmids. When a PCR product is obtained from one or more of the superpools, the process is repeated with individual pools of cosmids until it is determined which of the cosmids in the pool contains the DNA region of interest. Since each pool has 96 cosmids in an 8 X 12 grid, typically DNA from each row and column of the titer plate is prepared and the PCR reaction is performed on these. Positive reactions in a row and column pinpoint which cosmid contains the flanking sequence. The Moroney lab has recently identified cosmids carrying a putative ꞏ-type carbonic anhydrase (Mason and Moroney, unpublished), and Rubisco activase genes (Colombo et al., 2001) using this strategy. We have found this method to be much quicker than screening the cosmid library using hybridization techniques.

Discussion

Several strategies have been pursued to identify the components of the CCM in Chlamydomonas. One method, differential hybridization, has identified a number of genes that are induced or derepressed during acclimation from high CO₂ to low CO₂ conditions (Burow et al., 1996; Chen et al., 1997, Karlsson et al., 1998; Somanchi et al., 1998; Somanchi and Moroney 1999). More recently, thousands of ESTs from cells grown on high or low CO₂ have been partially sequenced (Asamiziu et al., 2000). Many of
these ESTs have been found to be in greater abundance in either the high CO₂ or the low CO₂ growth regimes. For most of these genes identified as up-regulated under low CO₂ growth conditions, a conclusive role in the CCM remains unknown because until recently, there has been no way to selectively knock out these genes in *C. reinhardtii*. More recently, the efficacy of RNAi has been explored. A second approach that can be utilized is to over-express one or more of these genes in a wild type strain, or in a mutant such as cia5 that has a repressed CCM (Moroney *et al.*, 1989). Again, the chances that the over-expression of a particular putative CCM gene can produce useful information are low. The two methods mentioned above have led to many discoveries in the study of the CCM, but in the absence of a method to selectively target and knockout genes of interest these discoveries are presently at a cul de sac.

An alternative experimental approach is the generation of mutants. The *Cah3* gene was found to be required for growth on low CO₂ by complementation of the cia-3 and ca-1 mutations (Funke *et al.*, 1997, Karlsson *et al.*, 1998). More recently, the transcription factor *Ccm1/Cia5* was identified using insertional mutagenesis (Fukuzawa *et al.*, 1998, Fukuzawa *et al.*, 2001). Random insertional mutagenesis method using *Ble⁸* can be used to tag genes that are required for growth in a low CO₂ environment or under other stress regimes. Using a reverse genetics approach, genes that have been knocked out by the insertion of the *Ble⁸* can rapidly be identified and assigned a specific role.

The *Ble⁸* cassette engineered by Purton and his colleagues was chosen as the selectable marker because it has a high level of expression upon integration into the *Chlamydomonas* nuclear genome (Stevens *et al.*, 1996, Lumbreras *et al.*, 1998). This modified gene, originally from *S. hindustanus*, yields transformant cells that are stably resistant to the antibiotic Zeocin. This contrasts with the *Chlamydomonas* endogenous
genes \textit{Nit1}, \textit{Arg7}, and \textit{Ars} where the transformants sometimes lose the ability to express the transgene after a number of generations. This foreign antibiotic resistance gene introduces unique sequences into the \textit{Chlamydomonas} genome that allows two different PCR based methodologies, iPCR and TAIL-PCR, to be used to rapidly identify the genomic DNA flanking the \textit{Ble} \textsuperscript{R} insert (Colombo \textit{et al.}, 2000).

The 120 slc mutants that grew slowly on low CO\textsubscript{2} exhibited a wide range of affinities for inorganic carbon (Fig. 3.2). Some had apparent affinities for inorganic carbon that were similar to wild type cells. Others displayed aberrant K\textsubscript{0.5}(C\textsubscript{i}) values (Figure 3.2). The range of K\textsubscript{0.5}(C\textsubscript{i}) values measured in these mutants suggests that a number of different genes might be disrupted by the insertional mutagenesis procedure. It is also likely that the screen at CO\textsubscript{2} levels lower than air helped to identify some mutants visually that might have grown normally in previous screens. A number of possible mutations could lead to the different K\textsubscript{0.5}(C\textsubscript{i}) values observed.

Slc mutants that have a very poor affinity for C\textsubscript{i} are likely to be missing some component of the CCM. Previously characterized examples of this type of mutant include cia-3 which is missing a chloroplast carbonic anhydrase (Karlsson \textit{et al.}, 1998) and cia-5 which is missing a transcription factor required for the expression of a number of CCM components (Fukuzawa \textit{et al.}, 2001, Xiang \textit{et al.}, 2001). Slc mutants that display a normal affinity might have defects in the photorespiratory pathway or in Rubisco activation. Slc-25, an example of this type of mutation, has a defect in Rubisco activase (Pollock \textit{et al.}, 2003). Slc-25 grows poorly on low CO\textsubscript{2} yet has a normal affinity for C\textsubscript{i}. It should be noted that the determinations of K\textsubscript{0.5}(C\textsubscript{i}) were made on cells that were acclimating to low CO\textsubscript{2} for fairly short times (4-12 h). If a cell were to build up a toxic
intermediate at low CO$_2$ we may see a change in the apparent affinity for C$_i$ only after an extended period of time.

The mutants that display an intermediate K$_{0.5}$(C$_i$) value represent a new class of mutant. These might have a defect in a single component of the CCM or a component that is redundant, a phenomenon seen with cyanobacterial C$_i$ transport mutants (Price et al., 2002). Perhaps these mutants can partially compensate for the loss of a CCM component knocked out by the Ble$^R$ insertion. It is encouraging that so many classes of mutants were obtained in this study. The characterization of these mutants should help clarify how the CCM operates in eukaryotic algae.

To successfully utilize random insertional mutagenesis to tag a gene of interest the number of insertion events must be kept to a minimum and the insertion must be linked to the mutant phenotype. Transformation conditions designed to introduce a minimum number of inserts in the host cell are presented in Chapter 2. An important modification of the transformation protocol of Shimogawara et al., (1998) is that the carrier DNA was omitted. Carrier DNA can also be inserted into the genome as well as the insert (Smart and Selman, 1991) and therefore to avoid the insertion of any DNA other than the plasmid carrying Ble$^R$ the carrier was not used. Leaving out the carrier DNA yields a lower rate of transformation, but the rate is still high enough to obtain a large number of transformants that contain one, two, or, very rarely, three insertion(s) required to screen for a specific mutant phenotype.

A more difficult problem to address is that only 30% of the analyzed mutants show linkage between the Ble$^R$ and the mutant phenotype. The reason for this level of linkage is not known, although it is not very different from that observed using T-DNA tagging approaches in higher plants (Dent et al., 2001). While this level of linkage
seems low, the method has generated many mutant strains that appear to have inserts in a gene required for optimal growth under low CO\textsubscript{2} conditions. Researchers using these insertional mutagenesis methods must screen several thousands of transformants and then genetically analyze them for linkage of the \textit{Ble}\textsuperscript{R} insert to the mutant phenotype of interest before attempting to obtain the flanking DNA.

Once linkage has been established, inverse PCR and TAIL-PCR provide two rapid methods of obtaining flanking sequence information in the transformants. We have recently used both methods successfully with a mutant that has an insert in the Rubisco activase gene (Colombo \textit{et al.}, 2001). Having two different methods increases the chances of recovering the flanking DNA sequences and identifying the gene that has been disrupted by the insertion. TAIL-PCR requires very little enzymatic manipulations and thus makes the analysis of several mutants at the same time feasible. Inverse PCR requires more enzymatic manipulations, but in concert with a good Southern blot to determine the target fragment size, this technique is very robust and worth the extra enzymatic manipulations.

An alternative method to obtain the genomic DNA sequence of the region flanking an insertion is to generate a subgenomic library from the mutant that contains the \textit{Ble}\textsuperscript{R} insert. Although this method can yield the flanking genomic region, it is a labor intensive procedure when compared to the PCR techniques described in this paper and it also requires that the \textit{Ble}\textsuperscript{R} gene be linked to the mutant phenotype.

Attempts were made to use the plasmid rescue technique to obtain the flanking DNA sequence. Plasmid rescue has been used successfully in a very limited number of projects (Davies \textit{et al.}, 1999). Plasmid rescue relies on the fact that the inserted \textit{Ble}\textsuperscript{R} DNA is physically linked to vector genes to make a competent plasmid in \textit{E. coli}, namely
the \textit{Amp}^R, and \textit{Ori}. Our results show that the vector portion of the inserted DNA is rarely complete. In the five transformants analyzed thus far the complete vector has never been present making the plasmid rescue technique useless in these cell lines.

If an interesting mutant arises that is not linked to the \textit{Ble}^R gene it is always possible to utilize an indexed cosmid library to attempt to complement the mutant phenotype as a way to identify the mutated gene. This method has been used successfully by several laboratories (Funke \textit{et al.}, 1997, Xiang \textit{et al.}, 2001). The major drawback of this method is that it is very labor-intensive, but it is a proven technique that could salvage information from the 70\% of cell lines that do not show linkage to the \textit{Ble}^R gene.

Many research groups using random insertional mutagenesis to tag genes have reported that deletions at least as large as 20 kb commonly occur during the incorporation of DNA into the \textit{C. reinhardtii} nuclear genome (Dent \textit{et al.}, 2001). If large regions of DNA are deleted upon insertion of the \textit{Ble}^R the task of identifying which gene is responsible for the given phenotype is much more complicated. Deletions observed in the genes disrupted by the \textit{Ble}^R using the electroporation protocol outlined in this paper were relatively small and did not exceed 200 bp, suggesting that insertion events are occurring without the deletion of large amounts of genomic DNA. The method described in this paper, together with the ongoing genome project, present a practical methodology to study and identify CCM components in \textit{C. reinhardtii}. 

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CHAPTER 4
MOLECULAR AND GENETIC CHARACTERIZATION OF INSERTIONAL
BLE\textsuperscript{R} MUTANT SLC-230

Introduction

In an effort to identify regulatory and structural elements in the CCM of C. reinhardtii, 42,000 insertional mutants were generated by electroporating BLE\textsuperscript{R} into the strain D66 (nit2\textsuperscript{−} cw15 mt\textsuperscript{−}; Schnell and Lefebvre, 1993). Of these 42,000 transformants, 246 grew poorly on low CO\textsubscript{2} levels (100 ppm), indicative of a possible defect in the CCM or photosynthesis. One of these transformants, slc-230, was selected from this pool of mutants. slc-230 was further tested by tetrad analysis. Once linkage was shown demonstrating that Zeocin resistance was linked to the “slc” or “sick on low CO\textsubscript{2}” phenotype, further molecular characterizations were performed. First, inverse PCR (iPCR) was used to identify the locus of the BLE\textsuperscript{R} insertion. Next, northern blots and RT-PCR were used to demonstrate that the BLE\textsuperscript{R} insert identified by iPCR was, in fact, the functional insert giving rise to the mutant phenotype and to elucidate how the gene was regulated. Lastly, PCR was employed to screen the genomic cosmid library (Purton and Rochaix, 1994) to identify a complementary cosmid carrying the affected genomic region from a wild-type cell as well as to generate a screening strategy to quickly identify transformants.

Results

Phenotype and Tetrad Analysis

slc-230, after the selection on Zeocin and screening on low and high CO\textsubscript{2}, exhibited a phenotype typical of an slc mutant. slc-230 appeared to have a wild-type phenotype on TAP + light, TAP + dark, and MIN + high CO\textsubscript{2} (Fig. 4.1). Relative to D66
(the wild-type strain), slc-230 showed resistance on TAP + Zeocin, indicating it had a \textit{Ble}^R insert. The mutant also grew poorly on low CO\textsubscript{2} (Fig. 4.1).

Tetrad analysis was performed on slc-230 by back-crossing it to CC-124 (mt) as described by Harris, (1989). The progeny that resulted from 25 crosses demonstrated linkage of Zeocin resistance conferred by the \textit{Ble}^R cassette to the mutant phenotype (Fig. 4.2). The genetic linkage means that the functional \textit{Ble}^R insert was at, or extremely close to, the disrupted gene that lead to the poor growth phenotype. The resulting progeny that were linked showed a slightly different mutant phenotype than the parent mutant when re-screened; those progeny that demonstrated Zeocin resistance did not grow well in light under high CO\textsubscript{2} or low CO\textsubscript{2} on minimal media (Fig. 4.2) and seemed to be more sickly on high CO\textsubscript{2} than the original slc-230. One possibility for this is that slc-230 had accumulated a second change that partially adjusted for the original defect. It was noticed that slc-230 had a tendency to “revert” and needed to be re-selected every few months. From the growth results it appeared that slc-230 had a possible defect in dealing with photooxidative stress and might not be defective in an element of the CCM.

**Flanking Sequence of the \textit{Ble}^R Insert in slc-230**

Once it was established that the \textit{Ble}^R insert in slc-230 was linked to the sick on low CO\textsubscript{2} phenotype, iPCR was employed to determine the location of the insert. Products were obtained when iPCR was done on the genomic DNA from slc-230 digested with EcoR I or Sal I (Fig. 4.3). The EcoR I iPCR product was 1171 bp and the Sal I iPCR product was 1364 bp (Fig. 4.3). The resulting sequence from both iPCR products of the 3’ end of the \textit{Ble}^R insert showed that the insert was near the \textit{yptC5} gene.
Figure 4.1 Growth of slc-230 under different conditions. Here slc-230 is compared to other slc mutants (slc-208 and slc-170), wild-type (D66) and known CCM mutants (cia-3 and cia-5)
Figure 4.2 Linkage analysis of slc-230. This example of resulting progeny (4a, 4b, 4c, and 4d) from tetrad analysis by backcrossing slc-230 to CC-124 (mt') (wt) shows linkage of Zeocin resistance with the mutant phenotype. 4a and 4b show Zeocin resistance and an slc phenotype. This is a representative cross of about 25 crosses.
(Dietmaier et al., 1995), which has homology to genes encoding rab 7 proteins and is implicated in late endosomal maturation (Somsel-Rodman et al., 2000). When primers (y2794f and m01b) were designed using the sequence of yptC5 from Dietmaier et al., (1995) (Fig. 4.4) two cosmids (69A1 and 74D4) were identified from the genomic cosmid library to carry genomic DNA containing the locus of yptC5. Upon further sequencing of the region near yptC5, a second, overlapping open reading frame 3’ of yptC5 in the C. reinhardtii EST (Expressed Sequence Tag) database confirmed the existence of a second gene close to yptC5 (Fig. 4.5).

**Sequence Analyses**

This second open reading frame overlapping the 3’ end of yptC5 is homologous with EST’s in the Chlamydomonas database. The ESTs starting from the 5’ end of the message in the database are of three lengths (Fig. 4.6). 1031029H11.y2 is the longest EST and starts with CAAT. 1031029H11.y2 comes from the “Stress II” library EST’s. “Stress II” conditions include nitrogen starvation, exposure to ROS ($\text{H}_2\text{O}_2$), and heavy metals (Cd). Three other EST’s were about ca. 120 bp shorter than 1031029H11.y2. The EST’s came from the “Stress I” library which includes low CO$_2$, the TAP + light library, and the gametogenesis library. A final, shorter EST obtained from the gametogenesis library was also present (Fig. 4.6). In addition to these EST’s for the 5’ end there were additional EST’s from the 3’ end of the gene. 5’ RACE was done on RNA from wild-type cells (D66) grown under low CO$_2$ to determine the length of the Hdh1 transcript (Fig. 4.7B). It was found that the long EST was present in the mRNA and the cDNA library (Fig. 4.7B, lanes 1 and 3). The EST 110 bp shorter was also present (Fig. 4.7B...
Figure 4.3 iPCR results from slc-230. Sac I and EcoR I digests yielded PCR fragments that were sequenced using ABI’s Big Dye terminator method.
\textbf{Figure 4.4} Map of the \textit{Ble}^R cassette insertion in slc-230. The exons for \textit{yptC5} (green), \textit{Ble}^R (orange), and \textit{Hdh1} (yellow) are shown as arrows indicating direction of transcription. This figure also illustrates the location of restriction sites (EcoR I and Sac I) used in iPCR, and the PCR primers used in iPCR.
Figure 4.5 Map of wild-type yptC5-Hdh1 locus. The exons for yptC5 (green) and Hdh1 (yellow) are shown as arrows indicating direction of transcription. This illustration shows restriction sites used for iPCR, overlapping yptC5 3’ UTR and Hdh1 promoter region, and primer sites used to amplify the overlapping region.
**Figure 4.6** Close-up view of 5' end of Hdh1. This figure includes each of the y read EST's and the primers used in RT-PCR.
Figure 4.7 5’RACE of Hdh1. A, To confirm the length of the Hdh1 mRNA, 5’ RACE was performed on mRNA from low CO₂ grown wild-type (D66) cells, then a nested PCR was performed on the 5’ RACE products (Lanes 1 and 2) and the cDNA library (Lane 3). B, Lane 1 confirms the presence of the longest mRNA in the EST database starting with “CAAT” by using primers f1 and r2. Lane 2 confirms the shorter mRNA by use of the adaptor primer from Ambion and primer r2. Lane 3 confirms the presence of the longest mRNA in the cDNA library using primer f1 and r2. Courtesy of Ruby Ynalvez.
lane 2) showing that both messages are present in wild-type cells under low CO$_2$
conditions.

The resulting contig of all of the EST’s using CAP3 (Contig Alignment Program)
(http://fenice.tigem.it/bioprg/interfaces/cap3.html) was 1357 bp. Primers made on the 5’
end of the gene using the sequence from the final contig of EST’s were used to generate
PCR fragments from cosmid 69A1 to elucidate the genomic sequence. The cDNA and
genomic sequences were combined to give the sequence shown in Fig. 4.8. This was
confirmed by the genomic C. reinhardtii sequence at DOE Joint Genome Initiative
(scaffold 443 in v.1; scaffold 9 in v.2). The genomic transcript is 2715 bp. The transcript
contains 10 exons and the genomic region with these exons highlighted is shown in Fig.
4.5 and Fig. 4.8.

This second gene’s sequence of 247 amino acid residues (Fig. 4.9) showed
predicted homology to halo-acid dehalogenase-like hydrolases in Ferroplasma
acidarmanus, Methanothermobacter thermoautotrophicus, and Chlorobium tepidum and
was named “Hdh1” (Fig. 4.10). Using the pFAM database at NCBI the translated EST’s
of Hdh1 were predicted to have a possible general phosphatase function. Hdh1 has two
possible translation start sites, indicated in Fig. 4.6. The presence of the target peptide
was checked. The shorter of the two sequences was not predicted to have a targeting
sequence. However, the ChloroP program predicted the Hdh1 protein to be targeted to
the chloroplast with a score of 0.431 and a leader sequence of 32 amino acids. While this
is a relatively low score, none of these programs are trained for Chlamydomonas proteins
and this score is similar to scores obtained with other known C. reinhardtii chloroplast
Figure 4.8 Genomic nucleotide sequence of $Hdh1$. The possible translational start sites, the stop codon, and the polyadenylation signal are indicated in red. The exons are bold-faced uppercase and the introns are in lowercase. The arrow indicates the point of $Ble^R$ insertion.
Figure 4.9  Predicted amino acid sequence of *Hdh1*. The methionine residues in bold-faced text indicate potential translation start sites.
Figure 4.10  ClustalW alignment of the predicted amino acid sequence of Hdh1. This has boxshade output against predicted amino acid sequences from *Ferroplasma acidarmanus* (Fa), *Chlorobium tepidum* (Ct), and *Methanothermobacter thermoautotrophicus* (Mt). Residues that match in 50% of the sequences or better are in the “Consensus” line. Residues in uppercase indicate that residues matched in all four sequences, while residues in lower
proteins. It also may be significant that all of these predicted proteins match *Hdh1* after 32 amino acids possibly indicating a signal peptide for the chloroplast (Fig. 4.10) or possibly the mitochondria since some proteins *predicted* to be in the chloroplast go to the mitochondria.

Further PCR reactions were done on slc-230 genomic DNA to determine the extent and location of disruption by the *Ble*<sup>R</sup> insert. To do this a series of primer pairs were designed flanking the location of the insert (Fig. 4.11). These amplifications showed that the *yptC5* coding region was intact by priming with y2313f and y2912b, producing a fragment from the distal 3’ end of *yptC5* (Fig. 4.11 A). Sequence analysis of the fragment amplified by y2794f and Ble3b (the distal 3’ end of *yptC5* into the *Ble*<sup>R</sup> insert) confirmed the location of the insert in the 3’ UTR of *yptC5* (Fig. 4.11 B). Amplification of slc-230 DNA with primers m0097f and m01b showed that the distal 5’ end of *Hdh1*, 80 bp into the long EST was intact (Fig. 4.11 D). These PCR’s showed that there was no large deletions in the *yptC5-Hdh1* locus of slc-230. Fig. 4.11 C shows that an amplicon can be formed in wild-type with primers y2794f and m01b; however no amplicon can be found in slc-230 as the region between these primers contains the *Ble*<sup>R</sup> insert and it has been found that many times these inserts are concatamers in a tandem repeat arrangement (Pollock, 2002, personal communication).

**RT-PCR and Northern Blots**

During the sequencing of slc-230 DNA in the region containing the *Ble*<sup>R</sup> insert, it was determined that part of the 3’ UTR of the *Ble*<sup>R</sup> had been lost during the transformation and insertion. This raised the possibility that the *Ble*<sup>R</sup> insert was not
Figure 4.11  Map of selected yptC5 and Hdh1 primers and their PCR products. A, PCR products from D66 and slc-230 genomic DNA using the y2313f and y2912b primer pair; B, PCR products using y2794f and Ble3b; C, PCR products from slc-230 and D66 using y2794f and m01b, Note: slc-230 does not form product, as indicated by the dashed line; D, PCR products using cosmid 74D4, D66, slc-230, and slc-230 4b with m0097f and m01b.
encoding a functional protein. The absence of the 3’ UTR implies that the incomplete message was being transcribed and degraded, not translated into the gene product.

To answer the question as to whether or not the $Ble^R$ insert in the $yptC5$-Hdh1 locus was the functional insert a northern blot, using the second exon of the $Ble^R$ gene as a probe was used to compare $Ble^R$ transcript size and abundance in slc-230 to D66 and slc-25 (a $Ble^R$ insertional mutant in rubisco activase). The $Ble^R$ construct has a 3’ UTR that is derived from $RbcS2$ (Fig. 3.1). During the course of sequencing it was found that the $Ble^R$ 3’ UTR was truncated and this led to the hypothesis that the message for $Ble^R$ in slc-230 would be a different length than in other slc mutants. As predicted, the functional $Ble^R$ transcript in slc-230 had a shorter length than a normal $Ble^R$ insert (Fig. 4.12). This observation is consistent with the expectation that the insert would be a different length as it was seen by sequence analysis that the 3’ UTR of the insert was truncated in the process of insertion and includes part of the $yptC5$ 3’ end.

The locus of the functional $Ble^R$ insertion was also tested using RT-PCR. Because the 3’ UTR of the $Ble^R$ insert was interrupted by the 3’ UTR of $yptC5$, there should be part of the $yptC5$ transcript in $Ble^R$ mRNA. Further confirmation of the locus of the expressed $Ble^R$ was shown by amplifying with primers that lie in the $yptC5$ and the $Ble^R$ sequences. RT-PCR demonstrated that primers for the 5’ end of $Ble^R$ and the 3’ end of the $yptC5$ transcript amplified a message in slc-230 from mRNA of 585 bp, but not in slc-25, which has the normal insert (Fig. 4.13, lanes 1 and 2). This demonstrated that the insert at this locus was being expressed.

Additionally, to show that another $Ble^R$ insert in slc-230 was not being expressed in slc-230, RT-PCR was performed using primers for the 5’ and 3’ ends of $Ble^R$ (Ble5a
and BleD) to amplify the insert’s full length message. This amplification only worked for slc-25 and formed a spurious band in slc-230 (arising from the fact that BleD will prime itself in RbcS2) confirming that the only expressed BleR insert in slc-230 is at the locus of yptC5-Hdh1 originally identified by iPCR (Fig. 4.13 lanes 3 and 4). None of these bands arose from genomic DNA (Fig. 4.13, Lanes 5 and 6) as the primers used were engineered to straddle exons.

Qualitative RT-PCR was used to determine the presence of the long mRNA of Hdh1 from the EST database. The determination of the presence or absence of this transcript would indicate the extent of the interruption from the BleR insert. If the longest EST were the message then the insert would interrupt the first exon causing a knockout of Hdh1. If the longest EST were not present, then the BleR insert would be interrupting the promoter region of Hdh1. The result from the second scenario might result in expression of Hdh1, but at an altered level. Fig. 4.13 confirms the presence of the longest message in the EST database for Hdh1 as the primer GGF1 is located at the extreme 5’ of the longest EST corresponding to the Hdh1 gene (Fig. 4.6). The long message is present in CC-124 (mt) (wt) under high CO₂ and CC-124 (mt) acclimated to low CO₂ (Fig. 4.14, Lanes 1 and 2). Not surprisingly, this message is not present in slc-230 acclimated to low CO₂ (Fig. 4.14, Lane 3). These results support the hypothesis that the BleR has disrupted the proper transcript of Hdh1.

Northern blots were also utilized to estimate if the expression of yptC5 was different in slc-230 versus CC-125. As mentioned earlier, the BleR insert is in the very 3’ end of the yptC5 3’ UTR. A northern blot using a 528 bp fragment amplified from the 3’ UTR of yptC5 using yptC5 FN1 and yptC5 RN1 primers as a probe showed that the
**Figure 4.12** Northern blot showing altered $Ble^R$ message length. This northern blot uses the first intron of the $Ble^R$ cassette as a probe. slc-25 and slc-230 show a different predicted message length because of the truncated 3’ UTR in slc-230. The same RNA samples were probed with GAPDH as a positive control and loading control. “a” indicates the BleR transcript from slc-25 and “b” indicates the BleR transcript from slc-230.
Figure 4.13 RT-PCR showing the presence of the yptC5-Ble\textsuperscript{R} fragment in slc-230. A, Lane 1, slc-25 with byIf and byIb; Lane 2. slc-230 with byIf and byIb; Lane 3, slc-25 with Ble5a, and BleD; Lane 4, slc-230 with Ble5a, and BleD (note the spurious band arises as Ble5a primes in \textit{RbcS2}); Lane 5, slc-25 genomic DNA with byIf and byIb; Lane 6, slc-230 genomic with byIf and byIb. 2-log from NEB was used as a size standard (ST). B, Maps of the Ble\textsuperscript{R} gene with primer sites and the slc-230 locus of Ble\textsuperscript{R} insertion with primers used in RT-PCR
Figure 4.14  Qualitative RT-PCR of Hdh1 expression. Lane 1, primers GGF1 and GGR to amplify the message for Hdh1 were used on low CO₂ acclimated CC-124 (wt) RNA; Lane 2, primers GGF1 and GGR were used on high CO₂ acclimated CC-124 RNA; Lane 3, primers GGF1 and GGR were used on low CO₂ acclimated slc-230 RNA. GAPDH was amplified as a control; Lane 4, primers GAPDH FRT 2 and GAPDH RRT2 were used on low CO₂ acclimated CC-124 (wt) and; Lane 5, slc-230. 2-log from NEB was used as a standard (ST). Locations of the primer pairs for lanes 1-3 can be found in figure 4.6. Courtesy of Baran Tural.
abundance of \textit{yptC5} and the length of its message in slc-230 (Fig. 4.15, lane 2) were similar to CC-125 (wild-type) (Fig. 4.15, lane 1) and slc-25 (Fig. 4.15, lane 3). This northern blot additionally confirmed that the 3’ UTR of \textit{yptC5} doubles as the 3’ UTR of the \textit{Ble}^{R} insertion in slc-230 (Fig. 4.15, lane 2). Northern blots utilized in an attempt to determine if the quantity of \textit{Hdh1} mRNA in slc-230 was decreased, yielded no data as it appears that the transcript abundance of \textit{Hdh1} is very low in wild-type cells as well as slc-230.

In an effort to estimate the relative level of transcription of \textit{Hdh1} mRNA in wild-type, semi-quantitative RT-PCR was employed. Semi-quantitative RT-PCR showed that the \textit{Hdh1} transcript increased after 24 hr under low CO\textsubscript{2} conditions in wild-type (CC-125) cells (Fig. 4.16 lane 2)

\textbf{Complementation}

Because it was assumed that slc-230 was interfering with the expression of \textit{Hdh1}, it followed that re-introduction of the wild-type copy of \textit{Hdh1} to the slc-230 nuclear genome would restore the wild-type phenotype. Nuclear transformation was initially performed on slc-230 using the glass bead method (Kindle, 1990) to introduce the genomic copy of the \textit{yptC5-Hdh1} locus contained in cosmids 69A1 and 74D4. Both 69A1 and 74D4 were selected from the cosmid library as they both produced an amplicon in \textit{yptC5}. These two cosmids were then mapped by PCR (Fig. 4.17). PCR was done on genomic DNA (D66), cosmid 74D4, and 69A1 with primers designed 3’ of \textit{Hdh1} (83HU and 83HL, 53HU and 53HL, 5’ no. 2U and 5’ no. 2L), in \textit{Hdh1} (H-35U and H35L, m01f and m605b), in \textit{yptC5} (y0453f and y2912b), and 5’ of \textit{yptC5} (75yU and 75yL; 55yU and 55yL; 3’ no. 1U and 3’no.1L) (Fig. 4.17). These primers all worked on genomic DNA (D66) and cosmid 69A1, but the 75y (75yU and 75yL) and 55y (55yU and 55yL) sets did
not generate a fragment from cosmid 74D4 indicating the absence of that length of DNA in cosmid 74D4 (Fig. 4.17).

Cosmid 74D4 was found to complement slc-230. The resulting transformants from the introduction of 74D4 appeared in the lawn of slc-230 after approximately four weeks of growth on 50 ppm CO₂ (Fig. 4.18 B). When these colonies were re-screened they showed a wild-type growth phenotype under high and low CO₂ (Fig. 4.18 C and D).

Further characterization by using PCR to amplify the yptC5-Hdh1 locus demonstrated that the transformants contained the wild-type locus by priming with y2794f and m01b (Fig. 4.18 E) to form the diagnostic wild-type 486 bp fragment that straddles the region between yptC5 and Hdh1. This region cannot be amplified in slc-230 because of the interruption by the Ble⁸ insert (Fig. 4.11C). As mentioned before, the original slc-230 had a tendency to revert perhaps due to a second site suppressor. Although revertants might appear to be wild-type, this PCR strategy does not produce an amplicon in revertants.

Discussion

slc-230 is a Ble⁸ insertional mutant with an “slc” phenotype (Fig. 4.1). The phenotype is linked to Zeocin resistance as shown by tetrad analysis (Fig. 4.2) showing linkage between Zeocin resistance and the slc phenotype. The resulting growth phenotype from the progeny slc 230 4a and slc-230 4b might arise from two possible sources. One possibility is that there may be a second site repressor in the original slc-230 that, over the course of maintaining the culture, allows slc-230 to revert to a less sickly phenotype requiring occasional reselection. When slc-230 was crossed with wild-type strains this suppression might be crossed out resulting in progeny with the
Figure 4.15  Northern Blot showing the abundance of yptC5 and confirming the locus of the Ble$^R$ insertion.  A, northern blot using a part of the yptC5 3’ UTR as the probe, Lane 1, CC-125 (wild-type); Lane 2, slc-230; and Lane 3, slc-25 (Ble$^R$ insertional mutant in rubisco activase).  B, Picture of a formaldehyde gel fluorescently stained with ethidium bromide revealing the ribosomal RNA bands and showing even RNA loading. slc-230 (Lanes A2) shows two bands: and upper band for yptC5 and a lower band Ble$^R$ confirming that the active Ble$^R$ insert is in the 3’ end of yptC5 and that yptC5 is being expressed at normal levels and at the normal length.  Courtesy of Baran Tural.
Figure 4.16 Expression of *Hdh1* in CC-125 by semi-quantitative RT-PCR. A, semi-quantitative RT-PCR of *Hdh1* (upper bands) of CC-125 (wild-type) in high CO₂ (Lane 1) and 24 hours of low CO₂ (Lane 2) show a slightly increased amount of transcript. The lower bands in A are from GAPDH and the total RNA loads used in the RT-PCR (B) indicates use of equal amounts of RNA used as starting material for RT-PCR. *Courtesy of Baran Tural.*
Figure 4.17  Mapping of 69A1 and 74D4 by PCR. A PCR of genomic DNA (D66; Lane 1), 74D4 (Lane 2), and 69A1 (Lane 3) canvassing an 18.4 Kb region around the yptC5-Hdh1 locus. Red lines represent location and relative length of amplicons.
Figure 4.18 Complementation of slc-230 4b with genomic cosmid 74D4. Slc-230 4b was transformed using the glass bead method with cosmid 74D4 or no DNA. A, slc-230 4b transformed with no DNA; B, slc-230 4b transformed with cosmid 74D4 and grown in low CO₂. Darker colonies, like the one indicated by the arrow, were selected and further investigated; C, Growth of putative transformants from B compared with wild-type, cia-5, cia-3, and slc-230 4b on low CO₂; D, Growth of putative transformants on high CO₂; E, Confirmation by PCR that the putative transformants contain the wild-type DNA.
appearance of a defect in dealing with photooxidative stress. It should be noted that strain slc-230 4b was chosen for complementation purposes because it was the most yellow in color and therefore the best to screen in complementation experiments. Another reason the phenotype of the progeny of slc-230 and CC-124 (mt) (slc-230 4a and slc-230 4b), appear to be as yellowish and slow growing high CO$_2$ as low CO$_2$ than the original slc-230 (Fig. 4.2), is that they are exhibiting a phenotype more consistent with a defect in photosynthesis. The poor autotrophic growth may be due to an eventual accumulation of reactive oxygen species (ROS).

iPCR demonstrated that the $Ble^8$ insert was located near the junction of two genes: $yptC5$ and a novel gene, $Hdh1$. $yptC5$ encodes a rab7 protein and $Hdh1$ is predicted to encode a novel, single-copy gene that may have a possible phosphatase function with homology to haloacid dehalogenases in archaebacteria (Fig. 4.9). However, it should be noted that the pFAM database also indicated that $Hdh1$ has similarities to phosphohexomutase or epoxide hydrolase in addition to a general phosphatase. The Conserved Domain Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd) lists the haloacid dehalogenase-like hydrolase family as including L-2-haloacid dehalogenase, epoxide hydrolases and phosphatases. Homodimers of the L-2-Haloacid dehalogenase catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids to yield the corresponding D-2-hydroxyalkanoic acids in $Pseudomonas$ $sp.$ YL (Hisano $et$ $al.$, 1996). Epoxide hydrolases are a family of enzymes that hydrolyze a variety of exogenous and endogenous epoxides to their corresponding diols (Grant $et$ $al.$, 1993). A soluble murine epoxide hydrolase (sEH) seems to have substrates that appear to be 1,2-disubstituted aliphatic epoxides (Wixtrom and Hammock, 1985). And, the $\oplus(1,3)$
fucosyltransferase in *Heliobacter pylori*, which also is in this family, has homology to phosphoserine phosphatase (Martin *et al.*, 1997). All of these proteins remove a group attached to a carbon atom (epoxide, halogen, or phosphate) and replace it with an OH group. The inherent problem with the homology matches of *Hdh1* to predicted proteins in *Ferroplasma, Methanothermobacter*, and *Chlorobium* is that the homologies are simply to the predicted amino acid sequences from predicted open reading frames from the genome sequencing results of those organisms and there has been no actual molecular biology or enzymology performed on those particular genes.

The two genes, *yptC5* and *Hdh1* are coded on the same strand. EST’s indicate that the gene structure of the *yptC5-Hdh1* locus is such that the 3’ UTR of *yptC5* sometimes overlaps with the longest EST (1031029H11.y2) of *Hdh1* (Fig. 4.6). While unusual, the coding regions of GdcH, Pr46a, and Pr46b overlap in *C. reinhardtii* in the mating type locus (Ferris *et al.*, 2002). Additionally, there are two messages for *Hdh1* as confirmed by 5’ RACE (Fig. 4.7). A long message whose ATG includes a 32 amino acid leader that could be a signal peptide for the chloroplast or mitochondria and a shorter message. The long *Hdh1* message is present in wild-type mRNA messages, but not slc-230 mRNA as indicated by qualitative RT-PCR (Fig. 4.14). Assuming that the long EST is the correct message then the *Ble* insert is in the first exon of *Hdh1*. The shorter EST’s (Fig. 4.6) may be generated as they are preceded by a putative CAAT box and TATAA box and may be the result of an alternate transcription start. 5’ RACE confirmed the presence of both EST’s in mRNA from wild-type and the long EST was also found in the cDNA library (Fig. 4.7). It should be noted that the conditions that give rise to the longer message (Stress II) were not the same conditions that gave rise to shorter messages.
(Stress I and gametogenesis). However, the long EST was confirmed using RNA from both high and low CO₂ grown wild-type cells.

The functional insert, as determined using iPCR (Fig. 4.3), is located in the 3’ end of *Hdh1* (or the 5’ end of *yptC5* as the two genes overlap) (Fig. 4.4). The hypothesis that this particular insert is being expressed in slc-230 is supported by a northern blot using the first intron of *Ble* (Fig. 4.12), and RT-PCR (4.13). Sequence analysis of the *Ble* insert indicates that much of the 3’ UTR of the *Ble* gene was lost during insertion (Fig. 4.13B). This led to the question of whether the *Ble* insert in the *yptC5-Hdh1* locus was functional. Northern blot analysis showing an altered length for the *Ble* transcript in slc-230 (Fig. 4.12) indicated that the *Ble* message size in slc-230 was consistent with an altered length and contained part of the *yptC5* 3’ UTR, but that slc-230 did not contain a “normal” copy of *Ble* as could be found in slc-25.

The *Ble* insert appears to be affecting the expression of *Hdh1* and not *yptC5*. Qualitative RT-PCR confirms the presence of the long mRNA of *Hdh1* in wild-type (CC-124) under high CO₂ and low CO₂, but not in slc-230 (Fig. 4.14). Additionally, a northern blot indicates that the transcript size and abundance of *yptC5* is the same in both slc-230 and wild-type cells (Fig. 4.15). These two pieces of data would indicate that for the proximity of *yptC5* and *Hdh1*, only the expression of *Hdh1* is affected in slc-230 as there seems to be no *Hdh1* mRNA present and the *yptC5* mRNA in slc-230 appears to be the same as wild-type.

Semi-quantitative RT-PCR would also indicate that there is a slight upregulation of *Hdh1* under low CO₂ (Fig. 4.16) that may be consistent with a function as a regulatory phosphatase as opposed to a structural gene. The inability to detect the mRNA for *Hdh1* using northern blot analysis (data not shown) may also indicate that *Hdh1* is transcribed
in very low amounts consistent with the hypothesis that \textit{Hdh1} is a regulatory element. Low levels of expression and a predicted phosphatase function would be consistent with a regulatory element. Likewise, the CCM1 gene, or cia-5, encodes a regulatory element in the CCM and it is hypothesized that cia-5 is essential to control the induction of the CCM (Fukuzawa \textit{et al.}, 2001 and Xiang \textit{et al.}, 2001).

The hypothesis that the functional \textit{Ble}^{8}\textsuperscript{R} insert is located between the \textit{yptC5} and \textit{Hdh1} genes and that the interruption is affecting \textit{Hdh1} giving rise to the slc phenotype is further supported by the complementation studies (Fig. 4.18). Originally, two cosmids (69A1 and 74D4) containing the \textit{yptC5-Hdh1} locus were selected from the cosmid library by screening with PCR. Mapping the region 5’ and 3’ of the locus by PCR showed that both 69A1 and 74D4 contained a full copy of \textit{Hdh1} (Fig. 4.17). A cosmid (74D4) containing the genomic region including \textit{yptC5} and \textit{Hdh1} successfully complemented the slc-230 mutation (4.18). Although these experiments coupled with the qualitative RT-PCR (Fig. 4.15) support the idea that \textit{Hdh1} and not \textit{yptC5} is responsible for the mutant phenotype it does not clearly determine if \textit{Hdh1} is the gene needed to complement slc-230. Further complementations are in progress employing 74D4 and 69A1, digests of 74D4 that either obliterate \textit{yptC5} (YKO’s) or \textit{Hdh1} (HKO’s) as well as the genomic copies of \textit{yptC5} or \textit{Hdh1} in pBlueScript.

To better understand the nature of the effects of a mutation in \textit{Hdh1}, physiological studies need to be done on slc-230 to better elucidate the effects of the altered expression of \textit{Hdh1}. 
CHAPTER 5

PHYSIOLOGICAL CHARACTERIZATION OF INSERTIONAL $BLE^R$ MUTANT SLC-230

Introduction

slc-230 was shown to have a $BLE^R$ insert at the locus between yptC5, which encodes a rab 7 protein, and $Hdh1$, a gene that encodes a protein that may have an epoxidase, hydrolase or phosphatase function. Further data that suggested that this specific $BLE^R$ insertion gave rise to the slc phenotype was by complementation by transformation with cosmid 74D4 which carries the wild-type $Hdh1$ gene. In an effort to better elucidate the effects of the altered expression and thus the probable function of $Hdh1$, slc-230 was further characterized physiologically.

Since slc-230 grew poorly on minimal media, the most important set of physiological parameter that could be investigated was the determination of the rate of photosynthesis of slc-230 compared to that of wild-type cells. Also, investigation of the rate of photosynthesis of slc-230 would allow a determination of the mutant’s $K_{0.5}(C_i)$ which provides an estimation of the strain’s affinity for inorganic carbon ($C_i$). While doing the photosynthetic assays, it was observed that another symptom of slc-230’s phenotype was an altered chlorophyll ratio. Because of this altered chlorophyll ratio, pigment analyses by HPLC were performed. In addition, the pigments associated with the xanthophyll cycle and lipid composition were investigated, as the linked progeny of slc-230 appeared to be photosensitive.

Although the results of these physiological experiments do not completely characterize the function of $Hdh1$, they give a clearer picture of the symptoms of its altered expression and lead to a better idea of the possible function of $Hdh1$. 

90
Results

Oxygen Evolution

Because slc-230 grew poorly on minimal media, photosynthetic rates were estimated by measuring oxygen evolution with a Clark-type oxygen electrode. This photosynthesis assay allows one to measure the $K_{0.5}(C_i)$ of the cells and use that as an estimation of the affinity of the cells for $C_i$. At high CO$_2$ levels (5% CO$_2$), *C. reinhardtii* has C3 photosynthesis and has a low affinity (elevated $K_{0.5}(C_i)$) for $C_i$; however, after the culture is placed under low CO$_2$ conditions (.035% CO$_2$) the cells acclimate and have a higher affinity (lowered $K_{0.5}(C_i)$) for $C_i$. Therefore, the affinities of wild-type cells, slc-230, and cia-5 (a known CCM mutant) for $C_i$ were estimated. Each oxygen evolution experiment was done three independent times for each strain. The means were plotted with error bars representing 1 standard error. Standard error was used as it is similar to standard deviation, but more appropriate for smaller sample sizes. And the $K_{0.5}(C_i)$ was estimated.

Under high CO$_2$ conditions, wild-type strains D66 and CC-124 had $K_{0.5}(C_i)$’s of 170 $\mu$M $C_i$ (Fig. 5.1) and 250 $\mu$M $C_i$ (Fig. 5.2) respectively. slc-230 had a $K_{0.5}(C_i)$ of 212 $\mu$M $C_i$ (Fig. 5.3) and cia-5 had a $K_{0.5}(C_i)$ of 172 $\mu$M $C_i$ (Fig. 5.4).

It was found that after acclimating cultures to low CO$_2$ (4 hr at air levels of CO$_2$), that slc-230 had a $K_{0.5}(C_i)$ of 65 $\mu$M $C_i$ (Fig. 5.3) while wild-type strains D66 and CC-125 had lower $K_{0.5}(C_i)$’s of 25 $\mu$M $C_i$ (Fig. 5.1) and 15 $\mu$M $C_i$ (Fig. 5.2) respectively. cia-5, a known CCM mutant defective in a transcriptional element in the CCM, was found to have a $K_{0.5}(C_i)$ of 110 $\mu$M $C_i$ (Fig. 5.4) in the same set of experiments.

Additionally, these experiments showed that slc-230 had a maximal rate of photosynthesis similar to that of wild-type strains D66 and CC-124. Under high CO$_2$
conditions, D66 and CC-124 had maximal rates of photosynthesis at 120 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) (Fig. 5.1) and 110 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) (Fig. 5.2) respectively, while air acclimated cultures of D66 and CC-124 had maximal rates of photosynthesis of 120 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) (Fig. 5.1) and 110 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) (Fig. 5.2) respectively. Under high CO\(_2\) conditions, slc-230 had a maximal rate of photosynthesis of 130 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) and had a maximal rate of photosynthesis of 96 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) under low CO\(_2\) conditions for 4 hr (Fig. 5.3).

Although the rates of oxygen evolution were similar to that of wild-type initially at high CO\(_2\) and at 4 hr acclimation, extended exposure under diffusion conditions (simple diffusion of CO\(_2\) from the atmosphere into the culture during shaking as opposed to bubbling with air or CO\(_2\)) over a 64 hr period showed that slc-230 had a marked decrease in maximal rate of photosynthesis when compared to that of D66 (Fig. 5.5 and Table 5.1). Figure 5.5 shows that initially slc-230 was not statistically different from D66 with a mean maximal rate of photosynthesis of 110 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) while D66 had a mean maximal rate of photosynthesis of 150 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \). However, after 64 hr, the mean maximal rate of photosynthesis of slc-230 was only 10 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) while D66 was still relatively high at 90 \( \mu \text{mol O}_2 \ \text{mg chl}^{-1} \ \text{hr}^{-1} \) (Fig. 5.5 and Table 5.1). It was also observed that when CO\(_2\) concentration was measured in the flasks when the ambient CO\(_2\) was 484 ppm the flasks containing D66 had a mean concentration of 157 ppm and slc-230 had a mean concentration of 463.5 ppm indicating that D66 was more effective at pulling CO\(_2\) out of the local atmosphere.

**Pigment Analyses**

It was noted during the oxygen evolution experiments that slc-230 had an unusual ratio of chlorophyll a to chlorophyll b when the amount of chlorophylls was measured in
the cells. The chlorophyll content and distribution in slc-230 were markedly different than in D66. slc-230 had approximately 40% of the total chlorophylls and approximately a third of the chlorophyll a found in wild-type (Fig. 5.6). slc-230 had about half the chlorophyll b of wild-type (Fig. 5.6). slc-230 was also found to have a chlorophyll a: chlorophyll b ratio of approximately 3:2 ($\mu$g chlorophyll a: $\mu$g chlorophyll b) compared to the 2:1 ratio of D66 (Fig. 5.6). Further investigation of chlorophyll ratios, over a 96 hr period under diffusion conditions showed that at the end of the 96 hr time course there was no significant increase in the ratio of chlorophyll a to chlorophyll b in slc-230 while the ratio of CC-125 increased over time (Fig. 5.7). Additionally, the linked progeny of slc-230 were used in this experiment and were found to have the same pigment characteristic. Furthermore, since the cells used for oxygen evolution were assayed by chlorophyll content and not cell count one can estimate that the $V_{\text{max}}$ of slc-230 would be much lower than wild-type (Fig. 5.3) and that the decreasing $V_{\text{max}}$ over time (Fig. 5.5) would be even lower.

One of the predicted functions of *Hdh1* is that it acts as an epoxide hydrolase. This, with the supposition that slc-230 is a photosynthetic mutant, led to the postulation that slc-230 might have a defect in the xanthophyll cycle (Fig. 1.2), specifically, in violaxanthin de-epoxidase. Such a mutant in *C. reinhardtii*, npq1, has been generated by insertional mutagenesis and is well characterized (Niyogi *et al.*, 1997). Although the npq1 mutation is not fatal under bright light, a double mutant (npq1-lor1) deficient in quenching xanthophylls (the npq1 mutation) and lutein (the lor1 mutation) will expire under high light (Baroli *et al.*, 2003). The npq1-lor1 double mutant parallels slc-230 in its chlorophyll content and lowered photosynthetic rate over time. To this end, slc-230 and CC-125 were dark adapted for 24 hr then “flashed” at 500 $\mu$E m$^{-2}$ s$^{-1}$ for 90 min with
samples being taken every 30 min. When the xanthophyll ratios were calculated, it was found that slc-230 did not accumulate quenching xanthophylls (.45 A+Z/V+A+Z) to the proportion that CC-125 did (.72 A+Z/V+A+Z) (Fig. 5.8). Again, this set of data additionally shows that the linked progeny, slc-230 4a and slc-230 4b, have the same phenotype as slc-230 (Fig. 5.8). The actual measurements of the three xanthophyll species are shown for wild-type and slc-230 in Table 5.2.

The effect of nitrogen starvation on pigments was also briefly investigated as it has been found that nitrogen starvation affects pigment composition (Young and Beardall, 2003) and increases the proportion quenching xanthophylls (Verhoeven et al., 1997). It was found that slc-230 does not adjust its pigments like D66 under low nitrogen conditions. Notably, zeaxanthin was not present initially and did not increase under nitrogen starvation (Table 5.3).

**Thin Layer Chromatography**

An altered lipid composition might also effect the xanthophyll cycle as MGDG is required for the function of VDE. To investigate whether slc-230 has an altered composition of phospholipids, lipids were extracted from D66 and slc-230 under low light (50 μE m⁻² s⁻¹) and high light (500 μE m⁻² s⁻¹) regimens and visualized with iodine. When samples were run on two-dimensional thin layer chromatographs, there were no discernable shifts or change in quantity in the major phospholipids between the two strains (Fig. 5.9).

**Discussion**

slc-230 is a $Ble^R$ insertional mutant exhibiting an slc phenotype. Molecular studies of slc-230 indicate that the $Ble^R$ insert is located between $yptC5$ and $Hdh1$. By
Figure 5.1 Oxygen evolution of *Chlamydomonas reinhardtii* mutant D66. This graph shows the rate of photosynthesis vs. $C_i$ in the form of $\text{HCO}_3^-$. “[]” represents cells grown under high CO$_2$ (5%) in minimal media. “[]” represents cells that have been grown under high CO$_2$ then acclimated for 4 hr to air levels of CO$_2$ in minimal media. Error bars represent the standard error of the experiment.
Figure 5.2 Oxygen evolution of *Chalmydomonas reinhardtii* CC-124. This graph shows the rate of photosynthesis vs. $C_i$ in the form of $HCO_3^-$. “[]” represents cells grown under high $CO_2$ (5%) in minimal media. “[]” represents cells that have been grown under high $CO_2$ then acclimated for 4 hr to air levels of $CO_2$ in minimal media. Error bars represent the standard error of the experiment.
Figure 5.3 Oxygen evolution of *Chlamydomonas reinhardtii* mutant slc-230. This graph shows the rate of photosynthesis vs. $C_i$ in the form of $\text{HCO}_3^-$. “[]” represents cells grown under high CO$_2$ (5%) in minimal media. “[]” represents cells that have been grown under high CO$_2$ then acclimated for 4 hr to air levels of CO$_2$ in minimal media. Error bars represent the standard error of the experiment.
Figure 5.4 Oxygen evolution of *Chalmydomonas reinhardtii* mutant cia-5. cia-5 is a known CCM mutant defective in an element responsible for induction of the CCM. This graph shows the rate of photosynthesis vs. $C_i$ in the form of $\text{HCO}_3^-$. “[]” represents cells grown under high CO$_2$ (5%) in minimal media. “[■]” represents cells that have been grown under high CO$_2$ then acclimated for 4 hr to air levels of CO$_2$ in minimal media. Error bars represent the standard error of the experiment.
Figure 5.5 Maximal rate of photosynthesis during acclimation to limiting CO₂. Starting at 6 hr of acclimation the maximal rate of photosynthesis was estimated on cells under diffusion conditions (no bubbling of air) by measuring O₂ evolution in the presence of saturating C₁ (4000 μM HCO₃⁻). At 0 time cells grown on high CO₂ were placed under diffusion conditions (no bubbling) for the indicated period of time. “●” represents D66 cells and “○” represents slc-230. Error bars represent the standard error of the experiment.
Table 5.1 Comparison of maximal rates of photosynthesis of D66 and slc-230. The mean maximal rates of photosynthesis as estimated by O₂ evolution. The units are in μmol O₂ mg chl⁻¹ hr⁻¹. The time is in hours after switching from high CO₂ to low CO₂. The maximal rate of photosynthesis was estimated on cells under diffusion conditions (no bubbling of air) using 4000 mM HCO₃⁻ as Cᵢ. Data used to generate Figure 5.5. ND not determined.

<table>
<thead>
<tr>
<th>Time in Hr</th>
<th>D66</th>
<th>Std. Dev.</th>
<th>slc-230</th>
<th>Std. Dev.</th>
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<tbody>
<tr>
<td>6</td>
<td>148.8</td>
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Figure 5.6 Relative amounts of chlorophylls in D66 and slc-230. Chlorophylls expressed as µg chlorophyll per 10^6 cells of D66 and slc-230 when maintained under diffusion conditions for 96 hr. Error bars represent the standard error of the experiment.
Figure 5.7  The change in chlorophyll ratios over 96 hr under diffusion conditions of D66, and slc-230, slc-230 4a, and slc-230 4b. Error bars represent the standard error of the experiment.
Figure 5.8 Accumulation of quenching xanthophylls in CC-125 and slc-230, slc-230 4a, and slc-230 4b. Conditions were dark adaptation for 24 hr then illumination over 90 min with 500 \text{E m}^{-2} \text{s}^{-1}. Error bars represent the standard error of the experiment.
Table 5.2  Table of levels of xanthophyll cycle constituents over a 90 min time course. Each constituent of the xanthophylls cycle violaxanthin (V%), antheraxanthin (A%), and zeaxanthin (Z%) is represented in this table as a percent of the total xanthophyll cycle over a 90 min time course for slc-230 and CC-124. Conditions were dark adaptation for 24 hr then illumination over 90 min with 500 □E m⁻² s⁻¹.

<table>
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<tr>
<th>Time in min</th>
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<tr>
<td>V%</td>
<td>A%</td>
<td>Z%</td>
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Table 5.3  Comparative amounts of pigments in CC-124 and slc-230 4b. Diffusion conditions, diffusion + nitrogen starvation conditions at moderate light (75\( \mu \)E m\(^{-2}\) s\(^{-1}\)), and bubbling with High CO\(_2\) at moderate light (75\( \mu \)E m\(^{-2}\) s\(^{-1}\)) were used. V, violaxanthin; A, antheraxanthin; L, lutein; Z, zeaxanthin; ChlB, chlorophyll b; ChlA, chlorophyll a; \( \square \)-car, \( \square \)-carotene. Amounts given are in \( \mu \)g pigment 10\(^6\) cells\(^{-1}\).

<table>
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<tr>
<th>Strain</th>
<th>V</th>
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<th>Z</th>
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<td>0.013</td>
<td>0.000</td>
<td>0.373</td>
<td>1.936</td>
<td>2.828</td>
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<td>0.012</td>
<td>0.000</td>
<td>0.288</td>
<td>1.096</td>
<td>1.584</td>
<td>0.087</td>
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<td>0.006</td>
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<td>0.197</td>
<td>1.536</td>
<td>2.221</td>
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<td>0.118</td>
<td>0.005</td>
<td>0.000</td>
<td>0.113</td>
<td>0.823</td>
<td>1.358</td>
<td>0.022</td>
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</table>
Figure 5.9 2-D TLC templates of major phospholipids in slc-230 and D-66. No major changes in lipid composition between slc-230 and D-66 were detected. PI, phosphatidylinositol; DGDG, digalactosyldiacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DGTS, diacylglyceryltrimethylhomoserine; MGDG, monogalactosyldiacylglycerol.
sequence analysis, Northern blot analysis and semi-quantitative RT-PCR, a novel gene in the haloacid dehalogenase-like hydrolase family with homology to genes in archaebacteria, specifically, *Ferroplasma acidarmanus, Methanothermobacter thermoautotrophicus,* and *Chlorobium tepidum* has been partially characterized. Although the exact function of *Hdh1* remains to be elucidated, physiological investigations were done to observe the effects of the altered expression of *Hdh1*.

slc-230 grows poorly on minimal media (Fig. 4.1). Therefore photosynthesis studies were conducted to begin to elucidate the defect in the mutant. Oxygen evolution experiments indicated that slc-230 is defective in an element that is either part of the CCM or is required for the induction of the CCM in *C. reinhardtii*. slc-230 has an elevated \( K_{0.5}(C_i) \) of 65 \( \mu M \) HCO\(_3^-\) compared to the \( K_{0.5}(C_i) \)'s of D66 and CC-124 (25 \( \mu M \) HCO\(_3^-\) and 15 \( \mu M \) HCO\(_3^-\) respectively). However, the phenotype of slc-230 is not as pronounced as that of cia-5 or cia-3 initially which may be consistent with the hypothesis that slc-230 dies due to accumulation of ROS.

Time course experiments also provided evidence that the protein affected by the slc-230 defect was required for acclimation to high light and/ or low CO\(_2\). During the course of these studies it was found that the rate of photosynthesis in slc-230 declined over a period of time. When slc-230 was grown on high CO\(_2\) and moderate light, slc-230 initially had a rate of photosynthesis similar to that of wild-type. However, when slc-230 was transferred from high CO\(_2\) to low CO\(_2\), over a time course of 64 hr, the photosynthetic capacity of slc-230 significantly decreased (Fig. 5.5). These oxygen evolution experiments indicate that *Hdh1* may be required for acclimation to either low CO\(_2\) or possibly high light. This is not improbable as Fukuzawa and colleagues proposed.
that there are hundreds of genes involved in the CCM (Asimizu et al., 1999) and Im and Grossman showed significant overlap of the genes expressed under high light and low CO₂ (2002). Clearly, the altered expression of Hdh1 over time results in over reduction the photosynthetic apparatus specifically resulting in less oxygen production from PSII.

Pigment analyses indicate that slc-230 has less chlorophyll than wild-type and an a lower chlorophyll a: chlorophyll b ratio than wild-type. Also, this proportion does not change over time under diffusion conditions in slc-230. It would seem that this lower ratio is because of a relative increase in chlorophyll B (Table 5.1). While, in wild-type, this proportion increases.

Additionally, slc-230 does not accumulate quenching xanthophylls (antheraxanthin and zeaxanthin) to wild-type levels under high light or under nitrogen starvation. npq1 does not accumulate any antheraxanthin or zeaxanthin whatsoever, but is not fatal in high light (Niyogi et al., 1997). The lor1 mutant does not accumulate lutein and is not fatal either (Baroli and Niyogi 2000). However, physiological data recently available on the double mutant of C. reinhardtii in npq1 and lor1 is parallel to that of slc-230. Neither the npq1 nor the lor1 mutations, individually, are fatal in C. reinhardtii; however, the double mutation is fatal in high light. The cellular chlorophyll content of npq1/ lor1 is 25% that of wild-type and this decrease is preceded a by a loss (15% after 48 hr) in photosynthetic capacity (Baroli et al., 2004). Hdh1 is not a violaxanthin de-epoxidase. Although, Hdh1 could have an epoxide hydrolase activity; and, a violaxanthin de-epoxidase with homology to higher plant VDE’s has as yet to be identified in C. reinhardtii even with the advent of extensive EST databases and the DOE Joint Genome Initiative. Lack of non-photochemical quenching, the removal of excess photochemical energy as heat to prevent the formation of triplet state chlorophyll, would
eventually result in lowered photosynthetic capacity consistent with the findings from oxygen evolution experiments. Furthermore, data (Table 5.2, Table 5.3) could suggest that the xanthophyll cycle in *C. reinhardtii* is either mediated by two enzymes (e.g. a violaxanthin de-epoxidase and an antheraxanthin de-epoxidase) or that the sequential steps from violaxanthin to antheraxanthin and antheraxanthin to zeaxanthin might be differentially regulated by the levels of protons in the thylakoid lumen. This is postulated because during exposure of slc-230 to bright light (500 μE) over 90 min, the ratio of antheraxanthin to zeaxanthin was higher than in wild-type, as though there was a build up of antheraxanthin (Table 5.2); and, in low light conditions there were levels of violaxanthin and antheraxanthin detected by HPLC in slc-230, but no zeaxanthin (Table 5.3) when compared to wild-type. However, the supposition that *Hdh1* functions as an epoxidase does not adequately explain the unusual ratio of chlorophylls. The other amounts of other carotenoid accessory pigments (β-carotene and lutein) appear to be at wild-type levels under low CO₂ conditions, but do not change as a result of nitrogen starvation for 24 hr (Table 5.3). It would be far more likely that *Hdh1* affects the xanthophyll cycle indirectly in a signal cascade.

Although the major phospholipids in slc-230 do not appear to be affected (Fig. 5.9), another possible function of *Hdh1* in that it removes peroxidated lipids. During times of high light, lipid peroxides are formed and as a result there are mechanisms to deal with this oxidative injury. Superoxide dismutase, glutathione peroxidase, and catalase scavenge harmful oxygen radicals and inhibit formation of lipid peroxides (Niwa and Sasaki, 2003). Lipid peroxides have an altered mass and polarity that are detectable using HPLC.
PSI and PSII consist of a reaction center and the light harvesting complexes LHCl and LHClII respectively. The LHC’s contain chlorophylls and carotenoids and have been shown to transfer absorbed light energy to the reaction centers. The level of expression of LHC’s can vary under different environmental conditions such as high salt in *Dunaliella salina* (Zhang *et al.*, 2002), light intensity (Teramoto *et al.*, 2002), circadian rhythm (Hwang and Herrin, 1994), and low CO₂ conditions (Somanchi, 1998). *Lhcb2*, *Lhcb3*, and *Lhca1* were all found to be upregulated under low CO₂ (Somanchi, 1998). And, LHC’s have also been implicated in regulating pigment synthesis (Plumely and Schmidt, 1995). *Hdh1* could also perform as a thylakoid integration factor responsible for getting LHC’s into the thylakoid. Lastly, The LHC II kinase is part of an integrated network of signal transduction. Light-dependent reduction of the plastiquinone pool affects a thylakoid bound kinase which in turn displaces PSII which causes less absorption of light (Buchanan *et al.*, 2003). A number of environmental cues affect how LHC II kinase acts (Allen and Race, 2002). It is conceivable that environmental conditions such as nitrogen starvation, low CO₂ levels, or presence of ROS could cause similar movement of PSII while the organism acclimates for the new condition. The implications of understanding these processes are central to the question of how plants adapt their photosynthetic machinery to changing wavelengths of light (Allen and Race, 2002). It is in this capacity that *Hdh1* may act as an effector for environmental cues. Deletion of these possible functions could give rise to the physiological symptoms observed.

The function of *Hdh1* is not clear. By these experiments, however, some of the results of the altered expression of *Hdh1* are shown. slc-230 has a lowered O₂ evolution that decreases over time indicative of over reduction of the photosystems. When
quantified slc-230 has a lower than wild-type amount of total chlorophylls and a lower chlorophyll a: chlorophyll b ratio that does not change over time under diffusion conditions. Lastly, slc-230 has a lower than wild-type accumulation of quenching xanthophylls under high light and nitrogen starvation.
CHAPTER 6
CONCLUSIONS

The experiments described in this dissertation have successfully generated \( \text{Ble}^R \) insertional mutants in the CCM and related systems and subsequently identified the locus of the \( \text{Ble}^R \) insertion in slc-230 to be between \( yptC5 \) and \( Hdh1 \). \( Hdh1 \) appears to be a novel, single copy gene in \( C. \ \text{reinhardtii} \) with homology to hydrolases in archaebacteria. The EST’s available for \( Hdh1 \) show approximately three lengths. The longest predicted mRNA was confirmed by RT-PCR and 5’ RACE (Fig. 4.14 and Fig. 4.7). Therefore the \( \text{Ble}^R \) insert is in the first exon of \( Hdh1 \) and RT-PCR confirms that this message for \( Hdh1 \) is not present in slc-230. In contrast, a northern blot showed that \( yptC5 \) is present in normal length and abundance in slc-230. Complementations with cosmid 74D4 would suggest that the wild-type phenotype can be restored in slc-230 with a genomic copy of \( Hdh1 \) as cosmid 74D4 contains a full copy of \( Hdh1 \) (and complements slc-230), but 74D4 also contains \( yptC5 \) so it is not it has as yet to be determined definitively that \( Hdh1 \) complements the slc-230 phenotype. RT-PCR has shown that \( Hdh1 \) is slightly upregulated under low \( \text{CO}_2 \) conditions, although, this transient increase may be a response to photooxidative stressors that presumably accompany acclimation of the cells to the switch to low \( \text{CO}_2 \). These results support the conclusion that the poor photoautotrophic growth observed in slc-230 is due to the disruption of \( Hdh1 \).

Furthermore, slc-230 has been characterized physiologically. Altered expression of \( Hdh1 \) affects pigmentation. The quantity of chlorophylls are lower and in a lower chl a: chl b ratio in slc-230. There is a lower accumulation of quenching xanthophylls in slc-
And, lastly, photosynthesis decreases over time, as shown indirectly by oxygen evolution; and, leads to cell death under photooxidative stress.

Photorespiration, the water-water cycle, the xanthophyll cycle, and the carbon concentrating mechanism (CCM) are adaptations in *C. reinhardtii* that prevent the over-reduction of photosystems and thus photoinhibition by dissipating the energy from the absorption of excess photons. From this work it appears that *Hdh1* is expressed at low levels, but is upregulated under low CO$_2$ conditions. The protein also appears to be localized to the chloroplast. Finally, its sequence is consistent with a phosphatase, hydrolase, or epoxidase function. One could hypothesize that it may have a regulatory function in response to environmental cues. *Hdh1* could be an effector of pigment modulation in the thylakoidal membrane that exerts control over the proportions of chlorophylls vis-à-vis CABs (Chlorophyll a and b Binding proteins) or the xanthophyll cycle under different light and CO$_2$ conditions. In this scenario, altered expression of *Hdh1* would result in no transferal of environmental cues and the cells might not modulate the antennae proteins to adjust to the new environmental conditions. If *Hdh1* signaled a shift from low light to high light one might initially observe an unusually low content of pigments. This condition would eventually lead to the over-reduction of PSII due to absorption of excess photons resulting in time to a lower affinity for C$_i$ and lowered photosynthetic capacity due to and accumulation of ROS. This hypothesis is favored as these symptoms have been observed in slc-230 and similarly in Baroli’s npq1/lor1 mutant (Baroli *et al.*, 2003). It is most probable that *Hdh1* is located in the stroma of the chloroplast as it is predicted to be a soluble protein and may affect steps in a signal cascade affecting pigmentation.
Physiological characterization has helped further the understanding of the nature of the altered expression of \textit{Hdh1}; however, the precise role of \textit{Hdh1} in photoprotection during acclimation to low CO$_2$ has as yet to be investigated. One of the problems associated with refining the conditions under which \textit{Hdh1} is expressed is the separation of environmental cues. It has been found that low CO$_2$ inducible proteins in \textit{C. reinhardtii} are not expressed without the presence of light. \textit{Cah1} (Fukuzawa \textit{et al.}, 1990), and \textit{Ccp1} and \textit{Ccp2} (Chen, \textit{et al.}, 1996) require light to be expressed under low CO$_2$. Likewise, the problem with clearly elucidating the effect of nitrogen starvation is difficult as it affects the entire cell, not just the chloroplast (Yamamoto, 2003, personal communication). It is probable that \textit{Hdh1} is expressed and exerts a control function under an amalgam of environmental cues as opposed to one specific environmental regulator. Physiological and molecular aspects of \textit{Hdh1} remain to be investigated. More physiology on slc-230 remains to be done. The nature of the symptoms of the physiology in slc-230 could be further described with a HPLC set up for quantitation of peroxidated lipids and a pulse amplitude modulated (PAM) fluorometer could measure the non-photochemical quenching in slc-230. Real Time PCR might be useful in quantitatively assessing the level of expression of \textit{Hdh1} under different environmental conditions. Overexpression of \textit{Hdh1} could be employed to test the hypothesis that it has a general phosphatase function.

The future of \textit{C. reinhardtii} as a model organism for the study photosynthesis and related processes is assured. At present, \textit{C. reinhardtii} remains as it has been and will be: cheap, easy, and quick to grow and maintain and easily transformed. More recent developments such as EST databases and the second draft of its genome have cemented \textit{C. reinhardtii} as a choice organism giving researchers the opportunity to acquire new
biochemical data. The future for those who study this organism should be rich and rewarding.
REFERENCES


## APPENDIX I

### PRIMERS AND PRIMER SEQUENCES

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APPENDIX II
HPLC CALIBRATION EQUATIONS

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<td>Amt. = (Area + 4.5226)/7802.4</td>
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HPLC Calibration Equations Courtesy of Dr. Kevin Carman and Soraya Silva
**APPENDIX III**

**RETENTION TIMES OF PHOTOSYNTHETIC PIGMENTS FOR HPLC**

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These retention times come from Jeffrey *et al.*, 1997
This is a sample HPLC chromatograph from D66 grown on high CO₂ in moderate light.

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APPENDIX V:
DNA MOLECULAR SIZE STANDARDS FOR AGAROSE GELS

Lambda HindIII Digest

DNA molecular size standards for agarose gels both obtained from NEB Beverly, MA.
VITA

James Edward Adams, IV, was born in Modesto, California, on November 25, 1968. He completed an associate of arts degree in 1989 from Las Positas Junior College, at Livermore, California. He completed a bachelor of science degree in 1996 at California State University, Stanislaus at Turlock, California, with a major in biology, a concentration in botany, and a minor in chemistry. James then completed a doctoral program under the supervision of Dr. James V. Moroney at Louisiana State University in 2004. His dissertation research dealt with insertional mutants of *Chlamydomonas reinhardtii*. After graduating from Louisiana State University, James will pursue postdoctoral training by joining the National Center for Hansen’s Disease Research under the direction of Dr. Richard Truman and Dr. Thomas Gillis at Louisiana State University, School of Veterinary Medicine. There he will genotype strains of *Mycobacterium leprae* and investigate the inflammatory response of armadillos to *M. leprae*. 