Investigation of the role of putative inorganic carbon transporters in the carbon dioxide concentrating mechanisms of Chlamydomonas reinhardtii

Bratati Mukherjee
Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/2722
INVESTIGATION OF THE ROLE OF PUTATIVE INORGANIC CARBON TRANSPORTERS IN THE CARBON DIOXIDE CONCENTRATING MECHANISMS OF CHLAMYDOMONAS REINHARDTII

A Dissertation

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

in

The Department of Biological Sciences

by

Bratati Mukherjee
B.Sc., Calcutta University, 1999
M.Sc., Calcutta University, 2001
August 2013
ACKNOWLEDGEMENTS

First of all, I would like to thank my PhD advisor, Dr. James V Moroney for giving me the opportunity to be a part of his research group. I have the greatest gratitude for his support and guidance through the years. I have learned not only to be a better scientist under his mentorship but also to be a better human being. Without his constant support and encouragement I would not be where I am today. I will continue to be inspired by his scientific brilliance, extreme positivity and generousness of spirit. It gives me great pride to have had him as my mentor for my PhD.

I would like to thank all members of my committee, for their help, throughout my research. Dr. David Longstreth, Dr. John Larkin and Dr. Sue Bartlett have shared their vast scientific experience with me and helped me in honing my scientific thinking. I continue to learn a lot from each of them. I would also like to thank Dr. Gregory Griffin for agreeing to be on my committee on short notice.

The Moroney lab has been my home for several years now and each of its members have been more like family to me. I have extreme gratitude towards my lab-mate, Dr. Yunbing Ma, for being a good friend and inspiring me through the years. Dr. Ruby Ynalvez and Ms. Cathy Mason made the first few years in the Moroney lab, a welcome place. I would like to thank Dr. Nadine Jungnick for being a source of great support and inspiration for the last couple of years. I would like to thank Wesley Frey for our wonderful brainstorming sessions. Robert DiMario, Julie Cronan, Kristin Bice, Tiffany Simms, Susan Laborde have been very helpful and kind all these years. All the other undergraduates in the lab who have helped out over the years all deserve special mention. Last but not the least, I would like to thank Dr. Krishen Cunnusamy, for being a friend, confidante, sounding board and providing strong emotional support all through
my PhD. I continue to be inspired by his extreme drive and work ethic. I thank him for always propping me up emotionally when I needed it the most.

I would not be here today without the endless sacrifice that my family has made and continues to make so that I may reach for my dreams. I thank my (late) father and mother for supporting me with everything that I set out to do since childhood. I thank my brother and sister-in-law for their constant encouragement and support through some of the most difficult times of my life. I would also like to thank my nephew, being away from whom all these years has been very difficult.

I would like to thank my friends, Prabha Krishnamachary, Sharbari Dey, Mahendra Shewalla and Dr. Swati Pakalapati for being there for me all these years. I would especially like to thank Dr. Robert Beaird, for being both friend and family for the last few years. His hugs have made many a bad day better! I would like to also thank my soul sisters, Dr. Sudarshana Basu and Artitreyee Ray for helping me through most of my life.

Last, but not the least, I would like to thank Dr. Sai Vinjanampathy, for being the partner I could not have done without. He chose to believe in me at times when I no longer believed in myself. His undying love and emotional support, enthusiasm for science and endless optimism about life continues to sustain me. I thank him for helping me through my PhD and helping me to look forward to tomorrow. I am in a better place today than I have ever been before, because of him.

To the endless stream of students, co-workers, LSU staff and people that I could not name, I thank you for making my stay in LSU and in Baton Rouge, a very fulfilling experience.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS.......................................................................................................................... ii

LIST OF TABLES......................................................................................................................................... vi

LIST OF FIGURES........................................................................................................................................ vii

LIST OF ABBREVIATIONS........................................................................................................................ x

ABSTRACT.................................................................................................................................................. xi

CHAPTER 1 GENERAL INTRODUCTION AND INTRODUCTION TO THE
THESIS......................................................................................................................................................... 1

Limitations of Rubisco................................................................................................................................ 1
Carbon stress ................................................................................................................................................ 2
The CCM.................................................................................................................................................... 3
Features of a CCM ...................................................................................................................................... 3
The cyanobacterial CCM.............................................................................................................................. 5
The *Chlamydomonas reinhardtii* CCM ....................................................................................................... 11
Induction/repression of the *Chlamydomonas reinhardtii* CCM................................................................. 16
Energization of the *Chlamydomonas reinhardtii* CCM ............................................................................ 21
Regulation of the *Chlamydomonas reinhardtii* CCM .............................................................................. 22
Signal transduction involved in the induction of the *Chlamydomonas reinhardtii* CCM ......................... 26
Structural changes associated with the *Chlamydomonas reinhardtii* CCM ........................................... 29
Carbonic anhydrases involved in the *Chlamydomonas reinhardtii* CCM .............................................. 30
Acquisition of inorganic carbon for the *Chlamydomonas reinhardtii* CCM ............................................ 38
*C*$_{i}$ transport across the plasma membrane ............................................................................................ 39
*C*$_{i}$ transport into the chloroplast ........................................................................................................... 42
*C*$_{i}$ transport into the thylakoid ................................................................................................................ 43
The role of the pyrenoid in the *Chlamydomonas reinhardtii* CCM ......................................................... 43
Introduction to the thesis............................................................................................................................ 45

CHAPTER 2 MATERIALS AND METHODS................................................................................................... 48

Cell culture and growth.............................................................................................................................. 48
Transformation of *C. reinhardtii* cells via electroporation .................................................................... 49
Nucleic acid preparations......................................................................................................................... 50
Analysis of protein..................................................................................................................................... 52
Isolation of Chloroplast Envelope and Plasma Membrane .................................................................. 53
GFP Fluorescence Imaging....................................................................................................................... 53
DIC-Dependent Photosynthetic Oxygen Evolution ................................................................................. 53
Intercellular Concentration of *C*$_{i}$ ........................................................................................................ 53
CHAPTER 3 INVESTIGATION OF THE POSSIBLE ROLE OF THE NAR1 PROTEIN FAMILY IN THE CHLAMYDOMONAS REINHARDTII CCM
- Introduction ........................................................................................................... 57
- Results .................................................................................................................. 60
- Discussion ............................................................................................................. 78

CHAPTER 4 THE POTENTIAL ROLE OF LC11, AS A BICARBONATE TRANSPORTER, IN THE CCM OF CHLAMYDOMONAS REINHARDTII ........................................................................................................................................................................ 86
- Introduction ........................................................................................................... 86
- Results .................................................................................................................. 89
- Discussion ............................................................................................................. 108

CHAPTER 5 THE ABSENCE OF THE PERIPLASMIC CARBONIC ANHYDRASE, CAH1, IN THE SEQUENCED WILD-TYPE STRAIN, CC-503 ........................................................................................................................................................................ 115
- Introduction ........................................................................................................... 115
- Results .................................................................................................................. 117
- Discussion ............................................................................................................. 123

CHAPTER 6 GENERATION OF INSERTIONAL MUTANTS IN CHLAMYDOMONAS REINHARDTII ........................................................................................................................................................................ 126
- Introduction ........................................................................................................... 126
- Results .................................................................................................................. 135
- Discussion ............................................................................................................. 153

CHAPTER 7 CONCLUDING CHAPTER OF THESIS ......................................................... 158

REFERENCES ........................................................................................................... 166

APPENDIX I C. REINHARDTII STRAINS USED IN THIS DISSERTATION ........................................................................................................................................................................ 181

APPENDIX II LIST OF SOME PRIMERS USED IN CHAPTERS 3, 4 and 5 ................. 182

VITA ........................................................................................................................... 183
LIST OF TABLES

Table 3.1  A few characteristics of the members of the *C. reinhardtii* NAR gene family...........................................................................................................................63

Table 6.1  List of genes chosen for the mutagenesis screen.................................................................138
LIST OF FIGURES

Figure 1.1 A schematic representation of the cyanobacterial CCM........................................6

Figure 1.2 A schematic representation of the *C. reinhardtii* CCM........................................14

Figure 3.1 A phylogenetic tree of some well-conserved FNT proteins....................................61

Figure 3.2 Alignment of the NAR1.2 protein sequence with some well-characterized prokaryotic
formate channels (FocA proteins).............................................................................................62

Figure 3.3 Alignment of the NAR protein sequences in *C. reinhardtii* ..................................64

Figure 3.4 A time course experiment showing NAR1.2 expression in the wild type strain D66..........................65

Figure 3.5 A time course experiment showing NAR1.4 expression in the wild type strain D66..........................66

Figure 3.6 RT-PCR data showing the suppression of NAR1.1 and NAR1.6 expression in the wild-type strain D66........................................................................................................67

Figure 3.7 *NAR1.2* expression levels in response to different nitrogen sources and different CO$_2$
levels........................................................................................................................................68

Figure 3.8 *NAR1.1* and *NAR1.6* expression levels in response to different nitrogen sources and
different CO$_2$ levels.................................................................................................................69

Figure 3.9 *NAR1.3* and *NAR1.5* expression levels in response to different nitrogen sources and
different CO$_2$ levels.................................................................................................................70

Figure 3.10 *NAR1.4* expression levels in response to different nitrogen sources and different
CO$_2$ levels................................................................................................................................71

Figure 3.11 Western blot analyses showing the enrichment of NAR1.2 on the chloroplast envelope fraction of whole cells.................................................................................72

Figure 3.12 SDS PAGE gel showing total protein from *E. coli* cells containing the MBP-NAR1.2-HA protein induced under different conditions .................................................................73

Figure 3.13 Western blot showing the presence of the HA tagged NAR1.2-MBP fusion protein in IPTG induced *E. coli* cells..............................................................................................74

Figure 3.14a A Ci uptake screening assay in a mass spectrometer involving transporters
expressed in the double deletion mutant DelCS.........................................................................76
Figure 3.14b A simple subtraction of the passive control (blue line) from the other traces as shown in the previous figure..........................................................77

Figure 3.15 RNAi knock-down attempts for NARI.2 ..........................................................79

Figure 4.1 The positions of the possible transmembrane domains of LCI1.................................90

Figure 4.2 ClustalW alignment of the LCI1 primary sequence................................................92

Figure 4.3 A time-course experiment showing the expression of LCI1....................................93

Figure 4.4 Western blot analysis showing the presence of LCI1 under low CO₂ conditions..............................................................93

Figure 4.5 Western blot analyses showing the expression of LCI1 under low CO₂ conditions in several common CCM mutants and wild-type strains..................................................................................95

Figure 4.6 Nitrate induced expression of LCI1........................................................................97

Figure 4.7 Accumulation of C₁ in the LCI1 transformants C2 and E4.....................................98

Figure 4.8 Expression of the LCI1-GFP chimeric protein.........................................................100

Figure 4.9 Live imaging of the LCI-GFP transformant cells showing the localization of the chimeric protein..........................................................101

Figure 4.10 Immunoblot showing the enrichment of LCI1 in the plasma membrane fraction..........................................................102

Figure 4.11 Construct used for RNAi knockdown of LCI1.........................................................104

Figure 4.12 The reduction of the LCI1 protein in the RNAi knock-down mutants L34 and L69..................................................................................105

Figure 4.13 The expression levels of other CCM proteins in the LCI1 knockdown mutants..................................................................................106

Figure 4.14 The growth phenotypes of the LCI1 RNAi knockdown mutants..........................107

Figure 4.15 The photosynthetic rates of low CO₂ acclimated cells of the LCI1 RNAi mutants..........................................................109
Figure 5.1 Western blot analyses showing the absence of the CAH1 protein in CC-503 cells.................................................................119

Figure 5.2 A schematic diagram of the region upstream of the CAH1 transcriptional start site, from two strains CC-503 and C9.................................................................120

Figure 5.3 Relative abundance of the CAH1 transcript in CC-503, compared to D66 and C9........................................................................................................121

Figure 5.4 Growth phenotype of CC-503, compared to C9 and D66.................................................................122

Figure 5.5 Comparison of the photosynthetic rates of CC-503 with other strains..................................................................................124

Figure 6.1 A schematic representation of the two approaches used in the insertional mutagenesis screen..................................................................................134

Figure 6.2 A diagram of the AphVIII gene cassette used in generating transformants..................................................................................136

Figure 6.3 Position of the forward (F1, F2, F3 and F4) and reverse primers (R1, R2, R3 and R4) in a sample target gene, MITC11................................................................141

Figure 6.4 A representation of the first 3 steps of the PCR-based mutagenesis screen..................................................................................143

Figure 6.5 An example of the first round of PCR screening (Step III).................................................................145

Figure 6.6 An example of Step III (band confirmation) and Step IV (2nd round of PCR)..................................................................................146

Figure 6.7 Identification of single pools (Step V, 3rd round of PCR)..................................................................................148

Figure 6.8 Identification of single mutant colonies (Step VI, 4th round of PCR)..................................................................................149

Figure 6.9 Growth phenotypes of some of the insertional mutants..................................................................................151

Figure 6.10 Phenotypic screens of some transformants under high and low CO2..................................................................................154

Figure 6.11 Summary of the PCR-based mutagenesis screen..................................................................................155
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIR</td>
<td>350 ppm (v/v) CO₂ in air</td>
</tr>
<tr>
<td>Cᵢ</td>
<td>Inorganic carbon</td>
</tr>
<tr>
<td>CCM</td>
<td>Carbon dioxide concentrating mechanism</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CBLP</td>
<td>G protein β subunit</td>
</tr>
<tr>
<td>DIC</td>
<td>Dissolved inorganic carbon</td>
</tr>
<tr>
<td>FNT</td>
<td>Formate nitrite transporter</td>
</tr>
<tr>
<td>High CO₂</td>
<td>5% CO₂ in air (v/v)</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LCI1</td>
<td>Low CO₂ inducible 1</td>
</tr>
<tr>
<td>LCR1</td>
<td>Low CO₂ regulated 1</td>
</tr>
<tr>
<td>LCE</td>
<td>Light-dependent CO₂ exchange</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>NAR 1</td>
<td>Nitrogen assimilation related</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
</tbody>
</table>
ABSTRACT

Photosynthetic microalgae optimize the utilization of inorganic carbon by active uptake and concentration of inorganic carbon ($C_i$) around the carbon-fixing enzyme Rubisco. This process, mostly induced under limiting carbon conditions is called the Carbon Concentrating Mechanism or CCM. The photosynthetic green alga Chlamydomonas reinhardtii serves as an excellent model organism for the study of eukaryotic CCMs. However, unlike the prokaryotic cyanobacterial CCM, which has a well-characterized $C_i$ uptake system, the $C_i$ uptake and transport system of the $C. reinhardtii$ CCM is not well understood.

Since CO$_2$ is a small neutral molecule, it is believed to be able to passively diffuse into the cell with or without any assistance from membrane bound transport proteins. However, the charged HCO$_3^-$ ion needs transporters to facilitate its uptake across each membrane barrier. The hydrophobic barriers posed by the plasma membrane, chloroplast envelope and thylakoid membranes in the path of the charged HCO$_3^-$ ion’s ultimate destination to the thylakoid lumen has led to the proposed existence of one or more transport proteins at each of these membrane locations.

The roles of the $C. reinhardtii$ NAR gene family, showing sequence homology to the Formate/Nitrite transporter family, were investigated for any changes with respect to the induction of the CCM. NAR1.2 was found to be the most interesting NAR protein with respect to the CCM. NAR1.2 was localized to the chloroplast envelope and is believed to be part of the chloroplastic $C_i$ uptake system.

The previously identified putative $C_i$ transporter, LCI1 was localized to the plasma membrane. The expression of this protein in a LCI1 deficient background showed a significant increase in $C_i$ uptake and $C_i$ affinity of cells even in the absence of a functional CCM. However,
the RNAi mediated knockdown of the protein failed to show any growth deficiencies or changes in photosynthetic rates at different pH levels. LCI1 is a transporter that is part of the plasma membrane $C_i$ uptake system.

In the quest for mutations in either previously identified or novel $C_i$ transporters, an insertional mutagenesis project was also undertaken. The sequenced wild-type strain, CC-503, was found to be a natural mutant for the periplasmic carbonic anhydrase, CAH1.
CHAPTER 1
GENERAL INTRODUCTION AND INTRODUCTION TO THE THESIS

Photosynthesis is a central process in the global carbon cycle. It serves as the single largest flux of organic carbon in the biosphere and helps in the assimilation of roughly 100 billion tons of carbon per year, accounting for almost 15% of the carbon in the atmosphere (Raines, 2011). A majority of photosynthetic organisms assimilate carbon via the C3 pathway. The C3 cycle also known as the Calvin-Benson-Bassham cycle, utilizes the products of the light reactions of photosynthesis, ATP and NADPH, to fix atmospheric CO\(_2\) into carbon compounds that are used to fuel the rest of plant metabolism (Stitt et al., 2010). The C3 cycle involves the initial carboxylation of the acceptor molecule ribulose-1, 5-bisphosphate (RUBP), using either atmospheric or dissolved carbon dioxide (CO\(_2\)), by the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco). This causes the yield of two molecules of 3-phosphoglycerate or 3-PGA (Tcherkez et al., 2006). It is this 3-carbon molecule, the first stable product of the carboxylation reaction of Rubisco that gives the C3 cycle its name. Despite its central role in carbon fixation, Rubisco is however, not a very efficient enzyme.

**Limitations of Rubisco**

The inefficiency of Rubisco stems not only from its low turnover rates but also from its catalysis of two competing reactions, carboxylation and oxygenation (Portis and Parry, 2007). None of the known Rubisco enzymes have a half saturation value for CO\(_2\) that saturates its carboxylase activity at the current atmospheric concentration of CO\(_2\) and at the temperature to which the active enzyme is exposed *in vivo* (Tcherkez et al., 2006). Therefore the presence of oxygen as a fairly abundant competitive substrate, causes the redirection of some of the fixed carbon into the photorespiratory cycle leading to the loss of at least 30% of the carbon fixed by
Rubisco (Raines, 2011). The large mass of the Rubisco molecule together with its very high abundance in a photosynthetic organism, seems to be a somewhat costly investment of energy and nitrogen resources to overcome, by sheer bulk, the deficiencies in its catalytic outputs (Raven et al., 2008).

**Carbon stress**

The Rubisco substrate, CO₂, is limiting for most actively photosynthesizing organisms, both terrestrial and aquatic (Spalding et al., 2008). This is because of the low concentration of CO₂ in the atmosphere compared to other gases such as nitrogen and oxygen. For aquatic photosynthetic organisms, the slow diffusion of CO₂ through water (10,000 times slower than air) compounds the carbon stress by causing major fluctuations in the external inorganic carbon available for uptake (Prins and Elzenga, 1987). Also, the alkaline pH occurring naturally in marine waters, or resulting from active CO₂ draw-down around actively photosynthesizing cells in fresh waters, causes a prevalence of charged inorganic carbon molecules such as HCO₃⁻ compared to the easily diffusible, CO₂. The evolution of Carbon Concentrating Mechanisms or CCMs seems to be an efficient way of overcoming the difficulties posed by fluctuating CO₂ levels around an already slow Rubisco. Since Rubisco remains the central carbon fixing enzyme in all photosynthetic organisms, the different forms of CCMs have all converged on the common aim of elevating CO₂ around Rubisco to alter the CO₂/O₂ ratios at the active site in favor of the carboxylation reaction thereby minimizing photorespiratory losses of carbon (Raven et al., 2008).
The CCM

Terrestrial plants have evolved CCMs in the form of C4 plants and CAM plants. C4 plants have adapted a modified cellular arrangement (Kranz anatomy) in their leaves creating a spatial separation of Rubisco allowing for the elevation of CO₂ in the cells containing Rubisco, with the help of preceding C3/C4 carboxylation/decarboxylation reactions (Raven et al., 2008). In case of CAM plants, the temporal separation of carboxylation and decarboxylation over the light-dark period of a diurnal cycle, helps elevate CO₂ around Rubisco by reducing the O₂/CO₂ ratio. In photosynthetic algae such as cyanobacteria and Chlamydomonas reinhardtii (C. reinhardtii), the active uptake of inorganic carbon, the conversion and trapping of CO₂ in the form of charged HCO₃⁻ within the cell and the sequestration of Rubisco in intracellular compartments such as the carboxysomes or pyrenoids have all contributed towards a successful CCM. However, unlike the C4 and CAM plants, which have a more ‘biochemical’ CCM, the biochemistry of most algal cells is strictly C3. They are therefore referred to as ‘biophysical’ CCMs (Raven et al., 2005). With the help of carbonic anhydrases that carry out rapid HCO₃⁻/CO₂ inter-conversions and the presence of an active inorganic carbon uptake system, the prokaryotic cyanobacterial CCM can concentrate inorganic carbon up to 100 fold compared to external levels while the eukaryotic C. reinhardtii CCM can manage up to a 20 fold increase in the concentration of inorganic carbon within its cells (Moroney and Ynalvez, 2007).

Features of a CCM

The key features of an algal CCM are the following: 1.) **Compartmentalization:** This involves separating the initial CA mediated conversion of CO₂ to HCO₃⁻ to prevent its leakage from the cell. It also helps make use of the different pH levels arising naturally within the
different cellular compartments of a photosynthesizing organism (for instance the alkaline pH of the stroma compared to the acidic pH of the thylakoid lumen) for driving the HCO$_3^-$/CO$_2$ interconversions in a favorable direction (Raven et al., 2005). 2.) **Uptake of inorganic carbon (C$_i$):** The need for an uptake machinery for the charged HCO$_3^-$ ion under limiting carbon conditions helps in the utilization of all forms of inorganic carbon as well as prevents the diffusive leakage of CO$_2$ from actively photosynthesizing cells (Berry et al., 1976). 3.) **Carbonic anhydrases (CAs):** The quick catalysis of CO$_2$ to HCO$_3^-$ and *vice versa* needs active CAs at different locations in the cell especially near the vicinity of Rubisco where the concentration of CO$_2$ needs to be elevated for a functional CCM (Berry et al., 1976). 4.) **Sequestration of Rubisco:** The isolation and concentration of Rubisco molecules within a specialized structure such as the carboxysome or pyrenoid makes it easier to produce a localized elevation of CO$_2$ around its active site to promote its carboxylation reaction and cut photorespiratory losses (Price et al., 2008). 5.) **Energization of the process:** Since the production of new proteins such as CAs, transporters etc. and the active uptake of charged inorganic carbon require an added expenditure of energy by the algal cell, CCMs require an energizing process. The energy required for this is mostly provided by the ATP generated from the proton motive forces resultant of the light reactions of photosynthesis. 6.) **Sensing the low CO$_2$ signal and CCM regulation:** The CCM is often an inducible process and hence the sensing of the lowering of CO$_2$ both intra and extracellularly is needed to drive the structural and biochemical changes that accompany CCM induction. The signals that trigger this induction have not been completely worked out and it is possible that more than one molecular signal might exist to bring about the induction of the CCM. Also a multitude of regulatory factors might be acting in parallel to control the activation of the genes and proteins central to the process and the low CO$_2$ adaptation of algal cells.
The cyanobacterial CCM

The cyanobacterial CCM is the most well characterized prokaryotic CCM to date (Figure 1.1). The main components of this CCM are the following: 1.) A $C_i$ uptake system 2.) Carbonic anhydrases and 3.) Carboxysomes.

$C_i$ transport in cyanobacteria is an active process that needs a direct coordination with photosynthesis. Energy sources for cyanobacterial $C_i$ uptake are provided either by ATP (for the $HCO_3^-$ transporter BCT1), NADPH or reduced ferredoxin ($CO_2$ uptake systems NDH-I3/I4) or coupling to an electrochemical sodium gradient ($HCO_3^-$ transporters SbtA or BicA). Five distinct cyanobacterial $C_i$ transport systems have been identified so far, three of which are involved in the uptake of $HCO_3^-$ ions and two in the uptake of $CO_2$ molecules. The $HCO_3^-$ transporters include the transporter BCT1 (Omata et al., 2002), SbtA (Shibata et al., 2002) and BicA (Price et al., 2004). BCT1 is a low $CO_2$ inducible, high affinity, ABC-type $HCO_3^-$ uniporter encoded by the CmpABCD operon. SbtA, on the other hand, is a high affinity, low $CO_2$ inducible $Na^+$-dependent bicarbonate transporter. BicA is a low affinity, high-flux, $Na^+$-dependent bicarbonate symporter belonging to the widespread SulP family that is related to the human SLC26 family of anion transporters. Both SbtA and BicA need a sodium ion for the uptake of bicarbonate whereas BCT1 does not.

The two cyanobacterial $CO_2$-uptake systems, are in an actual sense facilitators of $CO_2$ uptake, since the initial step involves passive diffusion of $CO_2$ across the plasma membrane followed by its conversion to $HCO_3^-$ inside the cell. Both systems are based on modified NADPH- dehydrogenase (NDH-I) complexes that use NADPH as the electron donor, to drive the Of the two systems, one is constitutively expressed (NDH-I4) whereas the other (NDH-I3) is
induced under limited CO\textsubscript{2} conditions and shows a higher affinity for CO\textsubscript{2} (Price et al., 2008). Two proteins, ChpX and ChpY, are an integral part of the NDH-I3/4 complexes and are believed to play a direct role in the conversion of CO\textsubscript{2} to HCO\textsubscript{3}\textsuperscript{−} (Ohkawa et al., 2000). The CO\textsubscript{2} uptake systems located internally on the thylakoid membranes can use either external CO\textsubscript{2} diffusing into the cell or internal CO\textsubscript{2} resulting from cellular respiration or leakage out of the carboxysome, as a substrate for ChpX/Y mediated conversion of CO\textsubscript{2} to HCO\textsubscript{3}\textsuperscript{−}. This can therefore prevent the diffusive loss of CO\textsubscript{2} from the cell (Maeda et al., 2002). However, the exact mechanism of this conversion is not yet known. These transporter systems are found in a number of cyanobacteria, although most species only have a few of the different types of transport proteins. Cyanobacteria concentrate HCO\textsubscript{3}\textsuperscript{−} to very high levels internally, and these transport proteins bring about this accumulation. The evidence that these proteins transport HCO\textsubscript{3}\textsuperscript{−} comes from studies of cyanobacterial strains that are missing one or more of these proteins. In general, if only one of these transport proteins is missing or defective, the cyanobacteria can still grow on low levels of
This is likely due to the fact that these transport proteins are somewhat redundant and a second transport protein can compensate for the loss of one of the transport proteins. However, if two or more transport proteins are lost, the cyanobacteria can no longer accumulate HCO$_3^-$ to high levels and can no longer grow photosynthetically on low levels of carbon dioxide. A cyanobacterial mutant in which all five of the C$_i$ transport systems are disrupted is unable to actively take up either CO$_2$ or HCO$_3^-$ and is unable to grow even in an environment saturated with CO$_2$. Additionally, this mutant shows growth inhibition under saturating levels of both light and CO$_2$, confirming the tight correlation between efficient light utilization and C$_i$ uptake in photosynthetic organisms with a carbon dioxide concentrating mechanism (Xu et al., 2008). A model showing how these various transporters participate in the concentration of carbon dioxide in cyanobacteria is shown in Figure 1.1.

The primary location of CAs in cyanobacteria is the carboxysome (Price et al., 1993). The carboxysome is a proteinaceous polyhedral structure that contains most of the cells’ Rubisco. Carboxysomes are divided into two categories based on the form of Rubisco they encapsulate; α-carboxysomes, found in mostly open ocean cyanobacterial strains, have Rubisco 1A whereas β-carboxysomes which are more common amongst fresh water cyanobacterial strains, contain Rubisco 1B. Both these categories of carboxysomes, though ultrastructurally similar, are very different in their protein compositions. There are different types of CAs in the two carboxysomes. The first CA found in the β-carboxysomes is soluble and shows significant similarity to bacterial and plant β-type carbonic anhydrases. Carboxysomal β-CAs, however, have a longer C-terminal end that is believed to play a role in proper carboxysome targeting and correct oligomeric assembly for catalytic activity (So et al., 2002). The physiological role of the carboxysomal CAs is to convert accumulated HCO$_3^-$ into CO$_2$. This creates a localized elevated
carbon dioxide concentration for Rubisco to utilize for photosynthesis. Mutations that disrupt the soluble carboxysomal CA cause a severe inhibition of carbon dioxide fixation in cyanobacteria at low concentrations of carbon dioxide (Fukuzawa et al., 1992). At high concentrations of carbon dioxide, the growth of cells without the soluble carboxysomal CA is close to normal. Although photosynthesis is inhibited at low carbon dioxide concentrations, cyanobacteria with mutations in the soluble carbonic anhydrase actually accumulate $C_i$ to higher levels than wild-type cells. These cells accumulate $C_i$ while appearing to be starved for carbon dioxide. If the wild-type gene for the soluble carboxysomal carbonic anhydrase is reintroduced into the mutant algae, normal rates of photosynthesis resume. Sulfonamide treatment of cyanobacteria produces similar physiological results. Cells treated with sulfonamides have inhibited carbon dioxide fixation but accumulate $C_i$ to very high levels.

The second type of CA in the carboxysome is actually a part of the carboxysome shell (So et al., 2004). The CsoCA gene encodes the carboxysomal CA (Saway et al., 2006). Since this protein had no significant similarity to any of the established classes of carbonic anhydrases, it was assigned a new class called the $\epsilon$ class of CAs. The sequence difference from other $\beta$-carbonic anhydrases was believed to reflect its dual role as a carbonic anhydrase and as a structural protein. Despite the sequence difference, the CsoCA protein was later shown to have a structural similarity to $\beta$-type CAs. Loss of the carboxysomal shell protein CsoCA also inhibits photosynthesis at low carbon dioxide concentrations (So et al., 2004). In this case, the carboxysome is disrupted in addition to the loss of CsoCA carbonic anhydrase activity. It is now thought that CsoCA might function in the delivery of carbon dioxide to the inside of the carboxysome. In this model, CsoCA would take $\text{HCO}_3^-$ from the outside of the carboxysome (the cytoplasm), and convert it to $\text{CO}_2$, which is then released inside the carboxysome (Figure 1.1). It
is clear from the mutant studies that both the soluble carboxysome CA as well as the carboxysomal shell protein CsoCA are required for the CCM of cyanobacteria.

The third CA found in carboxysomes belongs to the $\gamma$ class of CAs. This protein called the CcmM was found to be essential for carboxysome assembly and mutants lacking this protein could grow only under high CO$_2$ conditions (Ludwig et al., 2000). The N-terminal domain of this protein is similar to the $\gamma$-class of CAs whereas the C-terminal shows sequence similarity to the small subunit of Rubisco (Price et al., 1993). Because of its unique C-terminal, CcmM is believed to play a possible role in binding and stabilizing the large subunit of Rubisco within the carboxysomal shell. CA activity of CcmM from both *Synechococcus* sp. PCC7942 and Synechocystis sp. PCC6803 could not be detected in studies involving the recombinant protein (Cot et al., 2008). However, recently, the CcmM from *Thermosynechococcus elongatus* BP-1 was shown to be an active CA requiring encapsulation into the carboxysome’s oxidizing interior to be active (Pena et al., 2010). It is critical that the cyanobacterial CA, no matter what type, be localized to the carboxysome. If CA activity is produced in the cytoplasm by transforming the cyanobacteria with a gene encoding human carbonic anhydrase (Price and Badger, 1989), those cells have inhibited carbon dioxide fixation because the carbon dioxide can leak out of the cell before it can be fixed. In contrast to the carboxysomal CA deficient strain, cells with cytoplasmic activity are unable to accumulate C$_i$. The current model is that cyanobacteria concentrate HCO$_3^-$ and that this accumulated HCO$_3^-$ is converted to carbon dioxide in the carboxysome by the carboxysomal CA.

Some cyanobacteria have additional CA genes. One, an $\alpha$-type CA, has been found in *Synechococcus* and appears to be localized to the cell wall. The activity of this carbonic
anhydrase is quite low. The role of this cell wall localized CA might be to facilitate entry of C\textsubscript{i} into the cyanobacteria but there is no evidence for this role at this time.

The carboxysomes in cyanobacteria contain both CA and Rubisco. Anything that disrupts this packaging or inactivates the carbonic anhydrase leads to an inhibition of photosynthesis. As HCO\textsubscript{3}\textsuperscript{-} enters the carboxysome, it is converted into CO\textsubscript{2}. Since the HCO\textsubscript{3}\textsuperscript{-} concentration within the cell is high, the CO\textsubscript{2} concentration within the carboxysome is also high, higher than the environmental level of CO\textsubscript{2}. Since Rubisco is also located in the carboxysome it benefits from this localized high concentration of CO\textsubscript{2}. This structure also provides a CO\textsubscript{2} leakage barrier. Many cyanobacterial mutants have been characterized that require elevated carbon dioxide for growth. A number of these mutants have defects in genes that encode components of the carboxysome. One such mutant is the icf\textsubscript{A} mutant, which is defective in the carboxysomal CA (Fukuzawa et al., 1992). Other mutants have defects in the genes encoding carboxysomal shell proteins (Orús et al., 1995). Cells with mutations in the shell proteins have misshapen or empty carboxysomes. In cells that have empty carboxysomes Rubisco is no longer contained within the carboxysome and is found in the cytoplasm. Cyanobacterial cells that fail to package Rubisco into the carboxysome require high carbon dioxide for growth. Another example is a mutant of the RbcS gene that encodes the small subunit of Rubisco containing a 30 amino acid extension (Schwarz et al., 1995). Rubisco containing this larger small subunit is fully active but can no longer be packaged into the carboxysome. As a result, the Rubisco is in the cytoplasm and the cell requires elevated carbon dioxide for growth and photosynthesis.
The *Chlamydomonas reinhardtii* CCM

Work done with green microalgae, especially *Chlorella*, as early as the 1940s, showed that photosynthetic algal cells are capable of existing in two different physiological states depending on the CO$_2$ conditions that they were exposed to during growth. A high CO$_2$ concentration consists of 1-5% v/v of CO$_2$, while low CO$_2$ refers to a CO$_2$ concentration of 0.03% v/v or less. In case of *Chlorella vulgaris*, the low-CO$_2$ acclimated cells showed both a higher rate of photosynthesis and a higher level of CA activity when compared to the high CO$_2$ acclimated cells (Hogetsu and Miyachi, 1979). The increased level of CA activity, in low CO$_2$ acclimated cells, was reported in other microalgae and cyanobacteria as well. In the backdrop of these findings, investigations with *C. reinhardtii* in the late 1970s, revealed, that the photosynthetic exchange kinetics, measured in terms of oxygen evolution, differed between cells grown under different CO$_2$ conditions. Cells grown under low CO$_2$ conditions showed a much higher photosynthetic affinity for CO$_2$ than high-CO$_2$ grown cells (Berry *et al.*, 1976). The increase in CO$_2$ affinity was accompanied by a reduction of the O$_2$ sensitivity of photosynthesis, as measured by the effects on growth, glycolate excretion and inhibition of net photosynthesis, in low-CO$_2$ grown cells. Experiments were carried out to verify if the higher affinity for CO$_2$ (higher than Rubisco itself) shown by low CO$_2$ acclimated cells were either a result of a better form of the Rubisco enzyme or a more efficient form of photosynthesis. However, high and low-CO$_2$ grown cells of *C. reinhardtii* showed no difference in either the catalytic efficiency of the photosynthetic enzyme Rubisco or the pathway of carbon assimilation. Like the C4 pathway, low-CO$_2$ grown cells of *C. reinhardtii*, showed higher photosynthetic rates and a lower carbon compensation concentration than the C3 pathway. The energy requirements of this process were also later calculated to be close to the C4 pathway. However, when the levels of PEP carboxylase
and initial products of CO₂ fixation were experimentally determined, they showed no evidence of
an alternate initial carboxylation event that is characteristic of the C4 pathway (Berry et al.,
1976). Also the increased levels of CA activity could not alone explain the photosynthetic
efficiency of low CO₂ grown cells of C. reinhardtii since C3 plants showed a higher CA activity
than either C4 plants or low CO₂ grown cells of C. reinhardtii. Interestingly, the high-CO₂ grown
cells of C. reinhardtii showed a constant Kₘ (CO₂) over pH levels ranging from 6.5 to 8 while
the low-CO₂ grown cells of C. reinhardtii showed an increasing Kₘ (CO₂) and decreasing Kₘ
(HCO₃⁻) for the same pH range (Moroney and Tolbert 1985). This clearly showed that the low-
CO₂ grown cells of C. reinhardtii were able to use both forms of carbon under limiting-CO₂
conditions in a pH dependent manner (Berry et al., 1976). The internal concentration of
inorganic carbon (CO₂ + HCO₃⁻) under low CO₂ conditions far exceeded the levels that would be
expected with a passive accumulation driven by a pH gradient across the plasma membrane and
the internal chloroplast and thylakoid membranes. Further studies done with a varied collection
of inhibitors (Badger et al., 1980) revealed that the uptake of (CO₂ + HCO₃⁻) was energy
dependent and needs chloroplastic light dependent electron transport. Cell permeable inhibitors
of CA activity also negatively affected the high CO₂ affinity of cells (Badger et al., 1980). This
together with the discovery that the CO₂ and HCO₃⁻ reached a rapid equilibrium internally in low
CO₂ acclimated cells as opposed to high CO₂ cells further pointed to the activity of an efficient
carbonic anhydrase enzyme in that equilibration process. All of this evidence cumulatively led to
the proposal of the existence of an active carbon dioxide concentrating process in low CO₂
acclimated cells of C. reinhardtii that elevated the levels of carbon dioxide at the site of Rubisco,
reducing photorespiration and lowering the CO₂ compensation point. This process, named the
Carbon Concentrating Mechanism (or CCM in short), was believed to give low CO₂ grown cells
of *C. reinhardtii* the photosynthetic competence of C4 plants within the biochemical framework of a typical C3 photosynthetic pathway, without altering the molecular properties of Rubisco (Badger et al., 1980).

Ever since its discovery, the CCM in *C. reinhardtii* has continued to generate widespread interest and research that has already spanned several decades. In the 1970s, the earliest model of the *C. reinhardtii* CCM started off with the skeletal framework of a low CO$_2$ inducible process requiring active inorganic carbon transport and CA activity. The discoveries of new and improved genetic and molecular biological techniques over the last few decades have led to greater refinement of the model (Figure 1.2). New questions are being answered and newer pieces of the puzzle are being added every year to illuminate the finer details of this very complex process.

The following features are now considered integral to the CCM in *C. reinhardtii* and are briefly introduced below. They will be considered in more detail further on.

i.) **Induction/repression:** The CCM is induced at air levels of CO$_2$ (0.03%) or lower. When switched to high CO$_2$ conditions (5%) and growing phototropically, or when acetate is provided for mixotrophic growth, the CCM is repressed.

ii.) **Energization:** Induction under limiting CO$_2$ conditions is light dependent since it is an active process believed to be energized by the photosynthetic electron transport pathway. A proton motive force across the thylakoid membrane is thought to be necessary.

iii.) **Gene regulation:** Upon induction of the CCM, a number of genes, encoding proteins that function in the CCM are turned on by a transcription factor that functions as a ‘master regulator’. A few downstream transcription factors also play an important role. The activation mechanism of
the CCM proteins that are not synthesized de novo is not yet known. Also, the repression of transcription of CCM genes, and the inactivation mechanisms of some constitutive CCM proteins have not yet been fully studied. Although, several genes are upregulated in response to CCM induction they are not always solely playing a role in the CCM but are also involved in other metabolic and transport processes that are essential in the cells acclimation to low CO$_2$.

iv.) **Signal transduction:** The mechanism of CO$_2$ sensing that leads to the activation of transcription factors and thereby jumpstarts the CCM process is still not fully understood in this alga. There are a number of hypotheses that have only been partially backed up by experimental evidence. Similarly, the process of repression of the CCM under high CO$_2$ is not completely understood and is still being investigated.
v.) **Structural changes:** Upon induction of the CCM, the pyrenoid, which mostly consists of closely packaged Rubisco molecules, develops a thick outer starch layer. Several proteins have now been shown to form complexes, and provide an additional layer around the starch sheath, especially, around the gaps of the starch sheath. Also, several mitochondria migrate from central positions in the cytoplasm to the periphery of the cell between the plasma membrane and the chloroplast outer membrane.

vi.) **Carbonic anhydrases:** Several carbonic anhydrases with locations in almost every cell compartment and the periplasmic space are functional in the CCM. Of these, the thylakoid lumen CA has been shown to be critical to the proper functioning of the CCM under low CO\textsubscript{2}. A total of 9 carbonic anhydrases have been discovered so far and their roles in the CCM are still being studied (Moroney *et al.*, 2011).

vii.) **Inorganic carbon transporters:** Although CO\textsubscript{2} is believed to be able to passively diffuse in and out of the *C. reinhardtii* cell there are a few proteins that have been identified as probable CO\textsubscript{2} channels. Under limiting CO\textsubscript{2} conditions and high external pH levels that favor the formation of charged HCO\textsubscript{3}\textsuperscript{-} ions, the need for a plasma membrane bicarbonate transporter is necessary. Due to the presence of carbonic anhydrases in the cytoplasm and the chloroplast stroma, the need for other bicarbonate transporters at the chloroplast and finally the thylakoid membrane levels seem imperative. Although, several putative inorganic transporters localized to the plasma and chloroplast membranes have been studied, candidate thylakoid membrane localized, transporter/transporters, are yet to be identified.

viii.) **Compartmentalization within the pyrenoid:** This is the site of Rubisco accumulation and the thylakoid tubules that intersperse the pyrenoid are believed to be the centers of carbon
concentration and fixation. The pyrenoid is believed to be a dynamic structure with changes occurring in response to CCM induction that allow for the concentration of CO\textsubscript{2} around Rubisco and the recapture and prevention of CO\textsubscript{2} leakage out of the chloroplast.

As obvious from the above summary, the CCM of \textit{C. reinhardtii} involves an intricate network of a large number of components and processes that bring about the physiological, biochemical and structural changes that are key to the low CO\textsubscript{2} acclimation process. The following sections will deal with each of them in more detail.

\textbf{Induction/repression of the \textit{Chlamydomonas reinhardtii} CCM}

As evident from the earliest research done with \textit{C. reinhardtii} cells, the existence of two distinct physiological states namely the high and low CO\textsubscript{2} acclimated states, were easily distinguishable based on differences in photosynthetic efficiencies, and a host of other physiological and biochemical changes that occurred in the cells during either acclimation process. The high CO\textsubscript{2} acclimated cells grown at 1-5\% \textit{v/v} CO\textsubscript{2} and the low CO\textsubscript{2} cells grown at air levels of CO\textsubscript{2} or lower, had a prominent difference in the affinity for the inorganic carbon (C\textsubscript{i} = CO\textsubscript{2} + HCO\textsubscript{3}\textsuperscript{-}) available to them. The high CO\textsubscript{2} cells showed a photosynthetic K\textsubscript{1/2} (C\textsubscript{i}) similar to the K\textsubscript{m} (C\textsubscript{i}) of Rubisco whereas low CO\textsubscript{2} acclimated cells had a K\textsubscript{1/2} (C\textsubscript{i}) a hundred fold lower than that of Rubisco (Badger and Price, 1994). It was evident from the experimental data available at the time, that underlying this increased photosynthetic CO\textsubscript{2} affinity under a limiting CO\textsubscript{2} stress, was an increase in CA activity, and the ability to utilize all forms of inorganic carbon that was available to the cell depending on external pH. The latter ability provided an advantage that was no doubt tied in with the increase in CA activity. The lower rates of photorespiration pointed to the reduction in the oxygenase reactions of Rubisco, which could only be achieved by
increased levels of the carboxylation substrate, CO₂, even under limiting CO₂ conditions. Thus, it was obvious that the carbon dioxide concentrating mechanism or CCM, in the *C. reinhardtii* cells, was inducible under air levels of CO₂ and needed an active, dissolved inorganic carbon (DIC in short) transport, to bring in all forms of inorganic carbon. This then, aided by the carbonic anhydrases, would facilitate both the uptake (by maintaining a concentration gradient) and conversion (from bicarbonate) and hence concentration of the substrate CO₂ around Rubisco. Since the low CO₂ acclimated cells had neither a more efficient Rubisco nor an alternate pathway for CO₂ fixation it was clear that the inducible CCM was what was accountable for the photosynthetic efficiency and the increased ability to pull down and concentrate DIC from the surroundings by cells acclimated to CO₂ stress (Badger *et al.*, 1980). Since, under high CO₂ conditions and under conditions where acetate was added to the growth medium as a carbon source, the cells of *C. reinhardtii* showed an efficiency of CO₂ uptake that paralleled that of C3 plants and was lower than the low CO₂ acclimated cells. It was clear that the CCM was repressed under these conditions.

Although only air levels of CO₂ are being mentioned here as the possible inducer of the CCM, the actual CO₂ available to the cells might often be driven to levels below that of air depending upon the density of cells in the culture. Also, since the suggestion of another physiological state, designated a very low CO₂ state, was somewhat convincingly made with a CCM mutant *pmp1* (Van and Spalding, 2005). This together with the fact that most *C. reinhardtii* laboratories studying the CCM expose their cells on a regular basis to air levels and very low CO₂ levels (0.01%) in their growth studies, the existence of a very low CO₂ state is often taken into account. But since most CCM mutants lacking a key CCM protein show a growth phenotype at air levels and the CCM is readily induced both under air levels and very
low CO$_2$ conditions the term limiting CO$_2$ or low CO$_2$ is often used interchangeably. Over the years, a lot of study has gone into figuring out the changes in low CO$_2$ acclimated cells that accompanied the induction of the CCM. These acclimation responses include induction of CCM related genes and a lag in the growth of cells in culture when switched to low CO$_2$ conditions. Also observed was a relatively smaller cell size under low CO$_2$ compared to high CO$_2$ grown cells and a higher affinity to DIC.

A few structural changes also accompany the induction of the CCM. One of these changes involves the formation of a thick starch sheath around the central pyrenoid. The pyrenoid is an electron dense body interspersed by thylakoid tubules, that under low CO$_2$ conditions harbors about 90% of the cells Rubisco (Kuchitsu et al., 1988, 1991; Ramanazov et al., 1994; Geraghty and Spalding, 1996; Thyssen et al., 2001). Also, prominent among the structural changes are the relocation of the mitochondria from a central to a more peripheral position in the presence of the CCM.

Since the 1990s, the study of the changes in gene expression, in correlation to the induction of the CCM, have revealed the de novo induction of several genes in the *C. reinhardti* cell exposed to limiting CO$_2$ conditions. Needless to say, most of these genes were found to encode proteins, with prominent roles in the low CO$_2$ acclimation process, such as inorganic carbon (C$_i$) transport, carbonic anhydrase (CA) activity, and photorespiration. The expression of genes involved in a large number of metabolic processes affected by limited CO$_2$ availability or which actively help with the acclimation process are also up or down-regulated together with the prominent CCM genes. Genome wide transcript analyses has been undertaken in the past using techniques like Differential Display (Im and Grossman, 2002) to isolate the subset of genes turned on in high light that are also regulated by limiting CO$_2$ conditions. EST based Microarray
assays (Im et al., 2003; Miura et al., 2004;) were also performed to isolate genes that show a change in expression under CCM induction. The effect of different light intensities (high, medium and low light intensities) on the induced CCM were also studied recently with an EST based microarray assay (Yamano et al., 2008). In the microarray assay carried out by Miura et al (2004), of the 83 CO₂ responsive genes identified, 51 were upregulated by low CO₂ conditions whereas 32 were repressed under the same conditions. The prominent CCM genes that were upregulated in this global screen were the CA genes CAH1 (periplasmic) and CAH4 (mitochondrial), genes encoding membrane proteins, CCP1 (chloroplast membrane) and LCI1 (now known to be plasma membrane), genes of unknown function like LCI5 and photorespiratory genes like AAT1 and PGP1. The CCM gene LCI5, discovered in this screen, encodes a soluble protein found to be peripherally associated with the stromal side of the thylakoids. Interestingly, this protein was later reported to have multiple phosphorylations in a study aimed at revealing low CO₂ induced phosphorylations of thylakoid proteins (Turkina et al., 2006).

Utilizing available techniques like RNA gel blot analyses, macroarrays and microarrays a large number of genes involved or closely associated with the CCM had largely been identified and reported in the past 10 years (Im and Grossman, 2002; Im et al., 2003; Miura et al., 2004; Wang and Spalding, 2006; Yamano et al., 2008; Yamano and Fukuzawa, 2009). However, taking advantage of the recent advances made with RNA sequencing, a more extensive analysis of the global transcriptomic changes accompanying CCM induction has been made in C. reinhardtii. Since replicated deep RNA sequencing or RNA-Seq provides reproducible and high throughput analyses of the entire transcriptome with maximized coverage, the use of this technique has enriched our knowledge about the genes affected by the CCM induction. Two
Recent studies have concentrated on the global analysis of transcriptome changes associated with CCM induction using RNA-Seq on the Illumina platform and have significantly added to the list of previously identified genes in the process (Brueggeman et al., 2012; Fang et al., 2012). A statistically significant change (2 fold) in the expression of 5884 genes (around 38% of the C. reinhardtii genome), has been reported. Of these, 3828 genes were downregulated while 1088 genes showed an upregulation between 30 mins and 180 mins after a switch from high CO₂ to low CO₂ conditions (Brueggeman et al., 2012). A reduction of gene expression associated with photosynthesis, protein synthesis and bioenergetics pathways were observed probably the result of a global stress response to CO₂ deprivation. For example, a few C. reinhardtii genes downregulated under low CO₂ conditions are homologous to higher plant genes that encode proteins involved in a stress response exemplified by higher plants like Arabidopsis. Genes encoding LCI14 (a part of the chlorophyll a biosynthesis pathway in higher plants), LCI25 (homologous to a one-helix stress response protein in Arabidopsis) and LHCSR1 (a stress related chlorophyll a/b binding protein) are all downregulated under CO₂ deprivation. As expected, the transcription of most key CCM genes followed previously reported patterns of induction. For instance of the 9 reported CA genes, 4 showed a high upregulation (namely CAH1, CAH4/5 and CAH7) and none showed any significant repression, as previously reported (Ynalvez et al., 2008). However a key CA, CAH3, which showed no change in expression in previous quantitative RT-PCR measurements (Ynalvez et al., 2008), was found to be slightly upregulated in the recent RNA-Seq study (Brueggeman et al., 2012). CCM genes involved directly or indirectly with facilitating inorganic carbon uptake and transport such as LCIB and LCIC (~20 to 30 fold), HLA3 (~40 fold), NAR1.2 (~4000 fold), LCI1 (~3000 fold), CCP1 (~2000 fold), CCP2 (~120 fold) all showed high levels of induction in response to CO₂ stress consistent with previous reports. As mentioned
earlier, the levels of CO₂ to which *C. reinhardtii* cells are exposed, are believed to result in three physiological states of acclimation, namely high CO₂ (~ 5% v/v), low CO₂ or ambient air levels (0.03% v/v) or very low CO₂ (0.01% v/v). Therefore the identification of the induction/repression of genes when changed from high CO₂ conditions to either air levels of CO₂ or lower is critical to the understanding of the CCM induction process. However, the low CO₂ and very low CO₂ physiological states as proposed earlier (Spalding, 2008), could not be distinguished based on RNA-Seq data (Fang *et al*., 2012), suggesting that the lowering of CO₂ to air levels was sufficient in inducing most components directly or indirectly involved in the functioning of the CCM in *C. reinhardtii*. Under CO₂ deprivation additional factors like light intensity, temperature during growth and the *C. reinhardtii* strain used, might all affect the extent of transcription of many CCM genes under phototrophic conditions and must be taken into account in any global transcriptome study (Im and Grossman., 2002; Yamano *et al*., 2008; Fang *et al*., 2012). It should also be kept in mind that many other proteins that play a role in the *C. reinhardtii* CCM might be activated via post-translational modifications and might not be revealed in global transcriptome analyses. For instance, phosphorylations of many thylakoid proteins are seen in low CO₂ grown cells of *C. reinhardtii* (Turkina *et al*., 2006). Therefore more comparative proteomic studies of high and low CO₂ acclimated cells are also needed to unearth new CCM components.

**Energization of the Chlamydomonas reinhardtii CCM**

Low CO₂ acclimated cells of *C. reinhardtii*, were found to concentrate inorganic carbon internally up 20 fold higher than external levels. These levels are much higher than what could be accounted for by a pH gradient driven passive accumulation. This led to an early suspicion that the *C. reinhardtii* CCM requires an active expenditure of cellular energy in the form of ATP,
to fuel the uptake and internal concentration of inorganic carbon (Badger et al., 1980; Ogawa et al. 1985). The use of inhibitors like (3-(3, 4-dichlorophenyl)-1,1-dimethylurea) or DCMU, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone also known as FCCP and SF6847 have helped elucidate the energy dependence of the process (Badger et al., 1980). DCMU is a photosynthetic inhibitor, which inhibits plastoquinone binding to Photosystem II and thereby successively inhibits NADP reduction, photosynthetic electron transport and ATP production within the chloroplast. FCCP, a mitochondrial oxidative phosphorylation inhibitor together with another inhibitor SF6847 can act as uncouplers dissipating the proton gradient across membranes and affecting both mitochondrial and chloroplastic ATP synthesis. All inhibitors were successful in decreasing the ability of low CO\(_2\) acclimated cells to accumulate inorganic carbon to levels higher than the external environment (Badger et al., 1980). The inhibitory effect of FCCP and SF6847 points to the need for a proton motive gradient across membranes possibly fueling ATP synthesis. On the other hand, the inhibitory effect of DCMU suggests the direct dependence of the CCM on photosynthetic energy generation via photophosphorylation of ADP. Work with these inhibitors therefore proved valuable in establishing the CCM as an active process requiring light energy. Since the light dependent reactions of photosynthesis are key in generating the proton motive force across the thylakoid membrane that drive ATP generation, the availability and intensity of light no doubt affects the efficiency of CCM operation in *C. reinhardtii*.

**Regulation of the Chlamydomonas reinhardtii CCM**

Several environmental factors play a significant role in modulating the functioning of the CCM in *C. reinhardtii* such as external CO\(_2\) levels, nutrient availability, availability and intensity of light energy. Of these, the most important factor is the availability of inorganic carbon to the actively photosynthesizing cells. As mentioned earlier a large number of genes are actively
transcribed in a *C. reinhardtii* cell acclimating to a carbon deficient environment. Many of these genes have been shown to be regulated by a master regulatory gene called *CIA5*, which encodes a zinc finger like transcription factor. Another transcription factor, LCR1, reportedly acting downstream of CIA5, is the only other transcription factor identified so far as a regulator of CCM genes (Yoshioka *et al*., 2004).

The isolation and characterization (Moroney *et al*., 1989; Marek and Spalding, 1991) of a UV irradiation generated, high CO₂ requiring mutant called *cia5* (*C*₁ acquisition mutant #5), that lacked the ability to induce a CCM, was the first indication of the importance of the *CIA5* gene in the *C. reinhardtii* CCM. The inability of this mutant to acclimate to low CO₂ levels, indicated that the missing gene might be involved in activating the expression of key CCM genes either as part of a single direct signal transduction pathway or by its initial participation in several parallel gene activation pathways to achieve the same goal. Successful complementation of this mutant was achieved in two independent experiments (Fukuzawa *et al*., 2001; Xiang *et al*., 2001). The CIA5 protein sequence revealed two putative N-terminal zinc finger motifs and a glycine-rich region characteristic of transcriptional activators, which initially supported its possible role as a potential transcription factor. In fact the point mutation in the *cia5* mutant was found to have caused a histidine to tyrosine alteration in the first putative zinc finger motif (Xiang *et al*., 2001).

A CIA5 ortholog in the alga *Volvox carterii*, has a 51% similarity at the amino acid level to the *C. reinhardtii* protein. This includes similarities with the two zinc finger domains, a multifunctional protein-protein interaction domain characteristic of transcription factors (Plevin *et al*., 2005), a sumoylation site and a nuclear localization signal sequence of the *C. reinhardtii* CIA5 (Yamano *et al*., 2011). Although the expression of *CIA5* is unaffected by the level of CO₂ available to the cell, the expression of most low CO₂ induced CAs and inorganic carbon
transporters fail to be induced in its absence (Fukuzawa et al., 2001; Xiang et al., 2001; Miura et al., 2004). The CIA5 protein is believed to be activated under low CO₂ by post-translational modifications and this activation helps with its role in the downstream regulation of many CCM proteins. Several putative phosphorylation sites have been identified near the C-terminus of the protein (Xiang et al., 2001) and lend further strength to this hypothesis.

Although not much is known about the actual DNA sequences that it targets, it is still the only identified ‘master regulator’ so far, for many of the key CCM genes. The CCM genes turned on by CIA5 (as evident by their absence in the cia5 mutant) include some of the important CAs such as CAH1, CAH3, CAH4 and proteins involved in inorganic carbon uptake like HLA3, CCP1/2, LCII, NAR1.2, LCIB and LCIC (Moroney and Ynalvez, 2007; Wang et al., 2011). Also included in this group is the LCR1 gene encoding a protein that belongs to the myeloblastosis (myb) family of transcription factors and is involved in the downstream regulation of a small subset of CCM genes (Yoshioka et al., 2003). Recently, a global transcriptomic study using RNA-Seq technology to look at gene expression affected by carbon availability and CIA5 control, in different strains of C. reinhardtii (wild type strain 137C and the mutant strain cia5), revealed some interesting facts (Fang et al., 2012). This study revealed some details about CIA5 control that was already known from prior studies, while revealing some newer details of its possible role in controlling genes other than those with possible involvement in the CCM. Around 533 genes were found to show a pattern of upregulation under limiting CO₂ conditions and repression in the absence of CIA5 (Fang et al., 2012). These included 53 of nearly 106 previously CIA5 regulated CCM genes and included most of the key CAs (CAH1, CAH3 and CAH4/5) and inorganic transport facilitators (HLA3, LCII, CCP1/CCP2, NAR1.2, LCIB, LCIC, etc.) as mentioned earlier. Also, amongst the 533 CIA5-regulated genes mentioned above, were
several newly discovered genes, encoding proteins with transmembrane domains that could prove to be ideal candidates for possible inorganic carbon transport function under limiting CO$_2$ conditions. They were therefore included in the PCR-based mutagenesis screen as mentioned in chapter 6 of this thesis. CIA5 also proved to be an inducer of the photorespiratory pathway, turning on previously reported photorespiratory genes (Marek and Spalding, 1991; Tural and Moroney, 2005), but mildly repressed several Calvin cycle genes including the Rubisco small subunit gene (Fang et al., 2012). Interestingly, around 1396 genes showing a difference in expression in the global transcriptomic study by (Fang et al., 2012) were non-CCM genes that were regulated by CIA5. This fact opposes previous conjectures about the possible need for low CO$_2$ activation for CIA5 function (Xiang et al., 2001). Therefore, it is safe to assume that the upstream regulation of most key CCM genes is carried out either directly by CIA5 or indirectly, by possibly activating other parallely acting signal pathways that eventually regulate other genes involved in the process. Also the role of CIA5 in regulation of other physiologically processes that are independent of carbon availability within the *C. reinhardtii* cell, cannot be ruled out at this point (Fang et al., 2012).

One of the already identified downstream transcription factors that is transcriptionally regulated by CIA5 is called LCR1 (Low CO$_2$ Response regulator 1). In the absence of this myb-transcription factor, the expression of 13 low CO$_2$ expressed genes is repressed. The most important of these genes are those that encode the key periplasmic CA, CAH1, a putative inorganic carbon transporter, LCI1, and a possible soluble protein with no known function, LCI6 (Yoshioka et al., 2004). Two enhancer elements recognized in the promoter region of CAH1 through gel mobility shift assays by the Fukuzawa laboratory, were found to share the common DNA sequence GANTTNC (Kucho et al., 2003; Yoshioka et al., 2004). This was later
confirmed as the binding site for LCR1 (Miura et al., 2004). Since there is no direct evidence of CIA5 binding to DNA (Kohinata et al., 2008), the existence of LCR1 supports the hypothesis that CIA5 probably directly or indirectly induces the activation of other downstream transcription factors that bind to cis-regulatory regions in the promoter regions of key CCM genes. Future exploration of novel genes encoding possible transcriptional activators that regulate the expression of genes involved in the functioning of the CCM might reveal a lot more about the CCM regulatory process. The recognition of common cis-regulatory elements in the promoters of known and highly upregulated genes of the CCM might in turn help the process of identification of regulatory proteins bound to them via chromatin immunoprecipitation or ChIP assays (Brueggeman et al., 2012).

**Signal transduction involved in the induction of the Chlamydomonas reinhardtii CCM**

The structural, biochemical and metabolic changes that accompany the low CO₂ acclimation process in a *C. reinhardtii* cell arise from the possible activation/repression of a large number of genes in the nucleus. This large-scale regulation of gene expression is no doubt triggered by the perception of a low CO₂ signal by the cell that is rapidly transduced to the nucleus with the help of either a single signaling pathway or several pathways acting in parallel. The foremost ‘signal’ that sets these pathways into motion has not yet been completely characterized together with the receptors of this ‘signal’ and the pathway/pathways as a whole still remain a largely unresolved aspect of the *C. reinhardtii* CCM model. The cells must sense the change in either external or internal carbon levels, either directly, by the change in the amount of CO₂ diffusing into the cell or by the indirect effect on a cellular process caused by the changes in internal inorganic carbon concentration. *C. reinhardtii* cells induce the CCM by the quick detection of decreasing external CO₂ levels in the bulk medium irrespective of the pH.
levels (Matsuda and Colman., 1995; Bozzo et al., 2000., Giordano et al., 2005) and HCO$_3^-$ ion concentrations. This would point to the need of a possible detector located at the cell’s first diffusive barrier, the plasma membrane, in order to set off a downstream signal transduction mechanism leading on to the activation of genes within the nucleus. Although there is no current evidence of a plasma membrane bound external CO$_2$ sensor, the sensing of internal CO$_2$ by certain amino acid residues either within and outside the N-terminal zinc binding domains of CIA5 has recently been proposed (Kohinata et al., 2008). Critical cysteine and histidine residues of CIA5 namely C36, C41, H54 (mutated in the cia5 mutant), C77, C80, H90 and C93 were implicated in the response to limiting CO$_2$ levels by the induction of the CCM. Since, the amount of CIA5 protein present within the cell is unaffected by the internal CO$_2$ levels, some sort of activation of the protein under low CO$_2$ levels, is probably needed for it to migrate to the nucleus as a complex of approximately 290-580 KDa (Kohinata et al., 2008). The upstream mediator triggering the activation of CIA5 is still not known. Even in the case of CIA5 regulated genes there are often variations in the patterns of their induction. For instance, the periplasmic CA, CAH1 was reported to have a possible induction in the dark while other CIA5 regulated genes like CAH, CAH5, CCP1 and CCP2 required light for their induction (Villarejo et al., 1997). The same study also reported that the inhibition of the glycolate pathway subsequently inhibited the expression of CAH1, CAH4 and CAH5 but not CCP1 and CCP2. Also, the highly homologous putative inorganic transporter genes, CCP1 and CCP2 were found to be induced at different time points in the low CO$_2$ acclimation process (Chen et al., 1997). It was hypothesized that possible differences in their promoter regions despite sequence homology in the coding region, might be leading to differential expression due to the response to different transcriptional regulators, controlled by the same master regulator, CIA5 (Chen et al., 1997). These instances point to the
fact that even in the case where a single master regulatory gene seems to be responding to a particular signaling pathway, genes regulated by it might be responsive to other signals or parallel signaling pathways.

Another hypothesis regarding the signaling mechanism is the indirect signals generated from the build-up of certain metabolic byproducts by the internal depletion of inorganic carbon. A depletion of internal inorganic carbon levels might affect photosynthesis directly and as a result, either the redox potential of the photosynthetic electron transport system or the level of a photosynthetic metabolite might act as a direct trigger for the low CO$_2$ signal transduction pathway (Spalding and Ogren, 1982; Spencer et al., 1983; Dionisio et al., 1989; Fukuzawa et al., 1990; Villarejo et al., 1997). The direct effect of internal carbon and oxygen levels on the photorespiratory pathway is long known (Spalding and Ogren, 1982; Ramazanov and Cardenas., 1992; Villarejo et al., 1997). Therefore oxygen levels either acting as a direct trigger for the induction of genes like $CAH1$, or leading to the accumulation of photorespiratory metabolites like glycolate might also be playing a role in the sensing of low CO$_2$ levels (Spalding and Ogren, 1982; Ramazanov and Cardenas, 1992; Villarejo et al., 1997). The role of light as a possible signal for CCM induction has also been speculated. The effect of both photosynthetic light and blue light in inducing the expression of $CAH1$ (Dionisio et al., 1989) and other CCM genes (Dionisio-Sese et al., 1990; Borodin et al., 1994) has been reported. In case of the blue light, a flavoprotein was believed to be acting as the receptor for the light signal (Dionisio et al., 1989). The lack of sufficient knowledge of the seemingly complex signaling mechanism that brings about the induction of the CCM in $C.~reinhardtii$, fails to back any one signaling hypothesis. The relatively quick response of $C.~reinhardtii$ cells to changes in external levels of inorganic carbon can be conjectured at this point to be a combination of a dependence on the activation of CIA5
and other transcription factors, response to light and diurnal cycles and the changes to the photosynthetic redox states, photosynthetic and photorespiratory byproducts. The complexity of the signaling pathways that probably orchestrate the proper functioning of different aspects of the CCM can be best characterized by consistent efforts at unearthing and characterizing new regulatory genes. This together with the discovery and characterization of cis-regulatory elements in promoters of CCM genes might help understanding downstream elements of the signaling pathway that can gradually be traced to the source of the low CO$_2$ response signal.

**Structural changes associated with the *Chlamydomonas reinhardtii* CCM**

Low CO$_2$ acclimated cells of *C. reinhardtii* often show significant sub-cellular reorganization that seems to accompany the successful induction of the CCM. Prominent amongst these changes is the formation of a thick starch sheath enveloping the pyrenoid structure which hosts about 90% of the cell’s Rubisco (Kuchitsu et al., 1991; Ramazanov et al., 1994; Geraghty and Spalding, 1996; Borkshenious et al., 1998; Thyssen et al., 2001). Since the pyrenoid seems to be the epicenter for CO$_2$ accumulation and concentration lying at the heart of the CCM process, the starch sheath was initially thought to be a barrier to the leakage of CO$_2$ from the pyrenoid (Badger and Price, 1994). However, starchless mutants that were unable to form the starch sheath around the pyrenoid showed an uncompromised CCM function thereby failing to support the hypothesis about its importance as a diffusion barrier (Villarejo et al., 1997). Another prominently visible change in organeller distribution during the low CO$_2$ acclimation process is the migration of the cellular mitochondria from a central position towards the periphery of the cell (Geraghty and Spalding, 1996). The mitochondria are mostly ensconced in the space between the chloroplast and plasma membranes of the cell and the exact mechanism of their relocation is not fully known. They are believed to reach the periphery by squeezing
through gaps in the chloroplast possibly aided by the cytoskeletal network (Geraghty and Spalding, 1996). The time frame within the CCM induction process corresponding to the peripheral migration of the mitochondria coincides with the induction of many mitochondrial proteins (Erikkson et al., 1996; Geraghty and Spalding, 1996; Im and Grossman, 2002; Miura et al., 2004; Nakamura et al., 2005). This points to possible functions of the mitochondria in not only the possible energization of the inorganic transport machinery located on the plasma membrane as seen in the microalga Nannochloropsis gaditana (Huertas et al., 2002), but also a possible role in recycling photorespiratory glycolate to release much needed CO$_2$ back into the chloroplast (Spalding, 2008), in a low CO$_2$ acclimated cell. Together with mitochondrial migration, an increase in vacuolation (namely, a large central vacuole in 24 hr induced cells), is also seen and is believed to be associated with the metabolic changes that accompany the CO$_2$ acclimation process (Geraghty and Spalding, 1996).

**Carbonic anhydrases involved in the Chlamydomonas reinhardtii CCM**

There are 12 known CAs in *C. reinhardtii* covering all of the major CA evolutionary lineages, namely $\alpha$, $\beta$ and $\gamma$ CAs (Moroney et al., 2011). Of these, there are three $\alpha$-CAs (CAH1, CAH2 and CAH3), 6 $\beta$-CAs (CAH4, CAH5, CAH6, CAH7, CAH8 and CAH9) and three $\gamma$ CAs (CAG1, CAG2 and CAG3). The initial investigations into the low CO$_2$ acclimation of *C. reinhardtii* cells revealed that an increase in CA activity was an essential part of CCM induction (Berry et al., 1976; Hogetsu and Miyachi, 1977). The discovery early on of an external periplasmic CA (Colman and Grossman, 1984; Moroney et al., 1985; Fukuzawa et al., 1990) and an internal thylakoid lumen CA (Moroney et al., 1985; Funke et al., 1997; Karlsson et al., 1998) fit nicely into the earliest models of the *C. reinhardtii* CCM. Many more active CAs with varied cellular locations ranging from the cytoplasm to the mitochondrion have since been identified.
and characterized (Moroney et al., 2011). Although the physiological roles of many of the CAs have been characterized, the location and exact function of many of them remain unclear. The following sections will provide a summary of the current knowledge about the location and putative function of the 12 CAs in *C. reinhardtii* cells.

The α-CA family in *C. reinhardtii* consists of three members, CAH1, CAH2 and CAH3. CAH1, which encodes a periplasmic CA, is one of the first genes to be highly upregulated once the CCM is induced in low CO₂ grown cells (Spencer et al., 1983; Colman and Grossman, 1984; Moroney et al., 1985; Fukuzawa et al., 1990) and is completely suppressed under high CO₂ conditions (Fujiwara et al., 1990). Data from both quantitative reverse transcription studies (Ynalvez et al., 2008, Fang et al., 2012) and recent RNA-Seq experiments (Brueggeman et al., 2012) reveal a high level of expression of CAH1 (~1000 fold increase) within 3 hrs of low CO₂ acclimation. The expression of the CAH1 gene is under the direct control of the myb-transcription factor LCR1 which is believed to be acting downstream of the CCM ‘master regulator’ CIA5 (Yoshioka et al., 2004). The CAH1 promoter is the best characterized promoter of all the CCM genes and can be used as a low CO₂ inducible promoter for artificial expression of reporter genes (Yoshioka et al., 2004). Two regulatory regions have been identified in the CAH1 promoter region: a silencer region responsible for the high CO₂ repression of expression and an enhancer region composed of two cis-elements E1 and E2 that interact with nuclear proteins (Kucho et al., 1999; Kucho et al., 2003). Experimental support for the periplasmic location of CAH1 comes from the fact that it is present in abundance in the growth medium, especially during the growth of a cell wall-less strain (Kimpel et al., 1983). It has been detected in immunoblots of cell wall fractions (Ishida et al., 1993) and successful immunogold localization to the cell wall region of whole cells has been demonstrated using a CAH1 specific
antibody (Moroney and Ynalvez, 2007). The CAH1 protein has a leader sequence characteristic of secreted proteins (Ishida et al., 1993; Fujiwara et al., 1990) and undergoes substantial post-translational processing before being secreted to the periplasmic space (Ishida et al., 1993). Although a switch from low to high CO$_2$, rapidly suppresses the expression of the CAH1 gene, the CAH1 protein is found to persist within the cells for a few days after the switch (Moroney et al., 2011). CAH1 was initially believed to facilitate the uptake of C$_i$ by C. reinhardtii cells acclimating to a low CO$_2$ environment. This is because the inhibition of CAH1, through the use of cell membrane impermeable CA inhibitors, has shown a corresponding decrease in the C$_i$ affinity of low CO$_2$ acclimated C. reinhardtii cells (Berry et al., 1976; Badger et al., 1980; Tsuzuki and Miyachi., 1989; Moroney et al., 1985). However, work with cahl, a mutant lacking CAH1, failed to show any measurable difference in C$_i$ affinity or C$_i$-dependent photosynthetic O$_2$ evolution at different external pH levels between wild type and mutant cells (Van and Spalding., 1999). This experimental fact, together with the existence of a normally functioning CCM in CC-503, a natural C. reinhardtii laboratory strain lacking CAH1 (Moroney et al., 2011; Chapter 5 of this thesis), has undermining the importance of the perceived role of CAH1 in the C. reinhardtii CCM. CAH2, another periplasmic β-CA which shows a very close sequence similarity to CAH1, is believed to be the result of a gene duplication event. However unlike CAH1, the expression of CAH2 is very low and is only induced under high CO$_2$ conditions and therefore has no role in the C. reinhardtii CCM function (Fujiwara et al., 1990; Rawat and Moroney., 1991; Brueggeman et al., 2012).

The last member of the C. reinhardtii α-CA family, CAH3, is an indispensable component of the CCM. Unlike CAH1, mutants lacking CAH3 can barely survive under low CO$_2$ conditions and fail to provide Rubisco with CO$_2$ resulting in an overaccumulation of C$_i$ in the thylakoid
lumen (Spalding et al., 1983; Moroney et al., 1986; Funke et al., 1997; Karlsson et al., 1998; Hanson et al., 2003). Also, unlike CAH1, CAH3 is constitutively expressed, showing only a mild upregulation under low CO$_2$ (Karlsson et al., 1998; Im et al., 2003; Wang et al., 2006; Ynalvez et al., 2008; Yamano and Fukuzawa., 2009; Brueggeman et al., 2012; Fang et al., 2012).

However, like CAH1, the expression of the CAH3 gene is also regulated by the transcription factor CIA5, as evident by its absence from the cia5 mutant (Moroney et al., 1989; Marek and Spalding., 1991.; Ynalvez et al., 2008). CAH3 is located in the chloroplast (Karlsson et al., 1995, 1998; Moroney and Ynalvez., 2007) and might possibly be associated with Photosystem II (PSII) particles (Karlsson et al., 1998; Park et al., 1999). The lumenal localization of CAH3 is consistent with its two part N-terminal leader sequence to direct the protein past the chloroplast membrane to finally cross the thylakoid membrane into the lumen. Also, the second part of the leader sequence directing the protein to the thylakoid membrane has twin arginine motifs characteristic of other thylakoid lumen proteins (Karlsson et al., 1998). A recent study regarding the localization of CAH3, points to a higher concentration of the protein in the lumen of thylakoid tubules penetrating the Rubisco packed-pyrenoid structure (Sinetova et al., 2012). As a result, this places CAH3 at a closer proximity to Rubisco molecules and therefore ensures quicker fixation and less leakage under limiting CO$_2$ conditions (Sinetova et al., 2012).

The two proposed physiological roles for CAH3 within the *C reinhardtii* CCM, explain some of the phenotypes associated with the mutants lacking the protein. For instance, CAH3 is believed to be involved in catalyzing the conversion of HCO$_3^-$ ions (brought in from the chloroplast stroma) to CO$_2$, a reaction greatly favored by the acidic environment of the thylakoid lumen (Pronina and Semenko, 1992; Raven 1997; Karlsson et al., 1998; Moroney and Somanchi, 1999; Hanson et al., 2003). Therefore, in the absence of this CA, the pool of HCO$_3^-$ ions brought
into the thylakoid lumen would largely be unused in generating CO$_2$ leading to its overaccumulation in the cells, while Rubisco is still starved of its photosynthetic substrate (Spalding et al., 1983; Moroney et al., 1986). This causes CAH3 mutant cells to show a very poor growth under low CO$_2$ conditions while being able to grow under high CO$_2$ conditions. The second hypothesized role for CAH3 is in the stabilization of the PSII complex by CAH3 by the supply of HCO$_3^-$ ions to it (Shutova et al., 2008; Park et al., 1999; Villarejo et al., 2002). Therefore, according to this hypothesis, the absence of CAH3 causes a reduction of PSII activity in cells and slows down growth and affects C$_i$ dependent photosynthetic O$_2$ evolution (Villarejo et al., 2002). However a contrary report which points to a greater effect on CO$_2$ fixation than loss of PSII activity also exist (Hanson et al., 2003). CAH3 has also been shown recently to function in an epistatic manner to LCIB, a protein implicated in facilitating the transport of Ci into the thylakoid lumen under limiting CO$_2$ conditions (Duanmu et al., 2009). However, the exact role of CAH3 in the facilitation of C$_i$ transport is not yet known.

The next CAs to consider for their possible roles in the C. reinhardtii CCM belong to the β-CA family consisting of CAH4, CAH5, CAH6, CAH7, CAH8 and CAH9. The first β-CAs to be discovered, were CAH4 and CAH5. They were isolated from mitochondrial fractions and the proteins were subsequently found to differ by a single amino acid (Villand et al., 1997). The CAH4/5 genes are arranged in an inverted repeat (head to head) configuration on chromosome 5 indicating a possible gene duplication event. Both CAH4 and CAH5 have leader sequences consistent with mitochondrial localization and after the cleavage of the signal peptide have a mature protein roughly 21 kDa in size (Eriksson et al., 1996). The retrieval of CAH4 and CAH5 in the mitochondrial proteome further confirms their subcellular localization within the mitochondria (Cardol et al., 2005). The CAH4/5 genes are amongst the highly upregulated
CCM genes (Villand et al., 1997; Eriksson et al., 1998), showing an upregulation of more than 512 fold (Ynalvez et al., 2008; Brueggeman et al., 2012; Fang et al., 2012) after a switch from high to low CO₂. While the expression of CAH5 is completely repressed in the cia5 mutant, the expression of CAH4 is not completely repressed suggesting a possible difference in their regulation mechanism and response to CIA5 (Ynalvez et al., 2008; Brueggeman et al., 2012). However, both genes are repressed once cells are switched to a high CO₂ environment (Fujiwara et al., 1990). Interestingly, like CAH1, CAH4/5 expression exhibit a circadian rhythm of expression in synchronized cultures (alternating light and dark cycles) and are repressed by the inhibition of the glycolate cycle (Villarejo et al., 1997). Also like CAH1, the CAH4/5 proteins once produced by the C. reinhardtii cells under low CO₂, persist for a minimum of 4 days after the restoration to high CO₂ conditions (Moroney et al., 2011). The roles of CAH4/5 in the C. reinhardtii CCM are not completely understood. However, because of their association with the mitochondrion, they are believed to play a role in not only balancing the pH of the mitochondrial matrix but also in the anaplerotic carbon recycling processes as exemplified by reactions catalysed by PEP (phosphoenolpyruvate) carboxylase (Giordano et al., 2003). In the absence of adequate CO₂, the delicate balance between the carbon and nitrogen cycles is greatly disturbed and the need for recycling carbon skeletons for nitrogen assimilation is required as an overall stress response by the C. reinhardtii cells (Giordano et al., 2003). Keeping in mind the mitochondrial migration closer to the plasma membrane under limiting CO₂ conditions, the roles of these mitochondrial CAs might be related to the prevention of any CO₂ from the cells from leaking out and/or trapping any incoming CO₂ by the rapid conversion of CO₂ to charged HCO₃⁻ ions, that can be released into the cytosol for uptake by the chloroplast (Giordano et al., 2003., Moroney et al., 2011). In the same vein, rehydration of both respiratory and photorespiratory
CO₂ to HCO₃⁻ ions, by CAH4/5, could also yield more CO₂ as photosynthetic substrate for Rubisco (Giordano et al., 2003).

The third β-CA, CAH6 is another important chloroplastic CA (like CAH3) with a direct role in the C. reinhardtii CCM. The absence of a mutant for this CA prevents researchers from figuring out the direct physiological effects, that the absence of this CA might have, on a low CO₂ acclimated C. reinhardtii cell (Moroney et al., 2011). CAH6, like CAH3 is constitutively expressed showing only a mild upregulation under low CO₂ conditions (Mitra et al., 2004; Ynalvez et al., 2008; Brueggeman et al., 2012; Fang et al., 2012). Immunogold labeling has placed CAH6 in the chloroplast stroma of C. reinhardtii cells but it is still a matter of speculation if it is present as a free soluble stromal protein or is part of a protein complex with possible association to the thylakoid membrane (Mitra et al., 2004). The main role for the stromal CAH6 is believed to be the hydration of the CO₂ diffusing into the chloroplast stroma from either the cytosol or the pyrenoid, to HCO₃⁻ ions thus preventing its diffusive loss out of the cell under limiting CO₂ conditions (Mitra et al., 2004; Moroney et al., 2011). The alkaline environment of the stroma favors the hydration reaction of CAH6. The location of CAH6 within the chloroplast at any given time actually might reflect its exact role in the CCM (Moroney et al., 2011). For instance, its location within the chloroplast stroma, surrounding the pyrenoid or away from it would point to its role in CO₂ leakage prevention via its hydration of CO₂ to HCO₃⁻ ions. However, if present within the pyrenoid matrix it might contribute to the dehydration of HCO₃⁻ ions to yield more CO₂ in the vicinity of Rubisco. Thus, the generation of a CAH6 mutant and a more thorough localization within the chloroplast stroma in low CO₂ acclimated cells might throw more light into the actual physiological role/roles of CAH6 in the C. reinhardtii CCM (Moroney et al., 2011).
The last β-CAs to be considered are CAH7, CAH8 and CAH9. Of these, CAH7 and CAH9 have not yet been conclusively localized within the *C. reinhardtii* cell while CAH8 is believed to be associated with the plasma membrane (Ynalvez *et al*., 2008; Moroney *et al*., 2011). Both CAH7 and CAH8 have long C-terminal extensions with predicted hydrophobic regions consistent with transmembrane helices (Ynalvez *et al*., 2008). Although immunogold labeling has shown CAH8 to be associated with the plasma membrane, the actual location of the active site remains unclear (Ynalvez *et al*., 2008). The location of the active site outside the cell would indicate a CA function more in keeping with the periplasmic CA, CAH1, while a location on the cytosolic side might indicate a role in CO₂ trapping much like the chloroplast CA, CAH6. The lack of any leader sequences for CAH9 on the other hand predicts a possible cytosolic location (Moroney *et al*., 2011). CAH7 and CAH8 are constitutively produced in moderate amounts while the expression of CAH9 is very low in *C. reinhardtii* cells (Ynalvez *et al*., 2008; Moroney *et al*., 2011; Brueggeman *et al*., 2012; Fang *et al*., 2012). There is no significant level of induction of CAH8 under low CO₂ conditions indicating that any possible roles in the facilitation of C₅ uptake might be secondary to CAH1, which is highly upregulated under similar conditions. However, the redundancy of function between CAH8 and CAH1 needs to be examined in the future to possibly account for the lack of a compromised CCM in CAH1 mutants (Moroney *et al*., 2011).

The γ-CA family in *C. reinhardtii* consists of three genes, CAG1, CAG2 and CAG3. These genes are expressed constitutively and therefore show no response to changes in CO₂ levels. In higher plants, these CAs are believed to be associated with Complex I of the mitochondrial electron transport chain, found in the inner mitochondrial membrane (Klodmann *et al*., 2010; Sunderhaus *et al*., 2006). Not surprisingly, the 3 γ-CAs in *C. reinhardtii* cells are associated with the mitochondrial Complex I in proteomic studies done with whole mitochondria.
as well as mitochondrial electron transport complexes on the membrane (Cardol et al., 2004, 2005). The CA domain of Complex I as revealed by electron micrographs lies on the matrix side of the mitochondrion, and is believed to exist only in the mitochondria of higher plants and other phototrophic organisms (Braun and Zapaleta, 2007). The exact role, if any, that these γ-CAs play in the C. reinhardtii CCM remains to investigated (Moroney et al., 2011).

**Acquisition of inorganic carbon for the Chlamydomonas reinhardtii CCM**

The photosynthesizing C. reinhardtii cell can actively take up both CO$_2$ and HCO$_3^-$ ions when faced with carbon stress, a distinctive feature of its CCM process. Although at any given point active CO$_2$ uptake by the cell consists of the majority of the C$_i$ flux as evident from carbon disequilibrium and mass spectrometric studies (Marcus et al., 1984; Sultemeyer et al., 1989; Badger et al., 1994; Palmqvist et al., 1994), the active uptake of HCO$_3^-$ ions at the plasma membrane and the chloroplast level is also supported experimentally (Sultemeyer et al., 1989; Thielmann et al., 1990; Badger et al., 1994; Palmqvist et al., 1994; Amoroso et al., 1998). When considering C$_i$ uptake and transport, it is sometimes difficult to distinguish between active transport of CO$_2$ and HCO$_3^-$ ions on one hand and the passive uptake of CO$_2$ on the other. However, there is sufficient evidence at this point that supports the active uptake of HCO$_3^-$ ions as the internal concentration is too high to be accounted for by pH gradient alone (Marcus et al., 1984; Moroney et al., 1987; Palmqvist et al., 1994, 1998; Sultemeyer et al., 1989; Goyal and Tolbert, 1989; Badger et al., 1994; Amoroso et al., 1998; van Hunnik et al., 2002). Because of the fact that CO$_2$ is a small uncharged molecule with a partition coefficient of 1 (Prins and Elzenga, 1989), it can easily diffuse through the lipid barrier posed by the plasma and other cellular membrane barriers. The rapid CO$_2$ fixation carried out by Rubisco in the chloroplast of an actively photosynthesizing C. reinhardtii cell creates a concentration gradient that could be
driving the diffusive entry of CO\textsubscript{2} molecules through the plasma membrane, chloroplast membrane and finally the thylakoid membrane (Raven, 2010; Spalding \textit{et al.}, 2011). Thus, there is a possibility that although the generation of the CO\textsubscript{2} concentration gradient in the chloroplast might be an active process, the diffusive entry of the molecule across membrane barriers with or without the help from speculative CO\textsubscript{2} channels like Rhesus proteins (Soupene \textit{et al.}, 2004) and/or aquaporins (Calamita \textit{et al.}, 1997) indicate that CO\textsubscript{2} movement across membranes is most likely passive. The charged C\textsubscript{i} species, namely the charged HCO\textsubscript{3}\textsuperscript{-} ions on the other hand, unlike CO\textsubscript{2}, cannot cross the lipid barrier posed by the various membranes within the \textit{C. reinhardtii} cell, without the help of membrane transporter/transporters. The final fate of most of the C\textsubscript{i} pool within the cell, whether in the charged (HCO\textsubscript{3}\textsuperscript{-}) or uncharged form (CO\textsubscript{2}) within the cell, is the ultimate conversion and concentration of CO\textsubscript{2} near the Rubisco active site within the pyrenoid (Borkhsenious \textit{et al.}, 1998). As mentioned earlier with regards to the \textit{C. reinhardtii} acclimation to variable CO\textsubscript{2} concentrations, the C\textsubscript{i} species available to the cell is also dependent on external pH levels. Under limiting CO\textsubscript{2} conditions, the alkaline environment often makes HCO\textsubscript{3}\textsuperscript{-} ions the predominant C\textsubscript{i} species and an integral part of the functional model of the CCM are the C\textsubscript{i} transport proteins that account for an efficient HCO\textsubscript{3}\textsuperscript{-} uptake and transport system through the \textit{C. reinhardtii} cell. The following sections will summarize the current knowledge of C\textsubscript{i} transport, at each subcellular level within the \textit{C. reinhardtii} cell with an induced CCM.

\textbf{C\textsubscript{i} transport across the plasma membrane}

The \textit{C. reinhardtii} cell has been shown to take up both CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} ions across the plasma membrane barrier under CO\textsubscript{2} limiting conditions. While the movement of CO\textsubscript{2} is believed to be largely through passive diffusion, the movement of HCO\textsubscript{3}\textsuperscript{-} ions is an active transporter-mediated process (Spalding, 2008). So far, a couple of putative transporter proteins with a
plasma membrane location, namely HLA3 and LCI1, have been implicated in the uptake of C\textsubscript{i} into the *C. reinhardtii* cell (Duanmu *et al.*, 2008; Ohnishi *et al.*, 2011). Two Rhesus-like proteins called RHP1 and RHP2 with putative roles as CO\textsubscript{2} channels (Soupene *et al.*, 2004; Kustu and Inwood., 2006) are also localized to the plasma membrane (Yoshihara *et al.*, 2008).

The *HLA3* (High Light Acclimation 3) gene was first discovered as part of a restriction fragment differential display (RFDD) experiment that revealed a large array of *C. reinhardtii* genes induced by both high light and low CO\textsubscript{2} conditions (Im and Grossman., 2002). This gene was found to encode a putative ABC type transporter belonging to the multi-drug-resistance (MRP) sub-family of proteins and was not expressed in the *cia5* mutant indicating a similar regulatory control as other key CCM genes (Im and Grossman, 2002). The presence of a complete ABC-MRP domain arrangement of this protein made it a likely candidate as a single transporter on the plasma membrane not requiring any dimerisation or complex formation. Recently, the transcript levels of *HLA3* were successfully knocked down in *C. reinhardtii* cells, both singularly and in conjunction with other putative transporters (Duanmu *et al.*, 2009b) using RNA-interference (RNAi) mediated targeted RNA depletion. When knocked down on its own, there were significant decreases in photosynthetic C\textsubscript{i} affinity and C\textsubscript{i} uptake only under highly alkaline conditions, where HCO\textsubscript{3}\textsuperscript{-} is the predominant C\textsubscript{i} species. When the reduction in HLA3 was combined with a reduction in either *LCIB* levels (encoding a soluble C\textsubscript{i} uptake facilitator found in the chloroplast stroma) or in *NAR1.2* levels (encoding a putative C\textsubscript{i} transporter on the chloroplast membrane), the *HLA3* mutant cells showed very severe growth defects, with further decreases in photosynthetic C\textsubscript{i} affinity and C\textsubscript{i} uptake. These results point to the possible role of this transporter in the C\textsubscript{i} uptake process of the *C. reinhardtii* CCM.
The second putative C_i transporter on the plasma membrane is LCI1 (Low CO_2 Inducible 1). The gene encoding this protein was first discovered as a low CO_2 inducible gene and found to be highly induced like many of the other CCM genes and regulated by the CCM ‘master regulator’ gene, CIA5 (Burow et al., 1996; Im et al., 2003; Miura et al., 2004). The regulation of the LCI1 gene is directly regulated by the myb-transcription factor, LCR1 (Yoshioka et al., 2004). Recently, an attempt was made to artificially over-express the LCI1 gene under the control of a nitrate-inducible promoter, in an lcr1 mutant background. The aim of this experiment was to turn on the artificial LCI1 construct under high CO_2 conditions with the help of nitrate to get LCI1 expression in an environment free of other CCM components to see if it had any direct impact on C_i uptake on its own. Additionally, if the gene could be turned on under low CO_2 it would reveal its possible impact on the C_i uptake of the CCM. Nitrate induced expression of LCI1 under high CO_2 conditions in the lcr1 mutant background, increased the photosynthetic C_i affinity, C_i uptake/accumulation by cells and light-dependent CO_2 gas exchange (LCE) activity by the cells (Ohnishi et al., 2011). Although the increased C_i affinity and accumulation seen at a higher pH of around 7.8 (where C_i is predominantly in the form of HCO_3^- ions), pointed to a possible role as a bicarbonate transporter, the lack of differences between the increases in LCE activity at pH 6.2 and pH 7.8 in cells expressing LCCI under high CO_2, revealed an ambiguity about the C_i species preferred by this transporter. The LCI1 stimulated increase in LCE activity was also seen under low CO_2 conditions, despite the inability of the artificially expressed protein to reach wild-type levels. The LCI1 protein was also enriched in the plasma membrane fraction in cell fractionation experiments and localized to the plasma membrane of whole cells via a GFP fluorescence tag (Ohnishi et al., 2011). Based on the
above results, the LCI1 protein is now implicated as an integral part of the plasma membrane localized C\textsubscript{i} transport system.

Two Rhesus-like proteins, namely RHP1 and RHP2 which show similarity to the Rh proteins of human red blood cell membranes have been identified in \textit{C. reinhardtii} (Soupene \textit{et al.}, 2004; Kustu and Inwood., 2006). The \textit{RHP1} gene is highly expressed under high CO\textsubscript{2} conditions and the protein was localized to the plasma membrane (Yoshihara \textit{et al.}, 2008). The RNAi mediated knock down of \textit{RHP1} expression has shown an inability of mutants to grow under high CO\textsubscript{2} conditions but remain unaffected by low CO\textsubscript{2} conditions. This indicates its possible role in the uptake of CO\textsubscript{2} by the \textit{C. reinhardtii} cell under abundant CO\textsubscript{2} conditions. The low expression of RHP1 and RHP2 under low CO\textsubscript{2} conditions does not point to a large involvement in CO\textsubscript{2} transport for the CCM. However, their possible role as a CO\textsubscript{2} channel in the CCM cannot be ruled out completely, because of the continued presence of the proteins in small quantities, in low CO\textsubscript{2} acclimated cells.

\textbf{C\textsubscript{i} transport into the chloroplast}

As mentioned earlier, the entry of C\textsubscript{i} into \textit{C. reinhardtii} cells under CCM inducing conditions can occur in both the charged (HCO\textsubscript{3}\textsuperscript{-}) and uncharged forms (CO\textsubscript{2}). Once in the cytosol, the cytosolic CAs create a larger pool of HCO\textsubscript{3}\textsuperscript{-} ions by trapping and hydrating some of the incoming CO\textsubscript{2} molecules to prevent their leakage out of the cell. The CCM machinery taps into this cytosolic pool of HCO\textsubscript{3}\textsuperscript{-} to meet the CO\textsubscript{2} needs of photosynthesis under limited CO\textsubscript{2} conditions. The CO\textsubscript{2} molecules can enter the chloroplast via passive diffusion, an inward flux largely driven by the huge concentration gradient created by the fixation of CO\textsubscript{2} by Rubisco. However, the charged HCO\textsubscript{3}\textsuperscript{-} ions need the assistance of a chloroplast membrane bound
transporter. There are several proteins that have been identified as playing a role in the chloroplast membrane bound $C_i$ transport system. These include the putative transporters CCP1/CCP2, belonging to the mitochondrial carrier protein family, NAR1.2 belonging to the Formate/Nitrite (FNT) family of transporters and YCF10 belonging to the CEM family of proteins.

**$C_i$ transport into the thylakoid**

To date, transporters that might help the transport of $C_i$ across the thylakoid membrane have not yet been identified. However, LCIB (and its family of proteins namely, LCIC, LCID and LCIE) has been hypothesized to play a role in the facilitation of $C_i$ uptake. The pmp1 mutant and its allelic strain ad1 (air dier 1) missing the LCIB protein are the only single gene mutants identified so far that fail to accumulate $C_i$ when grown in atmospheric CO$_2$ and are believed to be deficient in $C_i$ uptake (Spalding et al. 1983). Although LCIB does not have any transmembrane domains, it is believed to be part of a complex that localizes around the pyrenoid under low CO$_2$ and helps in the recapture of any leaked CO$_2$ from the pyrenoid (Yamano et al., 2009). LCIB also acts epistatically downstream of CAH3 further complicating its proposed function in *C. reinhardtii*.

**The role of the pyrenoid in the Chlamydomonas reinhardtii CCM**

In eukaryotic algae, the pyrenoid may serve a function similar to the role of the carboxysome in cyanobacteria. Rubisco is localized to the pyrenoid in a number of green algae including *C. reinhardtii* (Rawat et al., 1996; Borkshenious et al., 1998; Morita et al., 1997). Isolated pyrenoids are composed largely of Rubisco. A number of morphological changes have also been observed when cells are transferred to a low CO$_2$ concentration. These changes include
the formation of a starch sheath around the pyrenoid as well as redistribution of Rubisco. This redistribution results in almost all of the Rubisco being localized to the pyrenoid as opposed to the chloroplast stroma. In *C. reinhardtii*, the thylakoid carbonic anhydrase catalyzes the interconversion of HCO$_3^-$ into carbon dioxide and results in a high local concentration of carbon dioxide, which Rubisco can use before it has a chance to leak out of the cell. The role of the pyrenoid is therefore to provide a compact location for the generation of carbon dioxide in the presence of Rubisco. Mutants that lack Rubisco have been shown to lack a pyrenoid structure (Rawat *et al.*, 1996). Recently, it was shown that Rubisco mutants engineered to have chimeric Rubisco enzymes where the *C. reinhardtii* Rubisco large subunit forms a hybrid with higher plant Rubisco small subunits not only lack pyrenoids but also have a defective CCM (Genkov *et al.*, 2010). The need to have a well-organized pyrenoid for the CCM to function efficiently has been further validated by the recent report of a novel carbon dioxide concentrating mechanism mutant, *cia6*. CIA6 is believed to encode a putative SET-domain methyl transferase. Mutants lacking this protein lack an organized pyrenoid despite having normal levels of Rubisco and grow very poorly under low CO$_2$ growth conditions (Ma *et al.*, 2010). Therefore, it is now clear that an organized pyrenoid is essential for the proper functioning of the carbon dioxide concentrating mechanism. The dynamic nature of the pyrenoid has also been further revealed by recent studies that show the presence of certain protein complexes such as the LCIB/LCIC complex that moves closer to the pyrenoid and surrounds it under low CO$_2$ conditions and together with the stromal carbonic anhydrase CAH6 plays a role in the possible recapturing of CO$_2$ leaking out of the pyrenoid (Yamano *et al.*, 2009). The mutants that lack LCIB have already been shown to have slow growth phenotype under low CO$_2$ conditions emphasizing the importance of CO$_2$ recapture outside the pyrenoid as essential to the function of the *C.*
*C. reinhardtii* CCM. Characterizing the proteins that play a structural role in the carboxysome in cyanobacteria and the pyrenoid is now an active area of research.

**Introduction to the thesis**

The *C. reinhardtii* CCM is a model for the in-depth study of eukaryotic CCMs. One of the important aspects of this CCM that needs to be worked out in more detail is the $C_i$ uptake system that is integral to the successful operation of this CCM. Since CO$_2$ is a small neutral molecule, it is believed to be able to passively diffuse into the cell with or without any assistance from membrane bound transport proteins. However, the HCO$_3^-$ ion needs transporters to facilitate its uptake across each membrane barrier. The hydrophobic barriers posed by the plasma membrane, chloroplast envelope and thylakoid membranes in the path of the charged HCO$_3^-$ ion’s ultimate destination to the thylakoid lumen has led to the proposed existence of one or more transport proteins at each of these membrane locations. The well-characterized $C_i$ uptake system in the prokaryotic model namely the cyanobacterial CCM has provided good support to the indispensability of an efficient $C_i$ uptake system in the operation of CCMs in general.

Over the years, a number of possible candidates have been investigated for their possible involvement in the *C. reinhardtii* CCM. As previously summarized, proteins such as LCI1, HLA3, NAR1.2, CCP1/CCP2 have been viewed as potential $C_i$ uptake proteins although none were identified as a potential thylakoid membrane protein. The thylakoid transporter is crucial because of the following reason. The thylakoid lumen CA, CAH3, is believed to be the key CA causing the rapid conversion of HCO$_3^-$ to CO$_2$ around the Rubisco sequestered in the pyrenoid under CCM inducing conditions. In the absence of this CA, *C. reinhardtii* cells show a drastic growth deficiency under CO$_2$ limiting conditions. For this CA to have HCO$_3^-$ ions to dehydrate,
there has to a transport protein that facilitates the entry of HCO$_3^-$ into the thylakoid lumen. Therefore identification of potential candidates that might be involved in C$_i$ uptake in this specific membrane location is still very much the focus of researchers studying this aspect of the 

C. reinhardtii CCM.

The work summarized in this thesis is aimed at two main objectives: 1.) the characterization of previously identified putative C$_i$ transporter candidates (Chapter 4 and 2) the possible identification of novel C$_i$ transporters, for instance a potential thylakoid transporter (Chapter 3 and 6). Investigation of a previously identified NAR protein family, composed of 6 members, belonging to the Formate/nitrite transporter (FNT) family of proteins is the focus of Chapter 3. The presence of so many members of a small anion transporter family, without known functions or membrane locations made them interesting targets for further investigation. In order to figure out their functional roles in the C. reinhardtii CCM, the expression levels of the NAR genes were measured to reveal any increases in response to low CO$_2$. Also, the possible nitrite transport role of these FNT proteins were investigated by measuring any change in gene expression upon changing the source of nitrogen from ammonium to nitrate. Since nitrate would be converted to nitrite in the cytoplasm, the resultant nitrite would require a potential chloroplastic nitrite transporter to cross the chloroplast membrane barrier. Of the NAR proteins previously identified, NAR1.2, was found to be the most important for a potential CCM role. The location of the protein was also confirmed to the chloroplast membrane. However, attempts to knock-down protein expression using RNAi were unsuccessful. In the future, focus will still be maintained on this class of proteins with the specific aim to find their membrane locations within the cell. This might yield NAR proteins, in addition to NAR1.2, that would prove to be interesting candidates in the study of C$_i$ transport into C. reinhardtii cells.
With a view to unearthing novel transporters involved in the CCM (with special emphasis on thylakoid membrane transporters) a large-scale mutagenesis screen was undertaken (Chapter 6). PCR-based reverse genetics screen was carried with roughly 22,000 insertional mutants with an aim to identify mutations in previously identified putative C₃ transport proteins. The absence of mutants lacking the key putative Cᵢ transporters increased the importance of this screen. A phenotype (growth deficiency under low CO₂) based forward genetics screen was also undertaken to identify any potential mutants in potential transporters that causes a disruption in CCM function. A total of 3 potential transporter genes (including a NAR gene) were found disrupted by the inserts, during the PCR-based screen of transformants.

LCI1, a potential Cᵢ transporter was investigated for its potential role as a Cᵢ transporter in the *C. reinhardtii* CCM (Chapter 4). The endogenous LCI1 and the GFP-tagged protein were found to localize to the plasma membrane. Artificial expression of the protein under high CO₂ conditions (CCM repressed), showed the ability of the protein to cause an increase in Cᵢ uptake. This provided evidence in support of LCI1 being an independently functional Cᵢ uptake protein and an integral part of the plasma membrane Cᵢ uptake system.

The attempt to generate knock-down mutants of Cᵢ transporters via RNAi, within the cell-wall less wild-type strain, CC-503, inadvertently led to the discovery of this strain as a natural mutant for the periplasmic CA, CAH1 (Chapter 5). This should help in the future investigation of CAH1 and other proteins that can compensate for its absence in the *C. reinhardtii* CCM.
CHAPTER 2
MATERIALS AND METHODS

Cell culture and growth

The laboratory strains used for different experiments mentioned in this thesis have been summarized in Appendix I. The cell walled wild-type strains CC-407 and C9 and the cell wall-less wildtype strains D66 and CC-503 were used at different times depending on the requirements of the experiments.

The *C. reinhardtii* laboratory strains were maintained on Tris-Acetate-Phosphate (TAP) media for mixotrophic growth before being transferred to Minimal (MIN) media (absence of acetate) for autotrophic growth. In case of autotrophic growth in liquid media, the flasks were bubbled with either 0.01% (v/v) CO$_2$ for low CO$_2$ growth conditions or roughly 5% CO$_2$ (v/v) for high CO$_2$ growth conditions, with continuous shaking and medium intensity light (roughly 100 µE/m$^2$/s). In case of growth on semi-solid medium, plates of MIN media containing 1.2% agar were inoculated with a small starting amount of cells (determined either qualitatively or quantitatively) in growth chambers maintained at either high or low CO$_2$ conditions. Both TAP and MIN media were prepared according to Sueoka (Sueoka, 1960).

For testing growth on solid media and under different pH levels, equal numbers or cells from different strains were spotted on MIN agar plates maintained at different pH levels by the addition of the following buffers: 25 mM MES (pH 5.8 and 6.2), 25 mM MOPS (pH 7.2) and 25 mM HEPES (pH 8.2). The plates were then allowed to grow in chambers maintained at high and low CO$_2$ conditions for 10 days under continuous illumination of roughly 100 µE/m$^2$/s.
For cell fractionation experiments, a 12 hours light/dark cycle was used during growth to allow for synchronous growth and uniformity of cell size. For the purpose of inducing the CCM in liquid cultures, cells were switched from high CO₂ to low CO₂. For induction of CCM genes either a short growth time of four hours under low CO₂ or a longer growth of 12 hours was used. In case of time course experiments to measure gene expression, the cells were harvested at different time intervals after the switch from high to low CO₂ had been made. In case of measuring protein expression, cells were mostly harvested after a day’s growth under low CO₂ conditions. Growth on TAP or high CO₂ were considered as the control growth condition for most mutant strains showing a growth deficiency under low CO₂ conditions.

In cases where nitrate were being used as the source of nitrogen in the medium instead of ammonium, the appropriate changes to the TAP and MIN media were made to substitute nitrate salts in place of ammonium during their preparation. Also nitrate induction of artificial gene constructs was carried out after harvesting, proper washing and re-suspension of cells in nitrate containing MIN media.

**Transformation of C. reinhardtii cells via electroporation**

The *C. reinhardtii* cell wall-less strain D66 was used for the introduction of artificial DNA constructs for the purposes of generating RNAi and insertional mutants, artificially expressing genes of choice as well as trying to determine the location of cellular proteins. Electroporation was the method of choice for the transformation of the wild-type strain (Shimogawara et al., 1998) because of its efficiency in generating large numbers of transformants as well as preventing large deletions at the sites of insertions as seen with the glass bead method.
Cells grown in either MIN or TAP liquid cultures were harvested at the mid-log phase and suspended in (TAP+sorbitol) to a concentration 2*10^8 cells/mL. Linearized plasmid DNA (1-5 ng) carrying the selective marker gene for paromomycin resistance was then mixed with 300 µL of the *C. reinhardtii* cells and incubated at 15°C for 15 mins in an electroporation cuvette. The cell and DNA mixture in the cuvette was then subjected to an electric pulse in a Gene Pulser II electroporator (Bio-Rad) with a modified 25-mF capacitor and a shunt resistor of 330 V. The electroporated cells were then allowed to recover in the dark, overnight in TAP+sorbitol. Selection for paromomycin resistant transformants was carried out by plating cells from each cuvette on TAP+paromomycin plates and allowing growth of colonies in dim light for 7 days (Pollock *et al.*, 2004).

To generate insertional mutants for a large-scale mutagenesis screen (summarized in chapter 6), D66 cells were transformed with the paromomycin cassette (AphVIII gene flanked by *C. reinhardtii* promoter and terminator sequences) from the plasmid pSL18 (Depege *et al.*, 2003) using the electroporation as mentioned above. The plasmid pSL18 was digested with KpnI-HF (High-Fidelity Enzymes, NEB, Ipswich, MA) and XhoI-HF (NEB, Ipswich, MA) to obtain the 1813 bp paromomycin cassette. A paromomycin concentration of 5 µg/mL was used in TAP+agar plates when screening for transformants. For the detailed protocol of the mutagenesis screen refer to the dissertation of Dr. Yunbing Ma (http://etd.lsu.edu/docs/available/etd-07042012-171543/).

**Nucleic acid preparations**

For genomic DNA extractions from *C. reinhardtii* strains, a protocol developed in Dr. William Snell’s laboratory (personal communication) was used for isolation of large quantities of
DNA in case of the insertional mutagenesis screen. The details of this protocol are available in Dr. Ma’s dissertation (http://etd.lsu.edu/docs/available/etd-07042012-171543/). For a quick prep of PCR-ready DNA in small amounts, a protocol devised by Dr. Steve Pollock (http://www.chlamy.org/methods/quick_pcr.html) was used. This method involves the lysis of cells and subsequent release of DNA, obtained by heating *C. reinhardtii* cells in the presence of Na-EDTA.

The extraction of RNA from *C. reinhardtii* cells was carried out using Trizol reagent (Invitrogen, Carlsbad, CA) and following the manufacture’s protocol with a few modifications (Ynalvez et al., 2008). Contaminating DNA was removed from RNA samples by DNase treatment (Roche Applied Science, Indianapolis, IN) and further purification of RNA carried out using a commercial kit (RNeasy kit, Qiagen, Chatsworth, CA). Total RNA was used for cDNA synthesis using the first strand synthesis reverse transcription kit (Roche Applied Science, Indianapolis, IN) including a CMV reverse transcriptase enzyme and oligo-dT primers. The manufacturer’s recommendations for amounts of RNA and the steps of the reverse transcription protocol were adopted and slightly modified when needed. Synthesized cDNA was used for used for quantitative PCR using a Sybr Green Premix (Takara, Shiga, Japan; now discontinued) and an ABI 7000 Real Time PCR System (Applied Biosystem, Foster City, CA).

Small-scale extraction of plasmid DNA was carried out either with the standard alkaline lysis method followed by ethanol precipitation (Sambrook et al., 2001) or with the help of a commercial kit (Qiagen, Chatsworth, CA). Large-scale plasmid preparations were carried out with a commercial plasmid maxi-prep kit (Qiagen, Chatsworth, CA).
Analysis of protein

To isolate total protein from *C. reinhardtii* cells, mainly for the purpose of SDS-PAGE (Sodium Dodecyl/ Lauryl Sulfate PolyAcrylamide Gel Electrophoresis), whole-cell proteins corresponding to 2 mg of chlorophyll were suspended in 50 mM Tris-HCl, buffer (pH 8.0), containing 25% glycerol (w/v) to a volume of 10 mL. The cells were solubilized by incubation at 100°C for 1 min in the presence of 2% SDS and 0.1 M DTT and subsequently centrifuged at 13,000g for 3 min. The supernatant containing solubilized proteins was subjected to SDS-PAGE with a 12% or 15% polyacrylamide gel that contained 6 M urea especially in case of membrane proteins (Ohnishi and Takahashi, 2001). In the case of protein immunoblot analysis for the cell fractionation experiments, samples containing equal amounts of protein solubilized in a buffer containing 5% SDS, 0.1 mM DTT, 0.1 M Na$_2$CO$_3$, 7 M urea, 0.02% mercaptoethanol, and 25 mM Tris were run on 12% or 15% SDS-PAGE gels. Separated polypeptides were blotted electrophoretically onto polyvinylidene difluoride membranes and probed with polyclonal antibodies against LCI1 (1/1000 dilution), CCP1 (1/1000 dilution; Ramazanov *et al*., 1993), NAR1.2 (1/1000 dilution) and D1 protein of photosystem II (1/1000 dilution; product number AS05 084 from Agrisera). The antibodies against LCI1 (AS-F and AS-M) were raised in rabbits using synthetic oligopeptides as the antigens, which were FDTQEGLKYTPVY (from Phe-43 to Tyr-56) or DAEESHAMPNVHVTSDGATKV (from Asp-172 to Val-192), respectively. The former oligopeptide was also used for affinity purification of the antibody against LCI1. The rabbit antibody against CCP1 was raised against the peptide CFKQVMSKHKGLYRGFTST. Signals were visualized using an enhanced chemiluminescence reagent (ECL; GE Healthcare) or one from Pierce.
**Isolation of Chloroplast Envelope and Plasma Membrane**

Intact chloroplasts were isolated from cells of CC-503 according to Mason *et al.* (2006). Isolated chloroplasts were then used for chloroplast membrane fractionation by the method of Clemetson and Boschetti (1988). The chloroplast envelope proteins were solubilized in buffer containing detergent. The procedure followed that for the isolation of plasma membranes adopted from Norling *et al.* (1996). Protein concentration was determined using the bichiniconic acid assay (Pierce) before loading on SDS-PAGE gels.

**GFP Fluorescence Imaging**

For GFP fluorescence imaging, live cells were immobilized in 1% low-melting-point agarose and observed using a laser scanning microscope LSM710 system at an excitation of 405 nm and emission at 490 to 550 nm for GFP image capture and at an excitation of 488 nm and emission at 600 to 700 nm for the chlorophyll image capture.

**DIC-Dependent Photosynthetic Oxygen Evolution**

The affinity of cells for C\textsubscript{i} was evaluated by measuring the rate of dissolved C\textsubscript{i}-dependent photosynthetic O\textsubscript{2} evolution. Cells were collected by centrifugation and then resuspended in 50 mM HEPES-KOH buffer, pH 7.8, or 50 mM MOPS-KOH buffer, pH 7.0, at 20 mg/mL chlorophyll. Photosynthetic oxygen evolution was measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK).

**Intercellular Concentration of Dissolved C\textsubscript{i}**

The intercellular concentration of dissolved C\textsubscript{i} was measured by the silicone oil centrifugation method (Fukuzawa *et al.*, 1998). Cells grown under low CO\textsubscript{2} were collected by
centrifugation and subsequently suspended at a cell density of 25 mg/mL chlorophyll in 50 mM HEPES-NaOH buffer (pH 7.8), that had been bubbled with nitrogen gas. 1 mL of cell suspension was bubbled with nitrogen gas for 10 min in an O₂ electrode until they no longer produced O₂. First, a 60-mL silicone oil layer (SH550/SH556 = 4/7 [v/v]) was overlaid on a 20-mL layer of the termination solution containing 1 M glycine-NaOH, pH 10.0, and 0.75% SDS (w/v). Then, the cells (300 mL) were further overlaid on the silicone oil layer. SH550 and SH556 were purchased from Toray Dow Corning Silicone and Nacalai Tesque, respectively. Cᵢ uptake was initiated by the addition of 10 mL of NaH¹⁴CO₃ at 50 mM final concentration, which was immediately followed by 20, 40, or 80 s of illumination with an actinic light source of 300 mmol photons/m²/s, and the reaction was terminated by centrifugation. Control cells that had been supplied with nonradioactive NaHCO₃ were collected as the zero time point. After centrifugation, the labeled cells were immediately frozen using liquid nitrogen and then suspended in 0.1 N NaOH. The alkaline cell suspension was divided into two aliquots. One was directly subjected to a liquid scintillation counting, which was described as total Cᵢ consumption. The other aliquot was added to 0.5 N HCl, desiccated to liberate ¹⁴C except for the fixed ¹⁴CO₂ and then subjected to a liquid scintillation counting to analyze the ¹⁴C level, which was described as CO₂ fixation. Cᵢ uptake/accumulation was calculated as the difference between the total Cᵢ consumption and CO₂ fixation. The Cᵢ uptake was corrected by estimating the cell volume as sorbitol impermeable space (SIS) using [¹⁴C]-sorbitol and ³H₂O as described previously (Heldt, 1980).

**Complementation of cyanobacterial mutants**

The cyanobacterial double deletion mutant, DelCS, lacking the high affinity transporters SbtA and BCT1, was used for the cyano-complementation experiments. This mutant strain was
generated in the laboratory of Dr. Dean Price in the wild-type background of the freshwater cyanobacterial strain, PCC7942. For this experiment, open reading frame (ORF) sequences from *C. reinhardtii*, coding for putative bicarbonate transporters, were used in phenotype-rescue experiments with the DelCS mutant. The *C. reinhardtii* genes NAR1.2, LCI1 and CCP1 were selected for this experiment. ORF sequences with and without predicted leader sequences (using SignalP, ChoroP and pSORT software) were cloned into the pENTR-D-TOPO entry vector (Invitrogen, Carlsbad, CA) and the resultant vector DNA sent to Dr. Price’s laboratory in Australia for further processing. In Dr. Price’s lab, the sequences were transferred to the destination vectors pSE2 and pSE4 by the LR recombinase reaction (Gateway cloning, Invitrogen). The pSE2/pSE4 vector DNA was used to transform the DelCS mutant cells and successful transformants then used for bicarbonate uptake measurements via membrane-inlet mass spectrometry (MIMS). High CO$_2$ grown cells (3% CO$_2$) were harvested and placed in BG11 media without nitrate (+20 mM NaCl) and 50 mM BTP-HCl (pH 8.2) for the assay. The available inorganic carbon or C$_i$ was depleted and then active CO$_2$ uptake inactivated by the addition of 500 uM Ethoxyzolamide or (EZ). This would force the use of bicarbonate ions for photosynthetic needs and encourage the active uptake of bicarbonate by the *C. reinhardtii* transporters if present and functional. Photosynthetic oxygen evolution was measured as a function of C$_i$ concentration and values compared between the mutant negative controls (DelCS cells transformed with empty vector), mutant positive controls (DelCS cells transformed singularly with either SbtA or the PCC7002-BicA transporter), and the selected transformants carrying the *C. reinhardtii* sequences.
**Generation of RNAi constructs**

Two approaches were taken to generate knock-down mutants via RNAi. The first involved the introduction of a short-hairpin loop comprising a sense-spacer-antisense construct cloned at the 3’end of the AphVIII gene (impacting paromomycin resistance) in the plasmid vector pSL72 (Pollock *et al.*, 2004). For this approach, portions of the cDNA unique to the genes of interest, to be targeted for the RNAi knockdown are chosen and cloned in both sense and antisense forms at the two end of the spacer in pSL72 (cyt6 intronic region). In case of dual constructs (for instance co-targeting of both NAR1.2 and LCI1 mRNA), the sense and antisense portions of the two genes were joined together by overlap PCR. Paromomycin resistant transformants were screened for knockdown of protein expression using Western blot analysis since good antibodies were available for both NAR1.2 and LCI1. In case of the artificial microRNA construct for the knockdown of the LCI1 protein, the protocol of Molnar *et al.* (2009) was followed closely, from the choice of the amiRNA target to the cloning of the construct into the pChlamyRNA3int plasmid obtained from the Chlamydomonas resource center.
CHAPTER 3
INVESTIGATION OF THE POSSIBLE ROLE OF THE NAR1 PROTEIN FAMILY IN THE CHLAMYDOMONAS REINHARDTII CCM

Introduction

Algal cells in an aquatic environment face a constant challenge of overcoming fluctuations in the availability of carbon dioxide in their vicinity (Raven et al., 2010; Spalding et al., 2011). Photosynthesis is a strong sink for dissolved carbon dioxide and readily depletes the amount of carbon dioxide surrounding an actively photosynthesizing cell (Talling et al., 1976). The diffusion of carbon dioxide in water is 10,000 times slower than in air making the replenishment around cells slower than the photosynthetic needs of the cell (Prins and Elzenga, 1989). Of the dissolved inorganic carbon (DIC) species available to the cell, the predominant forms are the neutral CO$_2$ molecules and the charged HCO$_3^-$ ion depending on the external pH levels (Giordano et al., 2005). At external pH levels above 6.5, most of the dissolved inorganic carbon is in the form of charged bicarbonate ions. Thus the ability to take up and transform bicarbonate to the Rubisco substrate CO$_2$ helps C. reinhardtii cells with a functional CCM to utilize both forms of inorganic carbon (C$_i$), carbon dioxide or bicarbonate, depending upon their availability (Marcus et al., 1984; Moroney et al., 1987; Palmqvist et al., 1994, 1998; Sultemeyer et al., 1988, 1989; Goyal and Tolbert, 1989; Badger et al., 1994; Amoroso et al., 1998; van Hunnik et al., 2002). The HCO$_3^-$ ion is rapidly converted back into the usable substrate CO$_2$ in the vicinity of Rubisco with the help of the carbonic anhydrase enzyme molecules (Raven, 2010; Moroney et al., 2011). C. reinhardtii cells have an active CCM at air levels of CO$_2$. The current operational model of its CCM indicates a passive diffusive entry of CO$_2$ and an active uptake of HCO$_3^-$, depending on their availability in the external medium. There is a rapid photosynthetic consumption of CO$_2$ by Rubisco in the chloroplast. This small neutral molecule can passively
diffuse through most of the membrane barriers in its path. Although the roles of CO$_2$ channels have been proposed in the facilitation of this movement, concrete evidence for their necessity in the *C. reinhardtii* cells is yet to be provided (Spalding *et al*., 2011). From the outside of the cell to its ultimate destination in the thylakoids, the charged HCO$_3^-$ ion however, would have to cross three membrane barriers, namely the plasma membrane, the chloroplast envelope and the thylakoid membrane. The successful operation of the *C. reinhardtii* CCM thereby makes it imperative for transport proteins to exist at each of these membrane barriers to facilitate the uptake and transport of the charged HCO$_3^-$ anion (Moroney and Ynalvez, 2007; Raven, 2010; Spalding *et al*., 2011). The putative candidates that could play a possible role in C$_i$ transport would be expected to have some or all of the following features: 1.) Be either induced or activated with the onset of the CCM; 2.) Cause a substantial decrease in C$_i$ uptake or transport when absent; 3.) Have either a gated or other electrogenic modes of operation; 4.) Show some structural and/or functional similarity with other small anion transport proteins; 5.) Show localization to any one of the three membranes and 6.) Be transcriptionally or translationally regulated during the low CO$_2$ acclimation process. Through the years, putative candidates have been identified and chosen for further investigations on the basis of these criteria.

The first membrane-bound putative inorganic carbon transporter genes to be revealed from genome-wide transcriptional screens were *LCIA* (now known as *NAR1.2*), *CCP1/2*, *LCI1*, and *HLA3* (Burow *et al*., 1996; Chen *et al*., 1997; Im and Grossman, 2002; Im *et al*., 2003; Miura *et al*., 2004). These genes were found to be highly upregulated under low CO$_2$ conditions in *C. reinhardtii* cells. Interestingly, the expression of the above mentioned putative transporter genes were suppressed in the CCM mutant, *cia5* (Moroney *et al*., 1989; Xiang *et al*., 2001; Miura *et al*., 2004; Fang *et al*., 2012). As mentioned in chapter 1, the mutant *cia5* lacks a functional *CIA5*
gene encoding a transcription factor that acts as a master transcriptional regulator, for key CCM genes (Moroney and Ynalvez, 2007). Comparison of the NAR1.2 sequence to other known transporter proteins, shows a sequence similarity to the Formate/Nitrite Transporter (FNT) protein family (Galvan et al., 2002; Miura et al., 2004; Mariscal et al., 2006). This family of proteins is found mainly in prokaryotes with fewer members in fungi, yeast, eukaryotic algae and protozoa (Peakman et al., 1990; Rexach et al., 2000; Suppmann and Sawers., 1994). Even prokaryotes such as E. coli express no more than three homologs, namely FocA, FocB and NirC (Suppmann and Sawers, 1994). No higher plant or mammalian FNT protein is known as yet. In C. reinhardtii cells there are now 6 known members of this family with diverse predicted membrane locations. The NAR family in C. reinhardtii, gets its name from Nitrate-Assimilation-Related proteins, as the first member NAR1.1 was believed to play a role in nitrite transport across the chloroplast membrane (Rexach et al., 2000; Mariscal et al., 2004). The fact that this family is structurally similar to a small anion transporting family, is predicted to have diverse membrane locations and at least one member (NAR1.2), shows a strong response to carbon limitation, caused it to be of potential interest in the quest for possible C_i transporters in the C. reinhardtii CCM.

In order to investigate the roles of one or more of the NAR transport family in the C. reinhardtii CCM, different experimental approaches were undertaken. The first approach involved the study of the transcriptional responses of each of these genes to CCM inducing conditions, either in the presence or absence of nitrite, in wild-type cells and the mutant, cia5, to identify the candidates that showed a low CO_2 response irrespective of the nitrogen species, indicating a possible involvement more pronounced in the carbon assimilation than the nitrogen assimilation pathway. Also suppressed expression in the CCM mutant, cia5, would indicate a
regulation mechanism in line with other CCM genes. The candidates that proved to be interesting from the first couple of experiments were then to be chosen for localization and knockdown studies to further analyze their roles in the C. reinhardtii CCM. Also, an attempt was made to try to rescue the ‘mutant phenotype’ of a cyanobacterial double mutant lacking two key bicarbonate transporters. This chapter deals with these experimental set-ups and the key results obtained from some of these experiments.

Results

The NAR gene family in C. reinhardtii:

The NAR gene family consists of six members showing sequence homology to the FNT family of transporters (Galvan et al., 2002; Rexach et al., 2000; Mariscal et al., 2006). Most of the C. reinhardtii NAR proteins have eukaryotic homologs amongst other algae, most commonly in another green alga, Volvox carterii (Figure 3.1). However, there is no significant homology of these proteins amongst higher plants, instead showing a more prominent evolutionary connection with protists. Recently, the crystallographic structure of a bacterial (Vibrio cholerae) FNT protein, FocA was reported (Waith et al., 2009). NAR1.2 shows only a 37% similarity to the V. cholerae FocA formate channel and around 35% similarity to the E. coli and S. typhimurium FocA proteins respectively as revealed from BLAST alignments of their primary sequences (Figure 3.2). When compared with the well-characterized Vibrio cholerae FocA protein, NAR1.2, which was previously shown to be a weak bicarbonate transporter (Mariscal et al., 2006), showed no conservation of the amino acids critical for formate binding (Figure 3.2).

The predicted membrane locations of some of the NAR proteins, indicates that at least 4 of them could be localized to the chloroplast membrane (see Table 3.1). The absence of clear chloroplast or mitochondrial signal sequences in the rest of the NAR proteins suggests a possible
location in the plasma membrane by default (Table 3.1). The presence of long C-terminal extensions can be seen for NAR 1.3 and NAR1.6 sequences (Figure 3.3).

Figure 3.1 A phylogenetic tree of some well-conserved FNT proteins. The C. reinhardtii NAR proteins, NAR1.2, NAR1.3, NAR1.4, NAR1.5 and NAR1.6 were compared with FNT proteins from other organisms. Included in this phylogram (using the neighbor joining method) are the well-characterized FocA (formate channel) proteins from prokaryotes like Staphylococcus typhimurium (St_FocA), Escherichia coli (Ec_FocA) and Vibrio cholerae (Vc_FocA) as well as the E. coli nitrite transporter, NirC (Ec_NirC). Amongst the eukaryotic FNT proteins most members belong to eukaryotic algae such as Volvox carterii (Vc_66209/105673/75029), Ectocarpus siliculosus (Es), Thalassiosira pseudonana (Tp), Thalassiosira oceanica (To) and Chlorella variabilis (Cv).
Figure 3.2 Alignment of the NAR1.2 protein sequence with some well-characterized prokaryotic formate channels (FocA proteins). FocA proteins such as the *Salmonella typhimurium* FocA (FocA-St), the *Escherichia coli* FocA (FocA-Ec) and the *Vibrio cholerae* FocA (FocA-Vc) proteins were used for comparison. The conserved amino acids are highlighted. In red boxes are the amino acid residues shown to be critical for formate binding in the *Vibrio cholerae* FocA. Underlined in red are the putative transmembrane domains of the *Vibrio cholerae* FocA protein and underlined in red are the transmembrane domains from NAR1.2 in *C. reinhardtii*.

**Responses to carbon and nitrogen:**

The response of the NAR gene family to different sources of nitrogen namely nitrate and nitrite was studied. Since these genes encode proteins showing sequence similarity to the Formate/Nitrite family of transporters it would be interesting to see if their genes showed the characteristic behavior of other nitrogen metabolism pathway genes in *C. reinhardtii*. 
Table 3.1. A few characteristics of the *C. reinhardtii* NAR gene family. The possible membrane locations of the proteins have been predicted using prediction programmes like pSORT and ChloroP, based on putative signal sequences. In case of NAR1.3 and NAR1.4, a chloroplastic or mitochondrial signal sequence was not detected by either prediction programmes and hence, a default plasma membrane location was chosen for these proteins. Only in case of NAR1.1 and NAR1.2, there is experimental evidence supporting the prediction data. Abbreviations used; CHL: Chloroplast membrane, PM: plasma membrane, Y: yes, N: No

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal Location</th>
<th>Amino acid Residues</th>
<th>Membrane Location</th>
<th>Response to nitrate</th>
<th>Response to low CO₂</th>
<th>Regulation by CIA5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAR1.1</td>
<td>9</td>
<td>358</td>
<td>CHL*</td>
<td>Y</td>
<td>N</td>
<td>No/NIT2</td>
</tr>
<tr>
<td>NAR1.2</td>
<td>6</td>
<td>337</td>
<td>CHL*</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>NAR1.3</td>
<td>4</td>
<td>553</td>
<td>PM</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>NAR1.4</td>
<td>7</td>
<td>407</td>
<td>PM</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>NAR1.5</td>
<td>12</td>
<td>341</td>
<td>CHL</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>NAR1.6</td>
<td>1</td>
<td>406</td>
<td>PM</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

Induction in the presence of nitrate, a condition under which there would be a need for nitrite to be transported across the chloroplast membrane combined with a repression under ammonium was expected to be one of the features of a gene involved in nitrite transport. On the other hand, any possible involvement in the C₅ transport process of the CCM might show a marked increase in gene induction under low CO₂ and/or control by the CCM master regulator, CIA5. The expression levels of all the NAR genes were first studied in the cell wall-less wild-type strain D66 (*cw15, nit2*), which is unable to utilize nitrate as a source of nitrogen because it lacks the regulatory gene *NIT2*, needed for the intracellular activation of the nitrate reductase gene, *NIA1* (Camargo *et al.*, 2007). In a time course experiment of low CO₂ acclimation, looking
Figure 3.3 Alignment of the NAR protein sequences in *C. reinhardtii*. This alignment is showing the conserved amino acid residues (highlighted) amongst the proteins. The presence of a large C-terminal end is unique to NAR1.3 and NAR1.6. Highly conserved residues are highlighted in blue.

at the expression of *NAR1.2* over a period of 2 days with continuous lighting, the gene expression shows an early upregulation with (within the first few hours of a switch from high to low CO$_2$) the onset of the CCM, peaking by the second hour of exposure to low CO$_2$ conditions (Figure 3.4). In contrast, *NAR1.4* shows a medium level of expression constitutive under both high and low CO$_2$ conditions during the same time course experiment (Figure 3.5). The time course experiment used the wild-type strain D66 and hence could only be grown in the presence
Figure 3.4 A time course experiment showing NAR1.2 expression in the wild type strain D66. This graph is showing the increase in abundance of the NAR1.2 transcript in low CO₂ with respect to high CO₂ levels. This was measured by qRT-PCR at different time points, after the switch from high to low CO₂. (L=Low CO₂, L₁ …L₄₈ = hours after switch from high to low CO₂). The transcript levels of low CO₂ grown cells are relative to the transcript levels of high CO₂ cells. It is to be noted that the gene is repressed under high CO₂ conditions in D66 (base level 1 on the X-axis) growing in the presence of ammonium as the nitrogen source. All values were normalized to the internal control gene CBLP, the expression of which remains unchanged between high and low CO₂ cells. Transcript values have been represented on a logarithmic scale.

of ammonium. Since, RT-PCR data showed that the expression of NAR1.1 and NAR1.6 was repressed in D66 (Figure 3.6), a different wild-type strain was chosen for further expression studies. Since the proteins being studied belong to the FNT class of transporters, it was decided to verify their responses to CCM conditions in the presence of nitrate. This required a C. reinhardtii strain where nitrate could successfully be taken up and converted to nitrite in the cytoplasm of the cell to be eventually taken up by the chloroplast. The cell walled-wild type strain CC-407, having all the genes necessary for nitrate metabolism, was therefore used for gene expression studies under both nitrogen and ammonium as a source of nitrogen in the medium.
Figure 3.5 A time course experiment showing NAR1.4 expression in the wild type strain D66. This graph is showing the constitutive expression of the \textit{NAR1.4} transcript, measured by qRT-PCR at different time points, after the switch from high to low CO\textsubscript{2}. (L = Low CO\textsubscript{2}, L\textsubscript{1} … L\textsubscript{48} = hours after switch from high to low CO\textsubscript{2}). The transcript levels of low CO\textsubscript{2} grown cells are relative to the transcript levels of high CO\textsubscript{2} cells (value shown as 1 on the Y-axis). All values were normalized to the internal control gene \textit{CBLP}, the expression of which remains unchanged between high and low CO\textsubscript{2} cells.

Expression of the NAR genes was also studied in the CCM mutant strain, \textit{cia5}. Quantitative RT-PCR (qRT-PCR) was used to study the expression of each NAR gene in CC-407 and \textit{cia5} in the presence of either ammonium or nitrate as the source of nitrogen under both high and low CO\textsubscript{2} growth conditions. The most interesting result from this experiment was the high expression of \textit{NAR1.2} in the cells under high CO\textsubscript{2} when nitrate was provided as the nitrogen source. In contrast, expression of NAR1.2 was completely repressed under high CO\textsubscript{2} with ammonium as the nitrogen source. When cells were switched to low CO\textsubscript{2}, expression was upregulated in both cases, irrespective of the nitrogen source.
Figure 3.6 RT-PCR data showing the suppression of NAR1.1 and NAR1.6 expression in the wild-type strain D66. Both genomic DNA and cDNA from low CO₂ acclimated cells of D66 (cw15, nit2) were used as template for PCR. The genomic DNA controls show the size of the expected fragment for each gene.

However, the extent of upregulation under low CO₂ was more pronounced in the ammonium grown cells and showed only a small increase in the nitrate grown cells (Figure 3.7). The expression of NAR1.2 was completely undetectable with both semiquantitative (data not shown) and quantitative RT-PCR, in the CCM mutant, cia5 under both high and low CO₂ conditions. It should be noted here that cia5 was generated in a (nit1 nit2) background and hence can only grow in the presence of ammonium. Hence, an absence of expression in cia5, for a gene normally repressed by ammonium cannot be considered as significant. However, in the case of NAR1.2, a gene normally induced under low CO₂ and in the presence of ammonium, an absence in cia5 points to a possible CIA5-mediated regulation of expression.
Figure 3.7 NAR1.2 expression levels in response to different nitrogen sources and different CO₂ levels. Wild-type CC-407 cells were switched from high to low CO₂ for 12 hours, in the wild type strain CC-407, and the CCM mutant, cia5 and transcript levels measured by qRT-PCR. The NAR1.2 expression seems to be upregulated under low CO₂ in the presence of ammonium to a greater degree than when nitrate is present as the nitrogen source in the medium. This is because the expression of NAR1.2 was repressed under high CO₂ with ammonium (base level 1 on the X-axis), whereas, it was strongly upregulated under high CO₂ with nitrate as the nitrogen source. The expression of NAR1.2 is suppressed in the mutant, cia5. The CCM mutant, cia5, having been generated in a (nit2) strain is able to grow only when ammonium is present as a source of nitrogen. All values are represented on a logarithmic scale after normalization with an endogenous control gene, CBLP.

The responses of the other members of the NAR gene family are very different. For instance, NAR1.1 and NAR1.6 are completely repressed under ammonium irrespective of carbon levels. In the presence of nitrate, they show low to moderate levels of expression under both high and low CO₂ (Figure 3.8). However, the expression of NAR1.1 is slightly down regulated under low CO₂ while NAR1.6 shows a greater level of down regulation (Figure 3.8). These genes are
Figure 3.8 *NAR1.1* and *NAR1.6* expression levels in response to different nitrogen sources and different CO$_2$ levels. The expression of the *NAR1.1* and *NAR1.6* genes were measured (via qRT-PCR), in the wild type strain CC-407, switched from high to low CO$_2$ for 12 hours. The expression of these genes seems to be suppressed under low CO$_2$ in the presence of ammonium. In the presence of nitrate as the nitrogen source in the medium, the expressions of both genes are down regulated under low CO$_2$ when compared to high CO$_2$. All values are represented relative to the endogenous control gene, *CBLP*. The formula used to calculate the relative transcript levels is $1000/2^{\Delta CT}$, where $\Delta CT$= difference between the $C_t$ values of either *NAR1.1* or *NAR1.6* and *CBLP*. The *CBLP* transcript value of 1000 is not shown in the plot area.

Repressed in *cia5*, and could be a result of ammonium repression as this mutant can only grow in the presence of ammonium as the nitrogen source. The genes *NAR1.3* and *NAR1.5* showed low to very low levels of expression under both high and low CO$_2$ conditions irrespective of the nitrogen source, in both the wild-type CC-407 and the mutant, *cia5* (Figure 3.9). *NAR1.4* is constitutively expressed at moderate levels under both high and low CO$_2$ conditions irrespective of the nitrogen source (Figure 3.10). It is also constitutively expressed under both high and low CO$_2$ conditions in the mutant, *cia5* under ammonium (Figure 3.10).
Figure 3.9 *NAR1.3* and *NAR1.5* expression levels in response to different nitrogen sources and different CO₂ levels. The expression of the *NAR1.3* and *NAR1.5* transcript levels (measured by qRT-PCR), in the wild type strain CC-407 and the CCM mutant strain, *cia5*, switched from high to low CO₂ for 12 hours. The expression of these genes seems to be constitutive under low CO₂ in the presence of either ammonium or nitrate in the medium. *Nar1.3* shows a low level of expression and *NAR1.5*, an even lower level of expression under all growth conditions. A down regulation of both genes is seen in the *cia5* mutant, grow with only ammonium as a source of nitrogen in the growth medium. All values are represented relative to the endogenous control gene, *CBLP*. The formula used to calculate the relative transcript levels is 1000/2^{ΔCT}, where \( ΔCT = \) difference between the Ct values of either NAR1.3 or NAR1.5 and CBLP. The CBLP transcript abundance value of 1000 is not shown in the plot area.

Localization of NAR genes:

The NAR1.2 protein was localized to the chloroplast membrane within the *C. reinhardtii* cell (Figure 3.11). The intact chloroplasts from low CO₂ grown cells were used to extract chloroplast membranes and probed with a peptide antibody raised to a C-terminal epitope. The polyclonal antibody showed cross-reaction with other *C. reinhardtii* proteins and was cleaned up
Figure 3.10 *NAR1.4* expression levels in response to different nitrogen sources and different CO₂ levels. The expression of the *NAR1.4* transcript levels (measured by qRT-PCR), in the wild type strain CC-407 and the CCM mutant strain, *cia5*, switched from high to low CO₂ for 12 hours. The expression of this gene seems to be constitutive under all conditions of growth in both the wild-type strain CC-407 and the mutant strain, *cia5*. All values are represented relative to the endogenous control gene, *CBLP*. The formula used to calculate the relative transcript levels is $1000/2^{\Delta CT}$, where $\Delta CT$ = difference between the Ct values of either NAR1.3 or NAR1.5 and CBLP. The CBLP transcript abundance value of 1000 is not shown in the plot area.

to some extent using different approaches like blotting out with *cia5* total protein, etc. Although, this semi-purified antibody solution was used in Western blots, an attempt was made to epitope-tag this protein for improved cellular localization data. Also, the epitope-tag approach was intended for the cellular localization of other NAR proteins for which specific antibodies were not available. The two tags used were GFP and 3XHA. The effectiveness of the HA tag was tested by over-expressing the HA tagged NAR1.2 in *E. coli* as a fusion protein with the *E. coli* Maltose Binding Protein (MBP). The over-expression of eukaryotic membrane proteins is not
Figure 3.11 Western blot analyses showing the enrichment of NAR1.2 on the chloroplast envelope fraction of whole cells. The total protein from synchronous cultures of the *C. reinhardtii* cell wall-less strain CC-400, acclimated to low CO$_2$ was probed with a NAR1.2 specific antibody (lane: Lw). This when compared to the envelope fraction isolated from cell fractionation experiments shows a clear enrichment of the protein (lane: Lce). It should be mentioned here that this antibody shows a weak binding to NAR1.2. Another chloroplast envelope localized protein CCP1 was used as a control for this experiment. The enrichment of CCP1 is not evident in lane Lce because of the denaturation of most of the CCP1 protein as seen as a smaller band (circled in red). Both proteins are absent from the *cia5* mutant.

often successful in *E. coli* over-expression systems. A small amount of expression was achieved of the MBP-NAR1.2-3XHA protein by increasing chances of solubility by growing cells at a lower temperature and overnight induction of expression (Figure 3.12). The protein expressed in *E.coli* could be detected both by anti-HA and anti-NAR1.2 antibodies (Figure 3.13). However, the expression of all tagged NAR proteins including NAR1.2, did not reach detectable levels in the *C. reinhardtii* cell (data not shown). There has to be a fresh exploration of different plasmid constructs.
Figure 3.12 SDS PAGE gel showing total protein from *E. coli* cells containing the MBP-NAR1.2-HA protein induced under different conditions. The expected size of the HA tagged NAR1.2 fused with the bacterial MBP is 77 kDa. The lanes do not represent equal protein loads. Lane 1, uninduced cells (37°C); Lane 2, induced cells (2 h, 37°C, 0.3 mM IPTG); Lane 3, induced cells (overnight, 37°C); Lane 4, uninduced cells (25°C, 1 mM IPTG); Lane 5, (overnight, 25°C, 1 mM IPTG). The red box and black arrow show the position of the overexpressed protein.
Figure 3.13 Western blot showing the presence of the HA tagged NAR1.2-MBP fusion protein in IPTG induced *E. coli* cells. The lanes are labeled 1 through 14 and do not represent equal protein loads. Lane 1 and 8, uninduced cells (37°C); Lanes 2 and 9, induced cells (2 h, 37°C, 0.3 mM IPTG); Lanes 3 and 10, induced cells (overnight, 37°C); Lanes 4 and 11, uninduced cells (25°C, 1 mM IPTG); Lanes 5 and 12, induced cells (overnight, 25°C, 1 mM IPTG, 10µL of total protein); Lanes 6 and 13, induced cells (overnight, 25°C, 1 mM IPTG, 5µL of total protein); Lanes 7 and 14, induced cells (overnight, 25°C, 1 mM IPTG, 2.5 µL of total protein). The red boxes highlight the induced protein (at 25°C) detected by the NAR1.2 antibody on the left and HA antibody on the right.

**Complementation of a cyanobacterial mutant lacking bicarbonate transporters:**

There are three main bicarbonate transporters discovered so far in the cyanobacterial laboratory strain *Synechococcus* PCC7942. This includes the high affinity bicarbonate transporter BCT1, encoded by the cmpABCD operon belonging to the traffic ATPase family (Omata *et al*., 1999) together with a second inducible, high affinity, putative Na⁺/HCO₃⁻ symporter called SbtA (Shibata *et al*., 2002). The third bicarbonate transporter, BicA, is a low affinity, high flux, Na⁺-dependent HCO₃⁻ transporter belonging to the well known SulP family (Price *et al*., 2004). A double deletion mutant (named DelCS) lacking the two inducible bicarbonate transporters SbtA and BCT1 was generated in the laboratory of Dr. Dean Price. The DelCS mutant was created in the wild-type background of the freshwater cyanobacterial strain, *Synechococcus* PCC7942. As mentioned earlier, SbtA is a high affinity bicarbonate transporter encoded by the cmpABCD operon and belongs to the traffic ATPase family (Omata *et al*., 1999). In the DelCS mutant, the A and B subunits of this BCT1 ABC transporter is deleted leading to the instability and destruction of the protein complex. The other protein disrupted in this double
mutant is SbtA, which is a high affinity, sodium dependent bicarbonate transporter (Shibata et al., 2002) and is believed to be functional as a sodium/bicarbonate symporter. The DelCS mutant was used for an attempted rescue of the mutant phenotype (impaired bicarbonate uptake) using putative bicarbonate transporter sequences from C. reinhardtii. The NAR1.2 ORF sequence was cloned with and without a predicted leader sequence into a GATEWAY (Invitrogen) entry vector (pENTR-D-TOPO). The entry vectors with the cloned C. reinhardtii sequences were sent to Dr. Price’s laboratory. The C. reinhardtii ORF sequences were sub-cloned into the appropriate cyanobacterial destination vectors and transformed into the DelCS double mutant. The subsequent HCO₃⁻ uptake dependent O₂ evolution measurements were also made in Dr. Price’s laboratory. At pH 8.2, where bicarbonate is the predominant Cᵢ species available to the cells, the cells carrying the C. reinhardtii transporter sequence, NAR1.2 (with/without leader sequence) failed to show any rescue of phenotype in the mutant DelCS (Figure 3.14a and 3.14b). The photosynthetic oxygen evolution was similar to the negative control indicating only basal levels of Cᵢ uptake. The passive diffusion of a small amount of CO₂ from the medium was largely believed to be responsible for the basal levels of Cᵢ uptake. In Figure 3.14-b, the traces for the photosynthetic oxygen evolution assay indicate that BicA (both versions, shown by the pink and green traces respectively) from the PC7002 strain, is able to revive the bicarbonate uptake in the mutant cells. SbtA allows for only weak uptake at low Cᵢ concentrations (blue trace). The values here were obtained by subtracting the basal level from each individual trace. The NAR1.2 carrying mutant transformants lacked any discernible increase in Cᵢ uptake above the basal levels of the negative control. The transformants carrying the putative transporter sequences from C. reinhardtii were later probed with specific antibodies to try and detect the presence of protein. Unfortunately, none of the transporters including NAR1.2 could be detected on a Western blot
Figure 3.14a A C\textsubscript{i} uptake screening assay in a mass spectrometer involving transporters expressed in the double deletion mutant DelCS. The transporter sequences were carried on the pSE2 or pSE4 shuttle vector and expressed in the PCC7942 double deletion mutant DelCS (DcmpAB; DsbtAB) which has both inducible HCO\textsubscript{3}\textsuperscript{-} transporters inactivated. BicA-1 and BicA-2 are two BicA transformants carrying the protein in different expression vectors. Both of these transformants show an increase in C\textsubscript{i} uptake from the basal level shown by the blue line. The SbtA/B complement is the black line (not much uptake, but some apparent at low C\textsubscript{i}); the empty vector control, is the blue line (passive CO\textsubscript{2} diffusion) and shows the same result as the NAR1.2 transformant.
Figure 3.14b A simple subtraction of the passive control (blue line) from the other traces as shown in the previous figure. Here the uptake kinetics are more obvious.

indicating the possible failure of transcription or translation of the ORF into a functional protein in order for the complementation to work.

**RNAi knockdown of NAR1.2:**

Since, NAR1.2 proved to be the most interesting gene in the study of the NAR gene family, an attempt was made to knock down its expression using artificial constructs to generate
hairpin loops. The reason for using hairpin loops was to use a relatively large portion of the NAR1.2 transcript for the RNAi mediated generation of a large population of target small interfering RNAs or siRNAs. This would increase the possibility of target mRNA destruction and generation of a mutant strain showing a significant amount of NAR1.2 knockdown.

A dual construct targeting both NAR1.2 and another putative transporter, LCI1 was also constructed. At the point that these experiments were carried out, the location and function of either of these proteins were still being worked out. Thus a dual construct aimed at overcoming any redundancy of function that might prevent a discernible mutant phenotype from being detected due to a single knock-down of these transporter proteins. The RNAi knockdown mutant generation met with very little success with the single NAR1.2 construct. A few transformants carrying the dual construct showed reduction in the LCI1 protein, but was very quickly silenced (within a few days) making it impossible to make physiological and biochemical measurements on time (Figure 3.15).

**Discussion**

*Chlamydomonas reinhardtii* is unique in having six members of the FNT family of transporters. Even in prokaryotes, where this family is more commonly present, no species are known having more than three members of this protein class at a time. Higher plants have no known members of the FNT class of transporters. The existence of more than one FNT protein in *C. reinhardtii* might reveal evolutionarily unique roles in small anion transport. Currently, there is one structurally well-characterized prokaryotic FNT protein, the FOCA protein in *Vibrio cholerae* (Waiget *et al.*, 2010). This protein is believed to function as a pentameric channel that can fit either formate or nitrite in its central cavity forming the constricted neck of its overall double-inverted funnel
structure. Another FocA protein, from *Salmonella typhimurium*, has been recently shown to be involved in the bacterial mixed acid fermentation pathway thereby playing a role in the transport of formate in and out of the bacterial cell (Lu *et al.*, 2012). Through electrophysiological

**Figure 3.15** RNAi knock-down attempts for *NAR1.2*. Plasmid maps for the hairpin constructs for RNAi mediated knockdown of *NAR1.2* (A.) and *NAR1.2+LCI1* (B.). The results of the dual knockdown (*NAR1.2+LCI1*) as measured by the levels of LCI1 protein in the transformants 152, 237 and 315, when compared to the wild-type strain D66 and the CCM mutant, *cia5* (C.) The α-Tubulin was used as a loading control. The wild-type levels of the LCI1 protein were restored in the transformants within a few days (3rd panel) from the slight reductions seen (especially in 152), right after transformations (2nd panel)
studies, the *Salmonella typhimurium* FocA has been shown to be able to transport not only formate and nitrite but also slightly larger anions such as acetate and lactate although with much lower affinity (Lu *et al.*, 2012). This FocA protein shows a mode of operation based on the external pH and the need for the cell to either move formate or other anions in or out of the cell. Under high pH conditions, it allows for the passive passage of formate out of the cell and acts as a water-filled passage much like an aquaporin. The selectivity for the anion comes from a largely charged interior with positive amino acids lining the lipid-spanning interior of the channel. However, under low pH conditions, when charged anions need to be taken up by the cell, the channel carries out an active uptake and acts as a voltage gated symporter. The channel then takes up both protons and formate anions via an active, electrogenic process. These findings might give us some insight into the probable mode of operation and anion specificity determination of the NAR FNT proteins in the *C. reinhardtii* cell even though the physiological difference between the two systems is vast. For instance, *NAR1.2* shows high upregulation under nitrate and high CO₂. Under these conditions, the protein might be needed to transport nitrite into the chloroplast. On the other hand, under low CO₂ and ammonium conditions, there is a greater need for the chloroplast to take up bicarbonate. This need might be met by NAR1.2 in conjunction with other chloroplast membrane transporters such as CCP1/CCP2. NAR1.2 was shown to be a high affinity nitrite and low affinity bicarbonate transporter through electrophysiological studies in *Xenopus* oocytes (Mariscal *et al.*, 2006). The NAR proteins show some amount of sequence homology with the *E. coli* and *S. typhimurium* FocA proteins, with NAR1.2 showing roughly 50% sequence homology with both proteins. Most of the critical amino acids believed to be essential for the functioning of either protein as an anion channel are, however, not conserved in all members of the NAR family in *C. reinhardtii*. 

80
So far, NAR1.2 is the most important candidate in terms of its possible role as a putative bicarbonate transporter across the *C. reinhardtii* chloroplast. We have corroborated earlier studies showing the very early and high induction of this gene in the *C. reinhardtii* CCM. The absence of the protein in the CCM mutant, *cia5*, has also been confirmed. Interestingly, we have found that the expression of *NAR1.2* is repressed under high CO$_2$ conditions, when ammonium is present as a nitrogen source in the medium. However, in the presence of ammonium as the sole nitrogen source in the medium, *NAR1.2* is highly induced under low CO$_2$ conditions. When nitrate is present as a nitrogen source in the medium, the expression of *NAR1.2* is induced even under high CO$_2$. This might imply a probable role in nitrite transport since the CCM is not operational under high CO$_2$. Under high CO$_2$, when the predominant form of C$_1$ used by the cell is CO$_2$, the increased transcriptional induction of *NAR1.2*, could indicate a possible role in the nitrogen assimilation pathway. As nitrate in the medium is converted to nitrite in the cytoplasm, this nitrite would need to cross over the chloroplast membrane barrier for its subsequent denitrification in the chloroplast stroma. Since, NAR1.2 belongs to the FNT family this could be a possible indicator of its nitrite transport function. But if a nitrite transporter is essential for the transport of nitrite across the chloroplast membrane the question arises as to why no such protein members have been unearthed in higher plant cells. Recently, CsNitr1-L, a member of the proton-dependent oligopeptide transporter (POT) family was found to play a role in transporting nitrite into chloroplasts of de-etiolated leaves of higher plants (Sugiura et al., 2007). However, no known member of the FNT family has yet been discovered in higher plants.

The NAR1.2 protein is also missing from the CCM mutant, *cia5*, under low CO$_2$ conditions, when grown in the presence of ammonium. This probably shows either a direct or indirect effect of CIA5 in regulating the transcription of NAR1.2. Two important studies have
led to some clues as to the possible role of NAR1.2 in the *C. reinhardtii* cell. In electrophysiological experiments with *Xenopus* oocytes, NAR1.2 was found to be a low affinity bicarbonate and high affinity nitrite transporter (Mariscal et al., 2006). Also, when the expression of this gene was knocked down together with another plasma membrane bound transporter, HLA3, cells showed a sick growth under high pH conditions indicating a possible reduction in the ability to utilize bicarbonate from the medium (Duanmu et al., 2009). NAR1.2 is localized in the chloroplast membrane as evident from membrane fractionation experiments (Figure 3.11). Thus, it could be involved in the transport of nitrite and the possible transport of bicarbonate as the need arises. Bicarbonate is a slightly larger anion compared to formate. This could explain the low affinity for this ion by NAR1.2 in the electrophysiological experiments. Also, if NAR1.2 carries out a passive non-electrogenic transport of bicarbonate its activity would not be effectively detected in electrophysiology experiments. Whatever its mode of operation, the regulation of NAR1.2 expression seems to pose an equally intriguing problem. NAR1.2 is expressed in the wild-type strain, D66 that lacks both *NIT1* and *NIT2* genes. *NIT1* encodes the cytoplasmic nitrate reductase whereas *NIT2* is believed to play a regulatory role in the expression of several genes required in the nitrate assimilation pathway including *NIT1* (Rexach et al., 2000). This might imply that *NAR1.2* is not directly regulated by *NIT2*. However, under conditions where nitrite would be produced and need to be transported across the chloroplast membrane, for instance in the presence of nitrate in the medium, NAR1.2 is also upregulated. Also, the absence of NAR1.2 in the *cia5* mutant under low CO₂ would indicate a CIA5 regulated expression as seen with other key CCM proteins. However, *NAR1.2* is expressed under high CO₂ in the presence of nitrate when the rest of the CCM is not operational. CIA5, though present, is probably inactive under high CO₂. This reveals a complexity in transcriptional regulation that
would be worthwhile to explore further. In the *S. typhimurium* FocA, the abundance of the anion to be transported triggers the induction and the mode of operation of the protein. Could *NAR1.2* be regulated in a similar way? The presence of nitrite in more abundance than bicarbonate under high CO$_2$ conditions might cause the upregulation of the gene. Again under low CO$_2$ when the presence of ammonium abolishes the need for nitrite transport, the transport of the more abundant bicarbonate ion takes precedence. However, the exact regulation of *NAR1.2* is still unresolved at this point and we can only speculate about the regulatory mechanics. The cyano-complementation experiments failed to show any rescue of the inorganic carbon (C$_i$) uptake phenotype. Since no protein was detected in the complemented mutants the reason could be either the failure at a transcriptional level or translational level. Future experiments could try to successfully express the protein in cyanobacterial cells before any rescue-of-phenotype studies are attempted.

Several approaches at knocking down *NAR1.2* expression using the RNAi approach have failed to provide any substantial knockdown of protein. However, off-target knockdowns as seen with others (Duanmu et al., 2009) led us to believe that such attempts in the future might be successful. So efforts are still on in the laboratory of Dr. James Moroney, carried out by the author to either knock-down or try to over-express NAR1.2 in the *C. reinhardtii* cell to see the impact on C$_i$ uptake. These attempts involve use of different promoter and enhancers to try to get significant levels of expression of artificial constructs in *C. reinhardtii* cells. Since knocking out a protein sometimes has a cleaner effect on the phenotype than a partial knockdown, attempts are also being made to create insertional mutants with a disrupted *NAR1.2* gene causing an absence of functional protein. However, it has to be noted that such a deletion mutant would have to be benign to the cell for it to be successfully selected. NAR1.2 will remain a focus in all future *C.
*reinhardtii* studies focused on C\textsubscript{i} transport carried out by the author since many questions still need to be answered.

In our studies of the response of *NAR1.3* and *NAR1.5* we have noted no substantial differences in expression of these genes under high or low CO\textsubscript{2} irrespective of the nitrogen source available. The low expression of both of these genes has been corroborated in recent RNASeq studies with transcripts from low CO\textsubscript{2} cells. Both genes seem to be down regulated in the mutant *cia5*. Both *NAR1.3* and *NAR1.5* were initially of interest because of their predicted chloroplast membrane location. In an insertional mutagenesis screen recently conducted in our laboratory (Chapter 6), with roughly 20,000 transformants, an interesting discovery was the unearthing of three insertional mutants for the *NAR1.3* gene. Since most of the insertions happened in the 3’ end of the gene, it has proven to be a mutational hotspot for genomic insertions. Since, the transformations involved low CO\textsubscript{2} grown cells with an induction time of four hours, it can be speculated that the 3’ region of *NAR1.3* probably has a more open chromatin structure under low CO\textsubscript{2} acclimation to allow for insertions to occur. This could indicate a possible transcriptional response to either a direct or indirect response to a low CO\textsubscript{2} stress. This makes it an interesting candidate for future studies.

*Nar1.4* is found to be constitutively expressed under both high and low CO\textsubscript{2} conditions and nitrate or ammonium conditions. *NAR1.1* and *NAR1.6* show a more defined nitrogen response than to lower levels of CO\textsubscript{2} in the medium. The NAR family of transport proteins in *C. reinhardtii* needs a complete characterization with respect to their cellular location and function. Though not all of them might play a key role in the CCM almost all of them probably have interesting roles to play in the anion transport across membranes. The author is currently attempting localization of these transporters in the *C. reinhardtii* cell through an epitope-tagging
approach. The search is still on for a putative thylakoid membrane transporter that brings bicarbonate ions into the thylakoid lumen to be converted back into CO$_2$ by the key carbonic anhydrase, CAH3. CAH3 has been found to be indispensible to the operation of the CCM and provides further proof for the need of a thylakoid membrane-located anion transporter to facilitate bicarbonate movement across the thylakoid membrane into the lumen. Hence, the NAR family with its diverse membrane locations and the possible ability to transport small anions such as the bicarbonate ion still remains an important family to study with respect to the *C. reinhardtii* CCM.
CHAPTER 4
THE POTENTIAL ROLE OF LC11, AS A BICARBONATE TRANSPORTER, IN THE CCM OF CHLAMYDOMONAS REINHARDTII.

Introduction

The availability of CO₂ is often one of the major factors that limit the photosynthetic performance of a photosynthetic organism. Normally, the environmental supply of carbon dioxide is lower than that needed for the optimal carboxylase activity of the key carbon fixing enzyme, Rubisco. This leads to an increase in the competing oxygenase reaction by Rubisco and loss of fixed carbon through the process of photorespiration. Aquatic photosynthetic organisms face a larger challenge in acquiring carbon dioxide to support photosynthesis than terrestrial organisms (Raven, 2010). This is because aquatic photosynthetic organisms must acquire dissolved inorganic carbon (DIC) either as neutral CO₂ molecules or HCO₃⁻ ions from the medium. The diffusion of CO₂ in water is 10,000 times slower than through air (Helder and Zanstra, 1977). This slow diffusion, compounded by the fact that most of the DIC exists in the ionized forms as the pH of the water increases, makes the aquatic photosynthetic organism face CO₂ stress quite often in its diurnal cycle. Needless to say, the use of CO₂ as a sole DIC source, under normal photosynthetic conditions, would lead to very low internal levels of CO₂ when compared to the more abundant O₂. This produces low rates of photosynthetic carbon fixation and high rates of photorespiration. However, most aquatic organisms possess the unique ability to concentrate DIC internally to levels that are 10 to 10,000 times higher than external concentrations. This process, that helps aquatic photosynthetic organisms to acclimate to low levels of external DIC, by concentrating carbon internally is better known as the Carbon Concentrating Mechanism or CCM (Raven, 2010). The CCM is biophysically similar to the C4 process in terrestrial plants. It can raise levels of CO₂ around Rubisco under conditions of CO₂
stress, while the cells still maintain a C3 photosynthetic biochemistry (Raven, 2010). The ability to acquire all forms of inorganic carbon for photosynthesis is a signature of most unicellular algal CCMs such as the prokaryotic cyanobacteria and the eukaryotic green alga, C. reinhardtii.

Evidence of an induced, inorganic carbon ($C_i$) transport system in the model eukaryotic C. reinhardtii CCM has existed for a long time and forms an essential part of the updated CCM model of C. reinhardtii (Badger et al., 1980; Giordano et al., 2005; Ynalvez and Moroney, 2007; Spalding, 2008; Yamano and Fukuzawa, 2009). Although a lot of putative inorganic carbon transport candidates might exist in C. reinhardtii cells, only a few, such as the plasma membrane bound HLA3 (Duanmu et al., 2009b) and the chloroplast membrane bound NAR1.2 (Mariscal et al., 2006) and CCP1/CCP2 (Pollock et al., 2003) have been characterized to some degree. The study of several key CCM mutants such as cia5 (Moroney et al., 1989), c16 (Fukuzawa et al., 1998), pmp 1-1 (Spalding et al., 1983), and lcr1 (Yoshioka et al., 2004) have given us a lot of insight into the functioning of the CCM in C. reinhardtii. Of the transporter proteins missing in the key CCM mutant cia5, HLA3 and NAR1.2 have recently been implicated in $C_i$ transport. When knocked down together in an RNAi mutant, the absence of both HLA3 and NAR1.2 transcripts caused a sick growth phenotype under high pH conditions when $HCO_3^-$ is the more abundant source of inorganic carbon (Duanmu et al., 2009b). The same was seen when HLA3 was knocked down on its own (Duanmu et al., 2009b). This, together with the fact that NAR1.2 has previously been identified as a high affinity nitrite but a low affinity bicarbonate transporter in electrophysiological studies with Xenopus oocytes (Mariscal et al., 2006), suggests the possible involvement of both NAR1.2 and HLA3 in the bicarbonate transport process in C. reinhardtii. A pair of nearly identical proteins missing in the cia5 mutant that might play a role in $C_i$ transport into the chloroplast is the chloroplast membrane localized transporters,
CCP1/CCP2. Reduction in the expression of these proteins causes cells to grow slowly under low CO$_2$ conditions (Ramazanov et al., 1993; Pollock et al., 2003). Other proteins that are not regulated by CIA5 but are believed to be involved in the transport of inorganic carbon are the chloroplast membrane protein CemA/Ycf10 (Rolland et al., 1997) and the plasma membrane protein, RHP1 (Soupene et al., 2004; Yoshihara et al., 2008). More evidence is needed to fully understand the mechanism of operation of these transport proteins.

This chapter deals with the investigation into the role of LC11, another putative transport protein that is missing in the CCM mutant, cia5. Named LCII (Low CO$_2$ Inducible gene 1), this gene is induced under low CO$_2$ conditions (Burow et al., 1996; Miura et al., 2004) and encodes a protein that does not show a distinct similarity with any known group of proteins. During the later study of another mutant, lcr1, lacking the myb-transcription factor LCR1, the expression of a number of genes was suppressed (Yoshioka et al., 2004). Some of the key genes that were identified to be missing from the lcr1 mutant were the periplasmic CA, CAH1, the putative transporter gene LC11, and the gene encoding an uncharacterized soluble protein, LC16. The lcr1 mutant showed a 30% reduction in growth compared to the wild-type background and a lowered affinity for dissolved C$_i$ under low CO$_2$ conditions (Yoshioka et al., 2004). Since the absence of CAH1 does not lead to reduced growth under low CO$_2$ conditions or affect the normal functioning of the CCM (Van and Spalding, 1999), its absence in the lcr1 strain was believed to be a non-contributing factor to the mutant phenotype. LC16 has no known transmembrane domains and is poorly expressed. The lowered C$_i$ affinity and the resultant slower growth of the lcr1 mutant under low CO$_2$ conditions, was believed to be due to the absence of the putative transporter protein, LC11 (Ohnishi et al., 2010). Therefore, LC11 was hypothesized to play a role in the acquisition of C$_i$ in low CO$_2$ acclimated C. reinhardtii cells.
With the aim of testing this hypothesis, our collaborators in Japan working in the lab of Dr. Hideya Fukuzawa expressed the LCI1 protein in high CO\(_2\) conditions, under the control of a nitrate inducible promoter. The light-dependent CO\(_2\) exchange activity, C\(_i\) affinity and C\(_i\) uptake were measured in the high CO\(_2\) cells expressing LCI1 in the absence of other low CO\(_2\)-induced CCM components. The same studies were repeated with the low CO\(_2\) cells expressing this protein in the presence of nitrate. When LCI1 expression was induced under high CO\(_2\) there was a corresponding increase in C\(_i\) uptake and affinity in the cells (Ohnishi et al., 2010). This pointed to a possible role in C\(_i\) uptake for this protein that was demonstrated even in the absence of other CCM components. Also the LCI1 protein was localized to the plasma membrane within the C. reinhardtii cell. The strong LCI1 antibody used for the localization studies was generated in our laboratory and the localization aspect of the LCI1 project was established by the data generated by the author of this thesis. The ability to increase C\(_i\) affinity and uptake without the help of other CCM components and the plasma membrane location of the protein implicates LCI1 in the transport of C\(_i\) as part of the C. reinhardtii CCM.

**Results**

**LCI1 is a protein unique to the C. reinhardtii CCM:**

LCI1 is a small protein, roughly 22 kDa (predicted size with the signal sequence), with four predicted transmembrane helices (see Figure 4.1). It shows no conserved domains in its primary sequence or any significant sequence similarities with known proteins. This makes it difficult to predict its possible function in the C. reinhardtii cell. The only protein that LCI1 shows a weak similarity to is the high osmolarity signaling protein called SHO1, which is found
Figure 4.1 The positions of the possible transmembrane domains of LC11. This figure was generated using a using the Phobius web server (http://phobius.sbc.su.se/index.html). This site uses a hidden Markov model (HMM) for prediction of transmembrane domains and signal peptide sequences, named Phobius by the authors (Kall et al., 2007).
in eukaryotes as diverse as the unicellular *Saccharomyces cerevisiae* (yeast) to higher plants (see figure 4.2). In yeast SHO1 acts as a plasma membrane localized osmosensor that activates the high osmolarity glycerol or HOG signaling pathway. This activation is carried out via a mitogen-activated protein kinase (MAPK) cascade in response to high osmolarity (Tatebayashi *et al.*, 2006).

**The expression of *LCII* is induced under CCM inducing conditions:**

*LCII* is expressed under low CO\(_2\) conditions in *C. reinhardtii* cells, while its expression is suppressed under high CO\(_2\) conditions and in the CCM mutant, *cia5*. The gene shows its highest level of expression as early as the fourth hour after cells are switched from high to low CO\(_2\) conditions (Fig. 4.3). This is similar to the expression of most key genes in the *C. reinhardtii* CCM. From the time-course experiment measuring the *LCII* transcript, it is clear that the expression remains more or less steady for the first two days under continuous lighting. The expression of the gene is suppressed under high levels of CO\(_2\) as shown in Western blots using total protein from high CO\(_2\) grown wild-type cells (Figure 4.4, lane 400H). The protein is also absent from the CCM mutant, *cia5*, under low CO\(_2\) conditions (Figure 4.4, lane *cia5*L). CIA5 regulates the expression of many genes that are expressed early on in the induction of the CCM. Since CIA5 is also believed to control the expression of the myb-transcription factor LCR1, which directly regulates the expression of *LCII*, the absence of LCI1 in the *cia5* mutant might be due to the indirect effect of the absence of LCR1. The detection of the LCI1 protein in the chloroplast membrane fraction (Figure 4.4, lane 400L\(_{cm}\)) of CC-400 cells, initially indicated that it might be a chloroplast membrane protein. However, follow-up experiments failed to show a chloroplast membrane enrichment that would be characteristic of a chloroplast membrane localized protein. The expression of LCI1 under low CO\(_2\) conditions remains conserved across
Figure 4.2 ClustalW alignment of the LCI1 primary sequence. The alignment of LCI1 is shown with other proteins that show a weak similarity to it. The alignment has been cropped to the end of the LCI protein sequence. The 11 protein sequences used for the alignment with LCI1 are:

- **Tb_unkn**: uncharacterized protein from *Treponema brennaborense*
- **Cs_kinase**: kinase-like protein from *Coccomyxa subellipsioidea* C-169
- **Mo_SHO1**: high osmolarity signaling protein SHO1 from *Magnaporthe oryzae*
- **Hw_SHO1A**: high osmolarity signaling protein SHO1A from *Hortaea werneckii*
- **Hw_SHO1B**: high osmolarity signaling protein SHO1B from *Hortaea werneckii*
- **Gt_osmo**: osmosensor protein from *Gaeumannomyces graminis* var. *tritici*
- **Al_unkn**: uncharacterized protein from *Arabidopsis lyrata*
- **Yi_unkn**: high osmolarity signaling protein SHO1 from *Yarrowia lipolytica*
- **Kp_SHO1**: high osmolarity signaling protein SHO1 from *Komagataella pastoris*
- **Sc_SHO1**: SHO1 protein from *Saccharomyces cerevisiae*

The alignment of LCI1 is shown with other proteins that show a weak similarity to it. The alignment has been cropped to the end of the LCI protein sequence. The 11 protein sequences used for the alignment with LCI1 are:

- **Tb_unkn**: uncharacterized protein from *Treponema brennaborense*
- **Cs_kinase**: kinase-like protein from *Coccomyxa subellipsioidea* C-169
- **Mo_SHO1**: high osmolarity signaling protein SHO1 from *Magnaporthe oryzae*
- **Hw_SHO1A**: high osmolarity signaling protein SHO1A from *Hortaea werneckii*
- **Hw_SHO1B**: high osmolarity signaling protein SHO1B from *Hortaea werneckii*
- **Gt_osmo**: osmosensor protein from *Gaeumannomyces graminis* var. *tritici*
- **Al_unkn**: uncharacterized protein from *Arabidopsis lyrata*
- **Yi_unkn**: high osmolarity signaling protein SHO1 from *Yarrowia lipolytica*
- **Kp_SHO1**: high osmolarity signaling protein SHO1 from *Komagataella pastoris*
- **Sc_SHO1**: SHO1 protein from *Saccharomyces cerevisiae*
Figure 4.3 A time-course experiment showing the expression of *LCI1*. Quantitative RT-PCR data showing the abundance of *LCI1* transcript after D66 cells were switched from high to low CO$_2$ (abbreviated as L on the Y-axis) conditions at the different time points (L1 through L48). The transcript abundance of *LCI1* is shown as a fold difference between high CO$_2$ grown cells (the base line of 1) and low CO$_2$ grown cells, at the same time points. The gene CBLP was used as an internal control as its expression remains unchanged under both high and low CO$_2$.

Figure 4.4 Western blot analyses showing the presence of *LCI1* under low CO$_2$ conditions. The total protein fraction was extracted from whole cells of the wild-type cell wall-less strain CC-400 (400L) and the mutant *cia6* (*cia6L*), under low CO$_2$ conditions (abbreviated as L). *LCI1* is present in the chloroplast membrane (cm) fraction of CC-400 (400L$_{cm}$). The protein is missing in the high CO$_2$ grown cells of CC-400 (400H) and its corresponding chloroplast membrane fraction (400H$_{cm}$). Although, present in the *cia6* mutant, *LCI1* is repressed in the CCM mutant *cia5* under low CO$_2$ conditions (*cia5* L). The mutant *cia6* lacks an organized pyrenoid and a lag in CCM induction while the mutant *cia5* lacks a functional CCM. The unknown chloroplast membrane protein cross-reacting with the *LCI1* antibody (highlighted in black box) is used as a loading control.
wild-type strains, both cell walled and wall-less. Also several individual mutants that lack other key CCM proteins such as CAH3 (cah3), LCIB (pmp1), CAH1 (cah1) have LC11. This is shown in immunoblots using an anti-LC11 antibody (Figure 4.5).

**LC11 expression can be induced artificially in the lcr1 mutant under both high and low CO2 conditions:**

The hypothesis that LC11 might have a role in C_i transport needed to be tested in a scenario where the protein, is independently able to change the C_i uptake ability and C_i affinity of cells in which it was expressed. Two different ways to achieve this would be to express the protein either in a *C. reinhardtii* mutant that is missing LC11 or heterologously in another eukaryotic system, like yeast cells. Since no *LC11* mutant has yet been generated in *C. reinhardtii*, the use of regulatory mutants like *cia5* or *lcr1* that lacked the protein was considered. Expression and exploration of function is always preferred in native systems versus heterologous expression in another eukaryote (like yeast), which is physiologically very different from an algal cell. Also, the added problem of correct processing of a membrane protein to ensure its insertion into the yeast cell membrane might also have to be taken into consideration in the case of heterologous expression. The mutant *lcr1* was chosen for this experiment, because unlike *cia5* it controls the expression of only a subset of genes involved in the CCM, with the three prominent ones being *LC11*, *CAH1* and *LCI6*. Therefore, the artificial expression of LC11 in the *lcr1* mutant, under high CO2 would be in the absence of CCM components while the expression under low CO2 would be in the presence of most other CCM proteins except for the ones controlled by LCR1. This would help us study the function of the protein and the changes that it
Figure 4.5 Western blot analyses showing the expression of LCI1 under low CO\textsubscript{2} conditions in several common CCM mutants and wild-type strains. Mutants such as \textit{pmp1} (lacking the C\textsubscript{i} transport facilitator in the chloroplast, LCIB), \textit{cia3} (lacking the thylakoid lumen carbonic anhydrase CAH3), \textit{cia5} (lacking the key CCM-regulatory transcription factor, CIA5) and \textit{cah1} (lacking the periplasmic carbonic anhydrase, CAH1) were used. All of the mutants, with the exception of \textit{cia5}, show the presence of LCI1 under low CO\textsubscript{2}. The LCI1 RNAi knock-down mutant L\textsubscript{65} (generated in a CC-503 background), shows a reduction in the amount of the protein when compared to the wild-type CC-503. The wild-type cell wall-less strains D66 and CC-503 and the cell walled strain C9, all show the presence of LCI1, under low CO\textsubscript{2} conditions.

brings to the C\textsubscript{i} affinity and uptake of the cells when it is expressed under both low and high CO\textsubscript{2} condition, both with or without the presence of the other key CCM components respectively. To achieve this goal, the \textit{LCI1} gene sequence was placed under the control of the nitrate-inducible promoter (Figure 4.6A) belonging to the nitrate reductase gene, \textit{NIA1}, from \textit{C. reinhardtii}. This promoter was fused to the minimal promoter of \textit{TUB2} encoding β2-tubulin (Davies and Grossman, 1994). The \textit{NIA1} promoter is repressed by the presence of ammonium as the nitrogen source but induced within a short period by the presence of nitrate as the sole source of nitrogen in the medium (Loppes and Radoux, 2002). The artificial construct carrying the gene under the
control of the \textit{NIA1} promoter was introduced into the \textit{lcr1} mutant and the subsequent presence of the LCII protein was detected under high and low CO\textsubscript{2} conditions in the presence of NO\textsubscript{3}\textsuperscript{-} ions, in the two \textit{lcr1} transformants C2 and E4 (see Figure 4.6B). This shows that LCII expression could be artificially induced in the transformants under nitrate induction. However, the artificial expression in these transformants never reached the same level as in the wild-type low CO\textsubscript{2} grown cells (Figure 4.6B, highlighted in circles). Expression of many transgenic constructs in \textit{C. reinhardtii} face the problem of not reaching wild-type levels because the random insertion into the genome often has positional effects on their expression. Interestingly, a faint band for LCII was detected in the \textit{lcr1} low CO\textsubscript{2} cells and the high and low CO\textsubscript{2} cells of the transformants in the absence of nitrate induction (Figure 4.6B). This could be indicating either a constitutive basal level of the protein in the \textit{lcr1} mutant cells or be the result of minor contamination during the running of the protein gel. It is to be noted that this low level of expression was not seen with other strains in the lab, wild-type or mutant (Figure 4.5). However, it is clear from Figure 4.6B, that there is an nitrate induced-increase in LCII expression, under both high and low CO\textsubscript{2} cells, in the \textit{lcr1} transformants C2 and E4. Hence, any differences in C\textsubscript{i} uptake properties in these transformants could be credited to the induced expression of LCII. The generation of the C2 and E4 transformants, which were undertaken in the laboratory of Dr. Fukuzawa demonstrates the feasibility of this approach in investigating the roles of other putative C\textsubscript{i} transporters. One of the prominent outcomes of this artificial expression of LCII is summarized in the following paragraph and confirms the ability of LCII to independently increase the C\textsubscript{i} accumulation in \textit{C. reinhardtii} cells.
Artificial expression of LCII increases the accumulation of C\textsubscript{i} under high CO\textsubscript{2}:

The accumulation of [\textsuperscript{14}C]-labeled C\textsubscript{i} by LCII transformants C2 and E4 was measured by using a silicone oil layer centrifugation method (Fukuzawa et al., 1998). It revealed an increase in C\textsubscript{i} uptake by the LCII transformants that is almost double compared to the lcr\textsubscript{I} mutant background (Figure 4.7). This shows a direct involvement of LCII in the uptake of C\textsubscript{i} by

![Figure 4.6 Nitrate induced expression of LCII. A.) A schematic representation of the chimeric construct for the artificial expression of LCII. The coding region of LCII was placed downstream of the nitrate inducible NIA1 promoter, fused to the minimal TUB2 promoter. The bleomycin resistant (selective marker: BleR gene) colonies were selected to check for the nitrate inducible expression of LCII. B.) Western blot analysis showing the expression of LCII in the wild type Q304P3, mutant lcr\textsubscript{I}, and NIA1::LCR1 transformants C2 and E4 when probed with a LCII specific antibody. The growth conditions involve high CO\textsubscript{2} (HC) or low CO\textsubscript{2} (LC) with either ammonium (NH\textsubscript{4}\textsuperscript{+}) or nitrate (NO\textsubscript{3}\textsuperscript{-}) supplied as the source of nitrogen. The LCII protein is detected in the wild-type Q304P3 cells under low CO\textsubscript{2} and minimally detected in the lcr\textsubscript{I} mutant. The expression level of LCII, when artificially induced under nitrate conditions shows only a slight increase when compared to the lcr\textsubscript{I} mutant background and the low CO\textsubscript{2}/ammonium grown cells of C2 and E4. However, under all nitrate-induced conditions, the artificial expression of LCII in C2 and E4 fails to reach the high levels of endogenous wild-type expression (for instance, see circled bands for Q304P3 and C2).]
Figure 4.7 Accumulation of $C_i$ in the LCI1 transformants C2 and E4. The LCI1 transformants C2, E4 and the lcr1 mutant cells were grown under high CO$_2$ and in the presence of nitrate (open circles) or ammonium (closed circles). These conditions were chosen because the transformants C and E4 showed the highest $C_i$ affinities under these conditions. The intracellular concentration of dissolved $C_i$ (top panels) and carbon fixation (bottom panels) were measured using the silicon-oil layer method described in chapter 2 of this thesis. SIS stands for Sorbitol Impermeable Space.

*C. reinhardtii* cells.

**Localization of LCI1 in the plasma membrane of the *C. reinhardtii* cell:**

The predicted signal sequence of LCI1 did not show a definitive alignment with either a chloroplastic or mitochondrial membrane located protein. The determination of the location of the LCI1 mature protein on a particular membrane within a *C. reinhardtii* cell would provide some additional insight into its role in the CCM. With this aim in mind, the protein was tagged
with a Green Fluorescent Protein (GFP) epitope tag, in order to be able to visualize the position of the protein in real-time within live *C. reinhardtii* cells. The *LCI1* gene was placed under the control of a nitrate inducible promoter (*NIA1*). The *C. reinhardtii* adapted GFP sequence (Fuhrman *et al.*, 1999) was cloned in right before the stop codon of the last exon.

A short artificial linker sequence was added to separate the C’ terminus of the *LCI1* protein from the GFP tag to allow for proper folding of the chimeric protein (see Figure 4.8A). The presence of the GFP tagged protein could be detected as a higher band on immunoblots where the total protein fraction from nitrate induced cells of *C. reinhardtii* (Figure 4.8B) was probed with a *LCI1* specific antibody. The *LCI1*: GFP was found to be localized to the both the nuclear and plasma membrane of the *C. reinhardtii* cells under live imaging using a confocal microscope (Figure 4.9). The plasma membrane in *C. reinhardtii* cells is often closely appressed to the chloroplast membrane making whole cell membrane localizations difficult. However, the presence of the GFP tagged *LCI1* in the open end of the chloroplast cup (the only region where there is substantial space between the plasma and chloroplast membranes), supports the plasma membrane location of the tagged protein (see arrows in the GFP panel of Figure 4.9). The fluorescence detected around the nucleus could be either due to erroneous insertion of the chimeric protein into the nuclear membrane or due to the detection of the chimeric pre-protein *en route* to the plasma membrane. The location of the mature *LCI1* protein was further investigated by probing immunoblots of membrane fractions from low CO₂-acclimated whole cells, with a *LCI1* specific antibody. The immunoblots showed an enrichment of the *LCI1* protein in the plasma membrane fraction (Figure 4.10). The same protein blot when probed with antibodies directed to chloroplast membrane proteins, CCP1/CCP2 and a thylakoid membrane protein, D1
Figure 4.8 Expression of the LCI1-GFP chimeric protein. A.) A schematic representation of the LCI1: GFP construct. The construct is showing the GGSGGR short linker between the C-terminus of the LCI1 protein and N-terminus of GFP (CrGFP: GFP, codon optimized for proper *C. reinhardtii* expression). B.) Western blot analyses showing the accumulation of the chimeric LCI1-GFP protein under nitrate induction, in the LCI1-GFP transformants. The endogenous LCI1 protein can be seen accumulating in the low CO₂ grown cells of wild type and the transformants under both ammonium and nitrate growth conditions.

showed only a small amount of chloroplast membrane contaminating the plasma membrane fraction, with no detectable contamination from the thylakoid membrane fraction (Figure 4.10). The GFP fluorescence data together with the plasma membrane fraction enrichment of the endogenous protein strongly indicated that LCI1 is a plasma membrane protein. The plasma membrane location of LCI1 fits its possible role as a Cᵢ uptake protein as part of the CCM.
Figure 4.9 Live imaging of the LCI-GFP transformant cells showing the localization of the chimeric protein. The cells were grown under nitrate conditions and observed with a confocal microscope using different wavelengths of light. The white arrows show the position of the plasma membrane at the open end of the cup-shaped chloroplasts as regions where it is not in close proximity of the chloroplast membrane. DIC: Differential Interference Contrast; GFP: fluorescing at 410-470 nm; chlorophyll auto-fluorescence at 600-700 nm. Bars correspond to 5 μm.
Figure 4.10 Immunoblot showing the enrichment of LCI1 in the plasma membrane fraction. The LCI1 protein was enriched in the plasma membrane fraction of low CO$_2$ grown cells of the cell wall-less strain CC-503. 10µg of total protein from whole cells grown under high CO$_2$ (HC/total) and low CO$_2$ (LC/total) were run together with the same amount of chloroplast envelope (ce) and plasma membrane (pm) protein fractions on a SDS-PAGE gel. Nitrocellulose blots were then probed with an LCI1 antibody, a CCP1 antibody (specific to the chloroplast membrane proteins, CCP1/CCP2) and a D1 antibody (a thylakoid membrane protein) to reveal the extent of cross-contamination between the different membrane fractions during the extraction process.

**RNAi knock-down of LCI1 expression:**

The use of RNAi mediated knock-down of gene expression is widely in use in *C. reinhardtii*. In *C. reinhardtii* cells targeted gene knock-outs have proved to be very difficult. The use of an artificial microRNA (amiRNA) construct that utilizes the endogenous RNAi pathway of *C. reinhardtii* for the targeted destruction of mRNA has been recently shown (Molnar *et al.*, 2009; Zhao *et al.*, 2009; Schmollinger *et al.*, 2010). This approach has proved to be an effective...
way to generate knock-down mutants (often under the influence of an inducible promoter) to better study the roles of proteins of interest (Schmollinger et al., 2010). The Web MicroRNA Designer platform (WMD2, http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl) was used to select a suitable amiRNA candidate within the LCI1 cDNA sequence, through a two-step selection process based on optimal hybridization properties to the target mRNA that additionally minimizes possible off-target effects to other genes in the C. reinhardtii genome (Ossowski et al., 2008; Molnar et al., 2009). A suitable candidate for the amiRNA construct was found in the 3’UTR (UnTranslated Region) sequence of the LCI1 cDNA sequence (Figure 4.11). A 3’UTR location of a possible RNAi target sequence is often desired when designing artificial RNAi constructs because of two important reasons. First, it provides the opportunity to complement a successful knock-down mutant in which a substantial amount of the target protein is missing, hopefully producing a discernible phenotype in the mutant cells. By artificially expressing the coding sequence of the target protein, without the 3’UTR sequence, the complementation construct can be protected from RNAi mediated destruction within the mutant cell, thereby opening up the possibility of ‘rescue’ of the mutant phenotype. Secondly, in the case of C. reinhardtii genes, the 3’UTR and 5’UTR sequences often yield unique gene-specific targets for an RNAi knockdown. This avoids off-targets and also the dilution of the target siRNA (through non-specific targeting) generated through the artificial introduction of hairpin loops or amiRNA constructs. The vector pChlamiRNA3int (vector map shown Figure 4.10A, Molnar et al., 2009) was used to clone in a small 18 bp miRNA target selected from the LCI1 3’UTR into the backbone of a naturally occurring C. reinhardtii miRNA, namely, creMIR1157.

In immunoblots using a LCI1 specific antibody, two transformants L34 and L69, showed a considerable knockdown of LCI1 protein (Figure 4.11A, lanes 4 and 5) when compared to the
Figure 4.11 Construct used for RNAi knockdown of LCI1. A.) A schematic map of the plasmid, carrying the amiRNA construct used to knock-down LCI1 in C. reinhardtii cells. The APHVIII cassette corresponds to the selectable marker gene APHVIII, conferring paromomycin resistance to the transformant cells, flanked by the C. reinhardtii dual promoter (HSP70+RbcS2) and the RbcS2 terminator. B.) The LCI1 cDNA sequence showing the position of the 18 bp LCI1 target sequence cloned into the pChlamiRNA3int (Molnar et al., 2009) vector.

wild-type D66 cells (Figure 4.12A, lanes 1-3) and the empty vector control (Figure 4.12A, last lane). This showed that the amiRNA technique to generate RNAi knockdown mutants was successful with this particular LCI1 construct. When looking at the expression of other important
Figure 4.12 The reduction of the LCI1 protein in the RNAi knock-down mutants L34 and L69. A.) L34 and L69 showed a reduction in LCI1 when compared to the wild-type D66 cells. The total protein from D66 whole cells was diluted from 10 µg to 2.5 µg to give a visual estimation of the extent of reduction of LCI1 in the mutants. Vc is the empty vector control. B.) The reduction in the levels of CCP1/CCP2 (a pair of near identical chloroplast membrane putative C\textsubscript{i} transporters) in two RNAi mutants, C1 and C15 when compared to L69. The CCP1/2 knock-down mutants were generated by Pollock et al., 2003). The LCI1 band is acting as a loading control, showing the lower loading of mutant C15.

CCM proteins, it was discovered that the RNAi knockdown mutants L34 and L69 were also missing the periplasmic CA, CAH1 (Figure 4.13, CAH1 panel). All the other proteins examined, namely the proteins believed to be involved in C\textsubscript{i} transport in the *C. reinhardtii* CCM namely CCP1/CCP2 and LCIB, the key CAs, CAH4 and CAH6 were present in the two mutants, L34 and L69. The growth phenotypes of L34/L69 when tested under low CO\textsubscript{2} conditions at different pH levels (Figure 4.14) showed very slight differences in growth compared to the wild-type D66.
Figure 4.1 The expression levels of other CCM proteins in the LCI1 knockdown mutants. The levels of other CCM proteins were examined via western blot analyses in L34 and L69 compared to the wild-type D66 cells. Different antibodies probes specific to different CCM proteins were used. The absence of LCI1 and the CAH1 protein in the L34 and L69 mutants are shown. The mutant, cah1, is a previously characterized mutant missing the CAH1 protein (Van and Spalding., 1999). The CCM mutant cia5 was used as a negative control and is missing the proteins LCI1, CAH4, CAH1 and CCP1/CCP2. The expression of LCIB (involved in the chloroplastic C\textsubscript{i} transport process) is slightly lower in the mutants compared to the wild-type. The presence of a band in the pmp1 mutant (Wang and Spalding., 2006) missing the LCIB protein is due to the cross-reaction of the antibody to another closely related protein of the LCIB family (Spalding lab, personal communication).
The growth phenotype of L34 and L69 was compared to the wild-type D66 and the CCM mutant cia5, at different pH levels and under low CO$_2$ conditions. Only a slight reduction of growth is visible at pH 7.2 and 8.2 when compared to D66. CCP1/CCP2 RNAi mutants (Pollock et al., 2003), C1 and C15, were included in the screen. The previously reported LCIB mutant pmp1 (Wang and Spalding., 2006; defective in chloroplastic C$_i$ transport; phenotype: poor growth under low CO$_2$, dead under air levels of CO$_2$) and the cia6 mutant (disorganized pyrenoid, sick under low CO$_2$, Ma et al., 2010) were included to compare extents of growth deficiencies in the knock-down mutants.

especially as the pH levels is increased from 6.2 (C$_i$ predominantly in the form of CO$_2$; L34/69 growth similar to wild type) to 8.2 (C$_i$ predominantly in the form of HCO$_3^-$ ions; L34/69 growth slightly deficient compared to wild type D66). A similar growth pattern is also seen with two other RNAi mutants showing a reduction in the CCP1/CCP2 proteins (see Figure 4.12B) namely the proteins believed to be involved in C$_i$ transport in the C. reinhardtii CCM namely CCP1/CCP2 and LCIB, the key CAs, CAH4 and CAH6 were present in the two mutants, L34 and L69. The growth phenotypes of L34/L69 when tested under low CO$_2$ conditions at different pH levels (Figure 4.14) showed very slight differences in growth compared to the wild-type D66 especially as the pH levels is increased from 6.2 (C$_i$ predominantly in the form of CO$_2$; L34/69 growth similar to wild type) to 8.2 (C$_i$ predominantly in the form of HCO$_3^-$ ions; L34/69 growth slightly deficient compared to wild type D66). A similar growth pattern is also seen with two
other RNAi mutants showing a reduction in the CCP1/CCP2 proteins (see Figure 4.12B) previously generated in the lab (Pollock et al., 2003). The rate of photosynthesis measured once, through an oxygen evolution assay at pH 7.2 (both L-34 and L-69 showing a slight growth deficiency at this pH) however, failed to show any significant differences between the wild–type D66 cells and the RNAi mutants (Figure 4.15). The generation of LCI1 RNAi mutants is helpful in establishing a reliable method of obtaining stable knockdown mutants in *C. reinhardtii*. The lack of a strong phenotype of the knock-down mutant however, may reflect a redundancy in function of some other protein transporters localized to the plasma membrane, for example HLA3. This might explain why the absence of LCI1 does not produce a significant deficiency in CCM function. Thus, this establishes the need for co-knockdowns of putative C\text{\textsubscript{i}} transporters at the same location within the *C. reinhardtii* cell, in order to produce strong enough phenotypes that would better expose the machinations of C\text{\textsubscript{i}} uptake and transport at each membrane level.

**Discussion**

The uptake and transport of inorganic carbon is an integral part of the *C. reinhardtii* CCM (Moroney and Ynalvez., 2007). In order to dissect this process, the transport proteins at each membrane level need to be identified and the interaction between them needs to be characterized. Recently, a number of reports regarding the putative inorganic carbon transporters at different cell membrane levels has provided more information about the bicarbonate transport process in the *C. reinhardtii* CCM (Pollock et al., 2003; Wang and Spalding., 2006; Duanmu et al., 2009a, 2009b; Ohnishi et al., 2010). HLA3 and LCI1 are two of the proteins that have been implicated in possible uptake of inorganic carbon by the *C. reinhardtii* cell at the plasma membrane under limiting CO\text{2} conditions (Duanmu et al., 2009a, 2009b; Ohnishi et al., 2010). The localization of LCI1 to the plasma membrane of the *C. reinhardtii* cell (as shown in Figures 4.10C and 4.11) is
crucial in examining its role as a potential C_i transporter in the CCM. The plasma membrane is the first barrier to the entry of the charged HCO_3^- ion. Bicarbonate ions provide the *C. reinhardtii* cell with an alternative source of inorganic carbon. This is especially true for an actively photosynthesizing cell under limiting CO_2 conditions.

![Graph](image)

Figure 4.15 The photosynthetic rates of low CO_2 acclimated cells of the LCI1 RNAi mutants. The DIC-dependent O_2 evolution rates were used to measure the rates of photosynthesis. The photosynthetic rates of the two LCI1 RNAi mutants, L34 and L69, were compared to the wild-type D66. Photosynthetic oxygen evolution was measured using a Clark-type oxygen electrode in the presence of different concentrations of dissolved NaHCO_3 (dissolved inorganic carbon or DIC) at pH 7.8. This experiment was performed only once (hence no error bars).
The current model for a functional *C. reinhardtii* CCM proposes the presence of one or more transporters at the plasma membrane to facilitate, especially, the entry of HCO$_3^-$ anions which would finally be converted to the Rubisco substrate, CO$_2$, within the cell. The location of LCI1 in the plasma membrane provides an ideal candidate for such a $C_i$ uptake/transport facilitator. The actual implication of LCI1 in the uptake of $C_i$ within the cell comes from the improvement shown in the physiological characteristics of transformants artificially expressing LCI1 (under high CO$_2$ and nitrate induction), when compared to the mutant *lcr1* (Ohnishi et al., 2010). The light dependent CO$_2$ exchange (LCE) activity as measured by the draw-down of CO$_2$ by photosynthesizing cells under nitrate induction showed a substantial increase in the transformants C2 and E4 under high CO$_2$ implying the direct involvement of LCI1 in increasing the uptake of $C_i$ independent of other CCM components that are repressed under the experimental conditions (Ohnishi et al., 2010). These data together with the increase in the photosynthetic rate and the increased accumulation of $C_i$ (Figure 4.9) clearly implicates LCI1 as being responsible for this increase in the transformant cells (Ohnishi et al., 2010).

The interesting approach undertaken to study the effect of the artificially induced LCI1 expression on cells with a repressed CCM under high CO$_2$ conditions should be useful in the future study of other putative $C_i$ transporters. The *C. reinhardtii* cell serves as an important model system for the study of eukaryotic CCMs. However, in the absence of reliable and reproducible systems to target the knockout and knock-down of specific genes to study their function, the artificial expression of genes under specific conditions, might prove to be a viable alternative approach in the study of yet uncharacterized transport proteins. Although successful in the case of LCI1, this approach also has revealed some draw-backs that should be taken into account when studying other putative $C_i$ transporters of the *C. reinhardtii* CCM. For instance,
artificial expression of LCI1 or other uncharacterized $C_i$ transporters in a wild-type/mutant background might suffer from the problem of never being expressed to wild-type levels. This is because of positional effects of the random insertion of the artificial construct into the $C. reinhardtii$ genome. This inability to sometimes reach wild-type levels of expression with artificial constructs might affect data interpretation in case of mutant complementation under low CO$_2$ conditions, when the endogenous protein is often the most abundant. The other problem with studying certain physiological aspects like the LCE activity of actively photosynthesizing cells under high CO$_2$ (to create an environment free of other redundant CCM components), might cause interference from the highly abundant and freely diffusing CO$_2$ molecules masking some of the bicarbonate uptake abilities of the expressed proteins. For instance, the increases in the LCE activity of the LCI1 transformants C2 and E4 were similar under both pH 6.2 and pH 7.8, when compared to the lcr1 mutant background. Normally the abundance of HCO$_3^-$ is more prominent at a pH level of 7.8 when compared to 6.2 (Ohnishi et al., 2010). However, the inability of the experiment to demonstrate a specific preference for one $C_i$ molecule over the other, because of the possible presence of CO$_2$ in excess, makes it difficult to label LCI1 specifically as a bicarbonate transporter (Ohnishi et al., 2010). The same problem might be encountered with any future studies using the same approach to study putative $C_i$ transporters. It is also worth mentioning here that like the putative $C_i$ transporter, NAR1.2 (see chapter 3), the complementation of a cyanobacterial mutant lacking two plasma membrane bound bicarbonate transporters was also attempted with LCI1. Introduction of the LCI1 ORF sequence, both with and without the putative leader sequence, failed to rescue the cyanobacterial mutant (data not shown). Immunoblots of the complemented cyanobacterial mutants failed to show the presence of the LCI1 protein (data not shown). Therefore, it could be speculated that the inability of either
the proper translation or a lack of stability of the translated LCI1 protein might be the reason behind the complementation failure.

An attempt was made to generate a knock-down mutant of LCI1 in order to verify its implicated role in C\textsubscript{i} transport in the \textit{C. reinhardtii} CCM. Although, two RNAi knockdown mutants L-34 AND L-69 were generated using an artificial miRNA construct, they failed to show any discernible phenotypes in terms of any significant deficiencies in growth or photosynthetic rates under low CO\textsubscript{2} conditions. It could be hypothesized that the redundant role of another plasma membrane bound transporter, for instance, HLA3, might be masking any effects that the absence of LCI1 might have, on the C\textsubscript{i} uptake of the \textit{C. reinhardtii} cell, under low CO\textsubscript{2} conditions. However, it should be noted here that silicone oil measurements of C\textsubscript{i} uptake and accumulation as well as more rigorous growth studies in liquid medium might reveal changes in the overall CCM physiology of the LCI1 mutants when compared to the wild-type cells. A positive fall-out of this experiment was the efficacy of the amiRNA approach in generating knock-down mutants in \textit{C. reinhardtii}. The author has successfully attempted the use of artificial hairpin constructs for generation of LCI1 target siRNA and generation of mutants with varying degrees of protein knockdown were achieved (data not shown). However, the amiRNA approach not only generated mutants with a greater extent of knockdown when compared to the artificial hairpin constructs but also required fewer transformant screenings to identify them. The most valuable feature of the amiRNA approach was the generation of mutants that were stable for more than two years as opposed to the hairpin constructs that were subject to quick silencing and subsequent loss of knock-down phenotypes within the \textit{C. reinhardtii} cell. Silencing of artificial gene constructs within the \textit{C. reinhardtii} cell is a recurrent problem faced by researchers and therefore any approach circumventing the problem is valuable. Generation of
stable knock-down mutants, especially with discernible phenotypes, can also be used for the study of other CCM proteins. The existence of successful reports where amiRNA constructs have been used to target two separate genes in Arabidopsis might make the application of such techniques in studying protein functions in C. reinhardtii a viable option (Park et al., 2009). Especially, in cases of redundant proteins, double or triple knock-down mutants of proteins (for instance, at the same membrane location like HLA3 and LC11) generated in this manner, might produce more prominent phenotypes than those produced by single knock-down mutants (Duanmu et al., 2009b). Needless to say, even an amiRNA construct is not free from off-targets. As seen with L-34 and L-69, together with LC11, the periplasmic CA, CAH1 is also knocked-down. Since, CAH1 mutants have been reported to have an unaffected CCM (Van and Spalding., 1999) its absence would not have contributed significantly to the phenotype, if any, of the LC11 mutants. The CAH1 transcript was found to be missing in the LC11 knock-down mutants (data not shown). A similarity of 8bp within the 18bp target LC11 sequence matched up with the 6th exon of the CAH1 cDNA sequence and its position in the crucial seed region of the amiRNA sequence was sufficient in either causing a substantial destruction of the CAH1 transcript via the RNAi pathway or by the direct blockage of translation by the miRNA (Gu et al., 2010). This brings to the fore, the need for generating complete knock-out mutants to circumvent the problems of dealing with the shortcomings of knock-down mutants. Knock-down mutants are still a viable option for studying the functions of some essential proteins, a complete absence of which might lead to lethality of cells. However, in case of proteins like LC11, where the knock-down of protein expression seems not to cause any major disruption of the process being studied (in this case the CCM), the generation of a knock-out mutant through some form of artificial gene disruption needs to be undertaken. This might help in generating double and triple mutants
achieved through crossing of mutant cells leading to mutant backgrounds lacking multiple target proteins. With this aim in view and with the specific purposes of validating the importance of LCI1 as a C\textsubscript{i} uptake protein and revealing its possible interaction with other membrane bound C\textsubscript{i} transporters, LCI1 was included in the CCM genes chosen for the insertional mutagenesis project summarized in chapter 6 of this thesis.

Endnotes

Figures 4.6, 4.7, 4.8, 4.9 and 4.10, are reproduced from the following published article (cited below with name of author of this thesis underlined) and are a “Copyright of the American Society of Plant Biologists.”

CHAPTER 5
THE ABSENCE OF THE PERIPLASMIC CARBONIC ANHYDRASE, CAH1, IN THE SEQUENCED WILD-TYPE STRAIN, CC-503.

Introduction

The photosynthetic green alga, *C. reinhardtii*, can successfully acclimate to fluctuating levels of external C\textsubscript{i}, without compromising its photosynthetic rates. It does so by the induction of an efficient C\textsubscript{i} uptake and utilization process that elevates the CO\textsubscript{2} levels around Rubisco, and is known as the Carbon Concentrating Mechanism or CCM (Badger *et al.* 1980). Key components of this mechanism are carbonic anhydrases (CAs), nine of which have been identified in *C. reinhardtii* so far (Moroney *et al.*, 2011). A periplasmic CA, CAH1, like many other key CCM proteins, is highly induced under limiting CO\textsubscript{2} conditions (Coleman and Grossman, 1984). Many studies have focused on the role of this CA in the *C. reinhardtii* CCM (Fukuzawa *et al.*, 1990; Ishida *et al.*, 1993) and some CAH1 mutants have been successfully generated and characterized (Van & Spalding, 1999).

CAH1 is found in the periplasmic space of *C. reinhardtii* (Fukuzawa *et al.* 1990a; Fukuzawa *et al.* 1990b). In fact, if cells from a low-CO\textsubscript{2}-grown culture are pelleted by centrifugation, CA activity could easily be detected in the supernatant (Rawat and Moroney 1991). Furthermore, the CAH1 protein can be detected in the cell wall fraction using immunoblots (Ishida *et al.* 1993). Immunogold localization studies using anti-CAH1 antibodies also clearly show labeling in the cell wall region (Moroney and Ynalvez 2007). In addition, the primary sequence of CAH1 has a leader sequence that is consistent with a secretory pathway signal (Fujiwara *et al.* 1990; Ishida *et al.* 1993).

CAH1 is an unusual CA in that it has two large and two small subunits. The assembly of CAH1 is a multi-step process. Work in the early and mid-nineties showed that CAH1 is
extensively processed after it is translated (Ishida et al. 1993). During translation, the CAH1 is directed to the ER lumen and the leader sequence is cleaved. Subsequently, an internal region of the protein is excised, leaving a large and a small subunit that remain associated in the periplasmic space through disulfide bridges. In addition, the protein is glycosylated (Fukuzawa et al. 1990b). After all of these post-translational modifications, the protein is sent to the periplasmic space. The final protein is a hetero-tetramer consisting of two large and two small subunits connected by disulfide bridges (Ishida et al. 1993; Kamo et al. 1990).

CAH1 is highly induced under limiting CO₂ conditions, under the control of the CCM master regulator gene, CIA5 (Fukuzawa et al. 2001; Moroney et al. 1989; Xiang et al. 2001). Additionally, LCR1, a MYB-type transcriptional factor, was reported to regulate CAH1, LCI1 and LCI6 via CCM1 under low CO₂ conditions (Yoshioka et al. 2004). Many studies have focused on the role of this CA in the C. reinhardtii CCM (Fujiwara et al., 1990; Fukuzawa et al., 1990a, 1990b; Ishida et al., 1993). Studies using acetazolamide, a soluble CA inhibitor, caused a small decrease in the affinity of the cells for Cᵢ (Badger et al. 1980; Berry et al. 1978; Tsuzuki and Miyachi 1979; 1989; Tsuzuki et al. 1980). The work was extended by making dextran bound sulfonamide (DBS), a membrane impermeant CA inhibitor (Moroney et al. 1985), demonstrating that DBS inhibited Cᵢ-dependent O₂ evolution to the same extent as acetazolamide. This led to the conclusion that inhibiting the periplasmic CAs also reduces the ability of C. reinhardtii to obtain Cᵢ from the medium (Moroney et al. 1985). However, later mutant studies have not supported the inhibitor work. In 1999, Van and Spalding characterized a strain, cah1, which has a deletion in the CAH1 gene (Van and Spalding 1999). When the Cᵢ-dependent O₂ evolution rates of cells grown at different external pH levels were measured, no significant physiological differences between cah1 and wild-type cells could be detected.
The strain CC-503 (nit1<sup>−</sup>, nit2<sup>−</sup>, cw-92, mt<sup>+</sup>), together with the other wild-type strains D66 (CC-4425, nit1<sup>−</sup> nit2<sup>−</sup>, cw15, mt<sup>+</sup>) and C9 (CC-408, mt<sup>−</sup>), are common laboratory strains used for experimental studies in the *C. reinhardtii* field. The cw-92 cell wall deficiency (Harris et al. 2009; Hyams and Davies 1972) in CC-503 facilitates the isolation of genomic DNA, thus making this strain the reference strain for *C. reinhardtii* genome sequencing (Merchant et al. 2007). For the same reason, nuclear transformation becomes more efficient when using this strain due to the minimal barrier from the cell wall. However, in the course of CCM related experiments performed with CC-503 as a wild-type strain, it was discovered that the CAH1 protein could not be found in the strain CC-503. This observation was quite surprising when one considers the great abundance of CAH1 when other wild-type strains are under the low CO<sub>2</sub> stress (Moroney *et al.* 2011). Attempts were made to explain the lack of CAH1 protein in CC-503, as well as to explore the possible physiological consequences from missing CAH1. Most importantly, this chapter highlights the fact that the sequenced strain CC-503 is a natural mutant for the periplasmic CA, CAH1.

**Results**

**The CAH1 protein is not present in CC-503**

The *CAH1* gene is known to be highly transcribed upon induction of the *C. reinhardtii* CCM (Moroney *et al.* 2011). For the wild-type strains D66 and C9, the presence of the CAH1 protein could be easily detected by immunoblotting in low CO<sub>2</sub> acclimated cells (Figure 5.1), which is consistent with previous observations (Kucho *et al.* 1999; Mitra *et al.* 2004). However, in CC-503, the CAH1 protein could not be detected (Figure 5.1). The lack of CAH1 in CC-503 resembles the situation in the two control mutants here, *cia5* and *cah1*. The *CAH1* gene is either not responsive to low CO<sub>2</sub> stress due to the *CIA5* mutation, or the *CAH1* gene itself is defective.
as seen in the *cahl* mutant (Van and Spalding 1999). While level of CAH1 was undetectable in CC-503, the level of the other important CCM components CAH4 and CCP1 was not affected. The CAH1 transcriptional regulator LCR1, (Yoshioka *et al.* 2004), is presumably functional in CC-503 as evident by the normal levels of LCI1, another protein that is regulated by the same gene (Figure 5.1).

**CC-503 has an extra repeat region in the CAH1 promoter**

The absence of CAH1 may be due to the presence of a mutation in the *CAH1* locus, which results in a lack of induction of *CAH1* in CC-503. In the *CAH1* promoter nucleotide transcription start site that was sufficient for the CO₂ responsive transcription of the *CAH1* gene (Kucho *et al.* 1999). This region was found to consist of two parts, a 358-bp silencer region from -651 to -294 and a 185-bp enhancer element from -293 to -109 with respect to the transcriptional start site. The silencer region was shown to repress the gene under high CO₂ conditions whereas the enhancer region induced gene expression under low CO₂ conditions in the presence of light (Kucho *et al.* 1999). The *CAH1* DNA sequence in CC-503 (available from the *C. reinhardtii* genome database, Version 4.0) was compared with the published sequence from C9 (Kohinata *et al.* 2005; Kucho *et al.* 1999). It was found that the 358-bp silencer region, the 185-bp enhancer region and the coding sequence were identical in both CC-503 and C9. However, there was one significant difference upstream of the 358-bp silencer region. In C9, there was a 78-bp region consisting of about 13 tandem repeats of the sequence AGGGGC. In CC-503, however, this repeat region was about 582-bp long, with 97 tandem repeats (Figure 5.2).
Figure 5.1 Western blot analyses showing the absence of the CAH1 protein in CC-503 cells. The western blot analysis was carried out using a CAH1 specific antibody. LCI1, CAH4 and CCP1 are CCM proteins that are induced under low CO₂ conditions. cah1: CAH1 mutant strain; cia5: CCM mutant strain.

Attempts to verify the presence of the repeat region by PCR amplification using primers flanking the repeat region failed in case of the CC-503 and revealed a slightly longer fragment than expected in the case of C9 (data not shown). The repeat could form what is called a G-quartet, which could pose a difficulty in the PCR amplification from the CC-503 genomic DNA. The presence of these repeats might also have interfered with the proper sequencing of this region.
which would mean that the actual number of AGGGGC repeats might vary from the values inferred from the sequences available from CC-503 and C9.

Figure 5.2 A schematic diagram of the region upstream of the CAH1 transcriptional start site, from two strains CC-503 and C9. The silencer region (red) and the enhancer region (blue) are identical between the two strains. However, there are 84 extra AGGGGC repeats upstream of the (-651 to -294) region in CC-503.

The CAH1 transcript is present in CC-503

The presence of the extra 79 repeats close to the CAH1 transcriptional regulator region could be a possible contributing factor to the absence of the protein by reducing or eliminating the transcription of the CAH1 gene. To test this possibility, an attempt was made to see if the gene was transcribed at the expected levels in CC-503.

The presence of the whole transcript was also confirmed with PCR, using primers covering the first nine exons (data not shown). These data suggest that even though CAH1 protein is absent in CC-503, the CAH1 gene is still transcribed at a comparable level to wild-type strains (Figure 5.3). Since the absence of the protein cannot be accounted for by a reduction in transcript levels, determining the cause for the absence of CAH1 will remain a focal point for future investigations.
Figure 5.3 Relative abundance of the \textit{CAH1} transcript in CC-503, compared to D66 and C9. The abundance of \textit{CAH1} transcript levels was measured by qRT-PCR under low CO$_2$ conditions. The \textit{CBLP} gene was used as an internal reference gene as it is of high abundance and its expression remains unchanged under varying CO$_2$ conditions. The relative transcript level is expressed as the $2^{\Delta \text{CT}}$, in which $\Delta \text{CT}= (\text{CT High CO}_2 - \text{CT Low CO}_2)$. $\Delta \text{CT}$ High CO$_2$ for instance, is calculated as CT value of \textit{CAH1} at high CO$_2$- CT value of \textit{CBLP} at high CO$_2$.

\textbf{CC-503 shows reduced growth under low CO$_2$ and low pH levels}

Previously, it was reported that in the absence of \textit{CAH1}, \textit{C. reinhardtii} cells fail to produce any significant differences produce any significant differences in either the ability to induce a fully functional CCM at low CO$_2$, even when pH levels are varied (Van and Spalding 1999). When the growth phenotype of CC-503 under different pH levels was tested in this study, it was noticed that the cells showed reduced growth at lower pH levels (pH 5.8) and a somewhat lesser reduction in growth at a high pH level (pH 8.2) (Figure 5.4). While the \textit{cah1} mutant cells could still grow at a low pH of 5.8, most of the CC-503 cells died at this low pH.
Figure 5.4 Growth phenotype of CC-503, compared to C9 and D66. The growth phenotypes of CC-503 cells were observed under low CO$_2$ conditions at different pH levels. The growth was compared to wild-type strains C9 and D66 as well as the mutant strains, cah1 and cia5. cah1: CAH1 mutant, cia5: CCM mutant. For spot tests, 4000 cells of each strain were pipetted onto minimum agar plates. The plates were incubated in low CO$_2$ conditions (100 ppm) for 10 days.

CC-503 showed no reduction in photosynthesis rate

The ability of the CC-503 strain to induce an otherwise normal CCM was indicated by the presence of other key CCM proteins at levels comparable to those in other wild-type strains (Figure 5.1), as shown in all laboratory generated CAH1 mutant strains. The rate of O$_2$ evolution in CC-503 at different pH levels did not deviate much from the other cell wall deficient wild-
type strain, D66 (Figure 5.5). At a low pH level of 6.2, CC-503 cells showed a photosynthetic response similar to the mutant cahl. This trend was consistent at pH levels of 7.2 and 8.2 (data not shown).

**Discussion**

Although artificially generated mutants for CAH1 exist, the discovery of CC-503, as a unique natural mutant, is interesting in many aspects. For one, it corroborates earlier findings that the absence of the periplasmic CA does not significantly affect the functioning of the CCM. This strain also provides a second mutant lacking this protein that could be targeted for future investigations into the role of this protein the *C. reinhardtii* CCM. One of the aspects that need to be looked into is whether another CA is compensating for the lack of the CAH1 protein in the *C. reinhardtii* cell.

A good candidate would be CAH8, a CA that has been localized to the plasma membrane of the *C. reinhardtii* cell (Ynalvez et al., 2008). However, the exact location of CAH8 active site (located in either the periplasmic or the cytoplasmic side of the membrane) is still undetermined as is the exact mechanism that is bringing about its attachment to the plasma membrane. It would be interesting to see whether in the CC-503 strain, the lack of the CAH1 protein causes any increase in the protein levels of CAH8. The TMpred program predicts that the active site would be on the outside of the cell membrane. If it were outside of the cell, then it would be expected that CAH8 activity is partially redundant with that of CAH1 and CAH2. The phrase “partially redundant” is used as CAH1 and CAH2 are found in layers of the cell wall quite far from the plasma membrane and are actually shed by *C. reinhardtii* during cell division. If CAH8 is outside of the cell, its presence could explain why CAH1 deletion strains show no reduction in
Figure 5.5   Comparison of the photosynthetic rates of CC-503 with other strains. The rates of photosynthesis were measured in low CO\textsubscript{2} grown cells of D66 (△), CC-503 (▼), cah\textsubscript{1} (■) and C9 (●) measured as a function of the dissolved inorganic carbon concentration (provided by solutions of NaHCO\textsubscript{3} ranging from 25µM to 2 mM). Experiments were carried out an external pH of 6.2 and each point represents the mean and standard deviation of three replicates.

DIC-dependent O\textsubscript{2} evolution. In this case the acetazolamide could be inhibiting three different isoforms while the deletion strains are only missing CAH1. If, however, the active site of CAH8 was inside the cell, then its role would be quite different than that of CAH1 and CAH2. In this case, CAH8 might be facilitating the entry of CO\textsubscript{2} into the cell by rapidly converting the CO\textsubscript{2} to HCO\textsubscript{3}\textsuperscript{-} as it enters the cell, maintaining the concentration gradient across the membrane.
CC-503 carries a $cw$-$92$ mutation leading to its extreme cell-wall deficient phenotype. This makes it a good candidate for transformation techniques such as electroporation. Also, this strain was also valuable in producing intact chloroplasts for harvesting chloroplast membrane fractions because of the ease with which these cells can be ruptured when passing through a syringe in the protocol modified from Mason et al. (2006).

The presence of a minimal cell wall is what prompted us to hypothesize that since the transcription of the $CAH1$ gene is not affected in CC-503, the protein might be just lost to the media during harvesting and washing of cells. Skipping the washing process did not help in attempts at isolating the protein. An attempt to try retrieving the protein from the media was unsuccessful. This attempt should be repeated in the near future using CAH2 as a control (in high CO$_2$ cells of $cia5$ which lacks CAH1), to make sure that the enrichment of proteins from the media was successful. The investigation of CAH1 levels in other strain carrying the $cw$-$92$ mutation might also reveal if the lack of the protein is somehow affected by this seemingly unrelated mutation. Also, the lack of the cell wall in this strain might be causing some of the growth defects seen in low pH levels. The discovery of this natural mutant lacking CAH1 will provide a good platform for future investigations into the role of this periplasmic CA in the $C. reinhardtii$ CCM.
CHAPTER 6
GENERATION OF INSERTIONAL MUTANTS IN *CHLAMYDOMONAS REINHARDTII*

**Introduction**

**The pros and cons of forward genetic approaches in mutant generation:**

Genetic mutations, either natural or artificially generated, have been the cornerstones in the studies of most biological processes. These mutations help reveal the genes that are often essential in the basic functioning of a process and help piece together the genetic networks of functional interaction and regulation. Researchers interested in developing working models for their processes of interest, often try to speed up their investigations by looking for ways to artificially disrupt genes that produce phenotypes pertinent to the scientific problem being studied. This approach helps them generate and characterize mutant phenotypes that possibly carry mutations in one or many genes that play a key role/roles in the proper functioning of a biological process. This approach, also known as a forward genetics approach, has been widely used in the development of useful models for many biological processes. In a forward genetics approach (dealing with a process for which natural mutations do not exist), the genotype of an organism is artificially disrupted either through physical insertion of foreign DNA or through chemical mutagenesis. The population of mutants thus generated, are then screened for the presence of a phenotype, which shows a deficiency in the biological process being studied. Further characterization of such mutants is then carried out leading to the identification of the gene/genes that are essential to the process. Such identifications can often be further validated when restoration of the wildtype gene/genes into the mutant genome rescues the mutant phenotype. Since, the success of a forward genetics approach depends on a “mutant phenotype”
that leads the investigator to the disruption in the genotype that caused it, it is essential that the mutant phenotype be distinct enough to be identified in the screening process. However, due to the redundancy of functional roles between multiple proteins involved in a particular process, or due to the absence of a measurable phenotype in the case of some gene disruptions, a forward genetics approach often fails in its purpose of revealing mutations that might be interesting in the investigation of a process. Researchers have often overcome these issues by taking on a reverse genetics approach to target specific genes that they suspect of being functionally important in the study of a biological process.

As the name suggests, a forward genetics approach starts from a phenotype and leads to the gene/genes responsible, a reverse genetics approach involves a targeted interference of the expression of a gene and the investigation of the resulting phenotype in the mutant. Although both approaches lead to the same end result of a better understanding of the genetic basis of a biological process, the choice of one over the other, often takes into consideration several factors that depend on the process being studied, and the organism within which it is being studied. In certain cases where the candidate genes for a process have been identified beforehand, specific targeting of such genes for artificial disruption or transcriptional/translational repressions for instance, via the endogenous RNAi pathways can be undertaken. The selection of target genes has been further facilitated by the abundance of genomic and transcriptomic data that is available to researchers mostly due to advances in high-throughput genome and transcriptome sequencing. This has lead to the identification of a large number of actively transcribed genes encoding proteins that are yet to be functionally characterized. Since data derived from modern transcriptomic sequencing techniques give an indication of the conditions under which certain genes have either induced or repressed expression within the organism, it is now easier to
identify genes that might be of interest in the study of specific processes. This helps a great deal in the selection of targets for reverse genetics approaches in organisms where such data is available. In organisms where homologous recombination is more frequent such as yeast and mice, swapping out target genes with selectable marker genes are commonly used in creating target knockout mutations. However, in organisms like higher plants and algae, homologous recombination events are extremely rare. When attempting to disrupt genes in such organisms, insertional mutagenesis approaches are commonly used that rely on random insertions of exogenous DNA such as transposons or selectable marker genes into the genome (Tam and Lefebvre., 1993). A sufficiently large number of transformants need to be generated to ensure a somewhat even coverage of the entire genome and increase chances of the genes involved in a particular process to be targeted. Insertional mutagenesis is often a powerful tool for forward genetic approaches but has recently also been successfully used for reverse genetics.

In cases where disruption of a gene might be lethal to an organism, a systemic knockdown of expression by targeting mRNA stability or protein translation via the RNAi pathway can also be attempted. These could involve the use of antisense technology with either natural antisense mRNA targeting specific gene transcripts or chemically modified antisense RNA (better known as morpholinos) that bind and translationally inhibit target mRNA thereby leading to suppression of gene expression. Antisense technology is commonly used together with other favored RNA interference (RNAi) mediated techniques that either directly use siRNAs and miRNAs or indirectly produce them from artificial DNA constructs generating small or large mRNA hairpin structures or preamiRNAs. Although RNAi mediated techniques have been successful in most model organisms studied so far, they have their own set of problems when it comes to interpretation of mutant phenotype. This is mostly due to the off-target effects seen with most
small RNA species often leading to the knockdown in expression of an off-target gene/genes that might contribute to a particular mutant phenotype. Also RNAi mediated knockdown mutations often show a wide spectrum of mRNA silencing. This, though useful in the study of essential genes by avoidance of lethality in mutants, often fails to produce distinctly measurable phenotypes in the study of a specific gene function. The variability in the silencing of the target gene also creates difficulties with reproducibility of certain phenotypes. Also, the silencing of the transgenic construct carrying the target siRNA might be subjected to silencing itself, often leading to a gradual loss of the knockdown phenotypes, over time. Attempts to overcome some of the problems faced with specificity in RNAi mediated knockdowns have been recently made by the use of zinc finger nucleases (ZFNs). This involves the use of custom designed chimeric endonucleases in artificial constructs that can be randomly inserted into the genome and when expressed specifically cleave target genes (Osakabe et al., 2010; de Pater et al., 2009; Tovkach et al., 2009). The use of chemical mutagenesis techniques have also been used to generate libraries of mutants that carry mutations in genes involved in the functioning of biological processes. Collectively, these techniques form the basis of the reverse genetics approach whereby specific targeted disruptions to a genotype are carried out to create mutant phenotypes that would help in elucidating the machinations of a biological process.

The success of forward and reverse genetics approaches in C. reinhardtii

In C. reinhardtii, forward and reverse genetics approaches have been widely used in the study of several important cellular processes. In recent years forward genetics approaches have led to the isolation and hence identification of genes involved in various processes in C. reinhardtii (Pazour and Witman, 2000; Galvan et al., 2007). Sometimes mutants with interesting phenotypes pertinent to the process being studied end up requiring a lot of time and effort in the
discovery of the mutated genetic locus leading to that phenotype. These days affordable whole genome-sequencing of *C. reinhardtii* mutant strains can sometimes speed up the process by providing an alternative approach to mutant locus identification (Dutcher *et al.*, 2012) when traditional methods such as chromosomal mapping or iPCR/adaptor PCR have failed to expose the mutated gene/genes. This has allowed forward genetics approaches such as generation of insertional mutants in *C. reinhardtii* to remain a viable option in the study of most processes in the alga. On the other hand, reverse genetics approaches that have been widely used so far in *C. reinhardtii* are the RNA interference (RNAi) based methods using either artificial hairpin creating constructs or an artificial miRNA construct that exploits a naturally occurring micro-RNA backbone. These methods aim at targeting and destroying the mRNA of a gene whose expression one wants to knockdown. Often these artificial constructs are placed under inducible promoters to create conditional knockdown of expression especially in cases of genes essential for survival of the cell. Careful design of artificial RNAi knock-down constructs targeting small portions of mRNAs in *C. reinhardtii* can sometimes make knock down mutants amenable to complementation experiments that ‘rescue’ the knock-down phenotype much like insertional mutants. Directing targets to the 5′/3′ UTRs, or targeting portions of the mRNA that would be unaffected by codon changes would exemplify a few such design modifications. These allow complementation of function by the expression of ORFs without 5′ and 3′ UTR sequences or the modified wildtype ORF sequence with altered redundant codons for some amino acids thereby effectively removing RNAi targets and complementing the levels of knocked down gene expression. Although RNAi based reverse genetics approaches have become increasing popular in *C. reinhardtii*, it is not always successful in generating reliable mutations for a particular process of interest. The frequent problems that are encountered with RNAi based methods such
as dilution of knockdown effect due to off-targeting, silencing of artificial constructs and hence the knockdown phenotype over time, different and sometimes irreproducible gradations of knockdown, absence of a strong phenotype even at lowered levels of a protein are also great deterreents in effective RNAi based knockdown in C. reinhardtii.

The current exploration of the zinc finger technology as a potential way to target genes for disruption as a more effective substitute to RNAi knockdown of gene expression is gradually attracting attention. This technique allows the use of hybrid proteins that are derived from the DNA binding domains of zinc finger proteins fused with the DNA cleavage domain of the endonuclease FokI, a type IIS endonuclease from the bacterium, Flavobacterium okeanokoites (Sugisaki and Kanazawa, 1989). The nuclease consists of two separate domains, an N-terminal DNA-binding domain and a C-terminal DNA-cleavage domain. The DNA-binding domain recognizes the non-palindromic sequence 5’-GGATG-3’ while the catalytic domain cleaves double-stranded DNA non-specifically at a fixed distance of 9 and 13 nucleotides downstream of the recognition site (Li et al., 1992). The fact that the FokI nuclease has a small DNA recognition domain it uses to recognize its target DNA and the fact that it functions as a dimer in solution helps the replacement of the two DNA recognition domains of each of its monomeric sequences with two target sequences flanking a portion of the C. reinhardtii gene being targeted. This ensures that the hybrid zinc finger nuclease sequences when expressed in a C. reinhardtii cell should be able to direct the nuclease to the appropriate target, a portion of which will then be cleaved thereby leading to a mutation in the target gene. Several such nuclease constructs targeting different portions of the gene can be made, to ensure thorough and complete knockdown of gene expression. Although it sounds a very effective mechanism on paper the actual success of the process in C. reinhardtii is yet to be validated. The problems of complexity
of the ZFN construct design, effective transgenic expression of randomly inserted constructs and the still lurking problems of decreased specificity of targets have plagued the success of this process in *C. reinhardtii* (laboratory of Dr. Donald Weeks, unpublished data).

Recently, the use of TAL effector proteins from bacterial plant pathogens like *Xanthomonas* sp. is also gaining some popularity in targeting specific gene sequences (Li *et al*., 2011). They help overcome the lack of specificity sometimes posed by artificial zinc finger nucleases by the presence of their modularly constructed DNA-binding domains that provide more sequence specificity when replaced with target sequences from a *C. reinhardtii* target gene. However, the difficulty of construction of these artificial TAL effectors and their overall effectiveness in *C. reinhardtii* still requires a lot of standardization before they become more widely exploited in reverse genetics studies.

Another reverse genetics approach called Targeting Induced Local Lesions In Genomes or TILLING manages to generate a large number of allelic mutations mostly chemically or irradiation induced in target genes. Relatively fewer mutants can encompass mutations across the genome when compared to insertional mutants making it a high-throughput process of mutant generation. However, the point mutations often generated in this manner miss critical amino acid residues leading to silent mutations or a gradation of phenotypes depending on the mutation. Because each mutant often has mutations in more than one gene, several stages of backcrossing and segregation analysis needs to be performed before a particular phenotype can be assigned to a particular mutated gene. This involves a consumption of time and resources that makes it a less favorable choice amongst *C. reinhardtii* researchers. Although the presence of a tagged mutant library for all *C. reinhardtii* genes would be very beneficial to the large number of researchers working with this model alga, problems of preservation of such mutant cultures require a large
investment of human and technological resources that are unavailable to the community. Therefore, advancements in the quick generation of interesting mutants by individual laboratories pertaining to their areas of interest might still be able to provide future researchers with a decentralized but still largely accessible collection of mutant strains.

**Insertional mutagenesis as a tool for a combined forward and reverse genetics approach:**

This chapter summarizes the progress made with a small-scale attempt at generating *C. reinhardtii* mutants lacking either previously identified or novel transport proteins that might play a role in carbon acquisition in the *C. reinhardtii* CCM. Using a combination of forward genetics and a PCR based reverse genetics approach (Gonzales-Ballester et al., 2011) a few mutants with disrupted transporter genes were identified (Figure 6.1). This mutagenesis project has revealed a few mutants whose functions need to be better characterized in the context of the *C. reinhardtii* CCM and paves the way for the generation of more mutants to further attempts at targeting the entire *C. reinhardtii* genome. The current *C. reinhardtii* CCM model emphasizes the role of bicarbonate transporter proteins to facilitate the transport of the charged bicarbonate anion across the plasma membrane, chloroplast membrane and thylakoid membrane barriers to the actual site of dehydration by the key carbonic anhydrase, CAH3 in the thylakoid lumen. Although, several candidates exist for this possible role in both the plasma and chloroplast membrane locations, the thylakoid membrane bicarbonate transporter still eludes us. The generation and phenotypic screening of more insertional mutants might identify possible bicarbonate transporter/transporters located on the thylakoid membrane that are the missing puzzle pieces of the current CCM carbon acquisition model.
Figure 6.1 A schematic representation of the two approaches used in the insertional mutagenesis screen. The *C. reinhardtii* cells were transformed with the paromomycin cassette (1) and plated on paromomycin plates (2) for the selection of paromomycin resistant colonies (3). The selected transformants were then plated on grids (4) and used for the forward and reverse genetic screens. In the reverse genetics strategy, DNA was extracted from the transformants (5), pooled together and target gene-specific primers used for a PCR-based screening for the disruptive insert (6 and 7). This leads to the identification of the single colony carrying a disruption in a gene of interest (8). In the forward genetics strategy, transformants were screened for a growth deficiency under low CO₂ (9) and techniques like iPCR used (10) to determine the exact gene disrupted by the insert in the phenotypically interesting transformants.
Results

The general strategy for mutant selection:

This mutagenesis screen involved both a PCR based reverse genetic screening (Gonzales-Ballester et al., 2012) to identify insertions in targeted genes as well as a phenotype based forward genetic screening to identify insertions in yet unidentified genes with potential influence on the proper functioning of the CCM (Figure 6.1). The combined application of these two different approaches to more than 20,000 odd transformants that were generated as part of this screen increased our chances of maximizing the number of mutants of potential interest.

The choice of insert for the mutagenesis screen:

The selective marker chosen for this screen (the AphVIII gene for resistance to paromomycin) emulated the already published PCR-based screen by Gonzales-Ballester et al. (2012) with only a slight modification. Unlike their screen, we retained the RbcS2 terminator at the end of Hsp70-RbcS2 promoter driven AphVIII gene cassette. This was done to prevent the occurrence of fusion proteins due to the non-termination of transcription in disrupted genes as well as provide a protective buffer to the 3’ end of the linear insert from DNA shearing losses. The paromomycin cassette was digested out of the vector pSL18 (Depege et al., 2003) using the enzymes KpnI and Xho1 (see Figure. 6.2) and used for transforming low CO₂ acclimated C. reinhardtii cells of the cell wall-less strain, D66(nit2’, cw15, mt+).

The selection of target genes for the mutagenesis screen:

The criteria for selection of target CCM genes for this mutagenesis screen took into account the following two considerations: 1) Known genes that were previously identified as
Figure 6.2 A diagram of the AphVIII gene cassette used in generating transformants. The cassette was digested out of the pSL18 vector background, using the restriction enzymes Kpn1 and Xho1. The AphVIII gene is flanked by the C. reinhardtii (HSP70 + RbcS2) dual promoter and the RbcS2 terminator. This gene is responsible for conferring paromomycin resistance to transformants expressing the protein. The primers RB1 and RB2 were used in the PCR-based screen in combination with gene specific primers.

putative bicarbonate or small anion transporters 2) Unknown genes that showed an upregulation of expression under low CO\(_2\) and also encoded transmembrane proteins with often predicted transport function not necessarily of only carbon species. The publication of RNASeq data (Fang et al., 2012; Brueggeman et al., 2012) identifying a large number of uncharacterized genes that show a strong upregulation under low CO\(_2\) conditions together with regulation by the CCM transcriptional regulator gene CIA5 in most cases, helped us in our selection of unknown genes.

The selected candidates were included in our mutagenesis screen with the hope that it might lead to the potential unearthing of novel components involved in the proper functioning of the CCM.

On account of a special interest in unearthing newer C\(_i\) transporter proteins (a potential bicarbonate transporter located on the thylakoid membrane for example), the RNASeq datasets were searched for potential transporter genes that showed transcriptional upregulation under low CO\(_2\) conditions. The protein sequences were run through prediction programmes to determine the
number of transmembrane helices and possible membrane destinations as indicated by sequences of putative signal peptides. A total of 71 genes were selected for the screen with nearly 22 genes encoding transmembrane proteins with either a known or suspected role in ion transport. These are summarized in Table 6.1 with the putative transport proteins circled in red. Also included in this list are proteins without transmembrane domains (Table 6.1, circled in yellow) for instance, the LCIB family of proteins, that have speculated roles in forming complexes that facilitate the uptake of inorganic carbon by the chloroplast and final transfer into the thylakoid lumen.

**Designing and testing primers for the target genes:**

Gene sequences from the most recent *C. reinhardtii* sequence database (Phytozome v4;http://www.phytozome.net/search.php?method=Org_Creinhardtii) were used to design primers for the target genes. Forward primers were designed at regular intervals of 1kb (labeled F1, F2…Fₙ where n refers to the last primer at the 3’ end of the gene) with the first primer/F1 at least 200 bp upstream of the 5’UTR to detect insertions that disrupt the 5’UTR. The reverse primer sequences were chosen similarly in the opposite direction, starting with R1, being at least 200 bp downstream of the 3’ UTR. Since 5’ and 3’ UTRs flank all *C. reinhardtii* genes, they have long been suspected to play potential roles in transcriptional regulation. Hence, we wanted to be able to detect insertions in both UTR regions. Primers were chosen that had similar melting temperatures (close to 60°C) with low probabilities for homo/heterodimerizations. Primers were tested in pairs (F+R), starting from the 5’ end of the gene using genomic DNA from the wild type strain, D66 (Fig. 6.3). Primers were redesigned when they failed to generate the right sized fragments in these test screens. We made sure that the insert primers; RB1 and RB2 (Gonzales-Ballester et al., 2012) for the AphVIII gene did not give any bands due to non-specific binding at
Table 6.1 List of genes chosen for the mutagenesis screen. Potential transporter genes circled in red. Soluble proteins with purported roles in C\textsubscript{i} uptake facilitation are circled in yellow. The triangles indicate the identification of the mutant in which this gene locus is disrupted by the \textit{AphVIII} insertion. The red triangle (▲) indicated that the insertion was found in the protein coding region of the gene. The green triangle (▲) indicated a 5'UTR insertion and the blue triangle (▲) indicated a 3'UTR insertion. The genes are listed in the order of the chromosome locations. The data of Cluster and Fold Change (F.C.) information were from Brueggeman et al. (2012). EST information was based on the data from the Chlamydomonas genome site (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html).
Table 6.1 List of genes chosen for the mutagenesis screen.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Name</th>
<th>Chromosome Location</th>
<th>Cluster</th>
<th>F.C.</th>
<th>EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>510853</td>
<td>NAR1.6</td>
<td>chromosome_1:2015037-2018721 (+)</td>
<td>n/a</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>511233</td>
<td>CGL41/RbcX</td>
<td>chromosome_1:4292836-4296074 (−)</td>
<td>n/a</td>
<td>3.34</td>
<td>~40</td>
</tr>
<tr>
<td>511332</td>
<td>4660.C</td>
<td>chromosome_1:4886649-4894847 (−)</td>
<td>9</td>
<td>n/a</td>
<td>13</td>
</tr>
<tr>
<td>510680</td>
<td>ABCt</td>
<td>chromosome_1:733666-752174 (−)</td>
<td>15</td>
<td>5.79</td>
<td>16</td>
</tr>
<tr>
<td>518934</td>
<td>HLA3</td>
<td>chromosome_2:3208217-3215491 (+)</td>
<td>15</td>
<td>5.33</td>
<td>~40</td>
</tr>
<tr>
<td>519635</td>
<td>LC19</td>
<td>chromosome_2:7341013-7348511 (−)</td>
<td>n/a</td>
<td>3.36</td>
<td>~50</td>
</tr>
<tr>
<td>519637</td>
<td>LC19 parologue</td>
<td>chromosome_2:7356802-7362006 (+)</td>
<td>8</td>
<td>2.08</td>
<td>20</td>
</tr>
<tr>
<td>519760</td>
<td>LCR1</td>
<td>chromosome_2:8320304-8324302 (−)</td>
<td>15</td>
<td>5.72</td>
<td>16</td>
</tr>
<tr>
<td>520703</td>
<td>LC1H</td>
<td>chromosome_3:1912474-1915352 (−)</td>
<td>15</td>
<td>11.57</td>
<td>~100</td>
</tr>
<tr>
<td>520827</td>
<td>PGP1</td>
<td>chromosome_3:2663806-2668960 (+)</td>
<td>n/a</td>
<td>n/a</td>
<td>~30</td>
</tr>
<tr>
<td>520884</td>
<td>GYX1</td>
<td>chromosome_3:2940092-2943406 (+)</td>
<td>n/a</td>
<td>n/a</td>
<td>2</td>
</tr>
<tr>
<td>520458</td>
<td>MTase</td>
<td>chromosome_3:521758-525622 (+)</td>
<td>15</td>
<td>4.51</td>
<td>4</td>
</tr>
<tr>
<td>521673</td>
<td>RDRP</td>
<td>chromosome_3:7132224-7143998 (+)</td>
<td>8</td>
<td>5.22</td>
<td>1</td>
</tr>
<tr>
<td>521712</td>
<td>NAR1.3</td>
<td>chromosome_3:7664317-7672585 (−)</td>
<td>3</td>
<td>n/a</td>
<td>~20</td>
</tr>
<tr>
<td>522029</td>
<td>THB4</td>
<td>chromosome_4:1182047-1186095 (−)</td>
<td>8</td>
<td>n/a</td>
<td>9</td>
</tr>
<tr>
<td>522030</td>
<td>THB3</td>
<td>chromosome_4:1186937-1191795 (−)</td>
<td>n/a</td>
<td>n/a</td>
<td>17</td>
</tr>
<tr>
<td>522119</td>
<td>CCP2</td>
<td>chromosome_4:1804462-1807550 (−)</td>
<td>14</td>
<td>6.9</td>
<td>~40</td>
</tr>
<tr>
<td>522120</td>
<td>LC1D</td>
<td>chromosome_4:1807721-1810885 (+)</td>
<td>14</td>
<td>6.48</td>
<td>1</td>
</tr>
<tr>
<td>522126</td>
<td>CAH1</td>
<td>chromosome_4:1849022-1853330 (−)</td>
<td>15</td>
<td>9.38</td>
<td>~150</td>
</tr>
<tr>
<td>522129</td>
<td>LC1E</td>
<td>chromosome_4:1874916-1877584 (−)</td>
<td>15</td>
<td>7.56</td>
<td>0</td>
</tr>
<tr>
<td>522130</td>
<td>CCP1</td>
<td>chromosome_4:1877695-1880829 (−)</td>
<td>15</td>
<td>10.99</td>
<td>~50</td>
</tr>
<tr>
<td>521926</td>
<td>SRC</td>
<td>chromosome_4:389097-400711 (−)</td>
<td>n/a</td>
<td>5.28</td>
<td>0</td>
</tr>
<tr>
<td>521927</td>
<td>SRC</td>
<td>chromosome_4:401538-413146 (−)</td>
<td>8</td>
<td>3.07</td>
<td>0</td>
</tr>
<tr>
<td>522486</td>
<td>Guanylate cyclase</td>
<td>chromosome_5:1403264-1414228 (−)</td>
<td>15</td>
<td>7.41</td>
<td>3</td>
</tr>
<tr>
<td>522626</td>
<td>CAH9</td>
<td>chromosome_5:2467646-2468704 (−)</td>
<td>13</td>
<td>n/a</td>
<td>6</td>
</tr>
<tr>
<td>522732</td>
<td>CAH4</td>
<td>chromosome_5:3320139-3322687 (−)</td>
<td>15</td>
<td>9.34</td>
<td>200-300</td>
</tr>
<tr>
<td>522733</td>
<td>CAH5</td>
<td>chromosome_5:3324418-3327142 (−)</td>
<td>15</td>
<td>9.34</td>
<td>200-300</td>
</tr>
<tr>
<td>523044</td>
<td>CEM1</td>
<td>chromosome_6:1544794-1547328 (−)</td>
<td>16</td>
<td>n/a</td>
<td>~20</td>
</tr>
<tr>
<td>523284</td>
<td>PGP3</td>
<td>chromosome_6:2792436-2796394 (−)</td>
<td>n/a</td>
<td>n/a</td>
<td>10</td>
</tr>
<tr>
<td>523507</td>
<td>LC123</td>
<td>chromosome_6:3840357-3845935 (−)</td>
<td>15</td>
<td>10.59</td>
<td>15</td>
</tr>
<tr>
<td>523557</td>
<td>RHP1</td>
<td>chromosome_6:4082786-4087794 (−)</td>
<td>3</td>
<td>n/a</td>
<td>35</td>
</tr>
<tr>
<td>536268</td>
<td>RHP2</td>
<td>chromosome_6:4088321-4094462 (−)</td>
<td>n/a</td>
<td>n/a</td>
<td>4</td>
</tr>
<tr>
<td>523796</td>
<td>MITC11</td>
<td>chromosome_6:5558933-5562561 (−)</td>
<td>8</td>
<td>2.94</td>
<td>2</td>
</tr>
<tr>
<td>524046</td>
<td>LC1C</td>
<td>chromosome_6:7141544-7144642 (−)</td>
<td>14</td>
<td>4.52</td>
<td>~100</td>
</tr>
<tr>
<td>524076</td>
<td>NAR1.2</td>
<td>chromosome_6:7279856-7282824 (−)</td>
<td>15</td>
<td>12.05</td>
<td>~60</td>
</tr>
</tbody>
</table>
Table 6.1 continued.

<table>
<thead>
<tr>
<th>August ID</th>
<th>Name</th>
<th>Chromosome Location</th>
<th>Cluster</th>
<th>F.C.</th>
<th>EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>524387</td>
<td>Adenylyl cyclase</td>
<td>chromosome_7:1263093-1265027(+)</td>
<td>15</td>
<td>5.02</td>
<td>~30</td>
</tr>
<tr>
<td>524677</td>
<td>NAR1.4</td>
<td>chromosome_7:3197820-3202384(+)</td>
<td>n/a</td>
<td>n/a</td>
<td>7</td>
</tr>
<tr>
<td>526207</td>
<td>CAH8</td>
<td>chromosome_9:3001549-3007313(+)</td>
<td>11</td>
<td>n/a</td>
<td>6</td>
</tr>
<tr>
<td>526296</td>
<td>ECA1</td>
<td>chromosome_9:3621094-3628678(+)</td>
<td>n/a</td>
<td>6.76</td>
<td>0</td>
</tr>
<tr>
<td>526295</td>
<td>ECA1</td>
<td>chromosome_9:3651805-3663233(-)</td>
<td>n/a</td>
<td>4.96</td>
<td>~30</td>
</tr>
<tr>
<td>526316</td>
<td>NAR1.4</td>
<td>chromosome_9:3796483-3799657(+)</td>
<td>n/a</td>
<td>n/a</td>
<td>2</td>
</tr>
<tr>
<td>509757</td>
<td>Acetyltransferase</td>
<td>chromosome_10:1250148-1255142(+)</td>
<td>15</td>
<td>7.16</td>
<td>11</td>
</tr>
<tr>
<td>509959</td>
<td>LCI5</td>
<td>chromosome_10:2491110-2493454(+)</td>
<td>8</td>
<td>2.3</td>
<td>~40</td>
</tr>
<tr>
<td>144255</td>
<td>Cia6</td>
<td>chromosome_10:2640236-2642514(-)</td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>509989</td>
<td>PGP2</td>
<td>chromosome_10:2669876-2671880(+)</td>
<td>n/a</td>
<td>n/a</td>
<td>22</td>
</tr>
<tr>
<td>510019</td>
<td>CGL2/CID11</td>
<td>chromosome_10:2809450-2814524(+)</td>
<td>15</td>
<td>5.09</td>
<td>20</td>
</tr>
<tr>
<td>510111</td>
<td>BOR1</td>
<td>chromosome_10:3307458-3316722(+)</td>
<td>16</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>510414</td>
<td>GPX5</td>
<td>chromosome_10:5434433-5436481(-)</td>
<td>n/a</td>
<td>n/a</td>
<td>~50</td>
</tr>
<tr>
<td>512404</td>
<td>MHX</td>
<td>chromosome_11:2142976-2152044(+)</td>
<td>14</td>
<td>3.43</td>
<td>15</td>
</tr>
<tr>
<td>512520</td>
<td>CAH6</td>
<td>chromosome_12:210288-213222(+)</td>
<td>n/a</td>
<td>n/a</td>
<td>~30</td>
</tr>
<tr>
<td>513120</td>
<td>Chromate Trans</td>
<td>chromosome_12:3365716-3370610(+)</td>
<td>8</td>
<td>4.13</td>
<td>4</td>
</tr>
<tr>
<td>513361</td>
<td>RMT2</td>
<td>chromosome_12:4690021-4695579(-)</td>
<td>n/a</td>
<td>n/a</td>
<td>8</td>
</tr>
<tr>
<td>513715</td>
<td>NAR1.5</td>
<td>chromosome_12:6839303-6844411(-)</td>
<td>15</td>
<td>n/a</td>
<td>17</td>
</tr>
<tr>
<td>513788</td>
<td>HP</td>
<td>chromosome_12:7237733-7239117(+)</td>
<td>15</td>
<td>10.62</td>
<td>0</td>
</tr>
<tr>
<td>513839</td>
<td>CGL2</td>
<td>chromosome_12:7473725-7477099(+)</td>
<td>15</td>
<td>2.59</td>
<td>2</td>
</tr>
<tr>
<td>513843</td>
<td>ABC1</td>
<td>chromosome_12:7496709-7504083(+)</td>
<td>n/a</td>
<td>1.34</td>
<td>3</td>
</tr>
<tr>
<td>513967</td>
<td>LCI6</td>
<td>chromosome_12:8266638-8269582(+)</td>
<td>n/a</td>
<td>2.04</td>
<td>~20</td>
</tr>
<tr>
<td>515107/515108</td>
<td>CAH7</td>
<td>chromosome_13:6481814-6487302(-)</td>
<td>n/a</td>
<td>n/a</td>
<td>23</td>
</tr>
<tr>
<td>515280</td>
<td>THB2</td>
<td>chromosome_14:1124707-1127451(+)</td>
<td>2</td>
<td>n/a</td>
<td>2</td>
</tr>
<tr>
<td>515281</td>
<td>THB1</td>
<td>chromosome_14:1128937-1131301(+)</td>
<td>15</td>
<td>n/a</td>
<td>~40</td>
</tr>
<tr>
<td>515848</td>
<td>ORF158</td>
<td>chromosome_15:1581786-1589614(-)</td>
<td>14</td>
<td>10.93</td>
<td>0</td>
</tr>
<tr>
<td>516263</td>
<td>RMT1</td>
<td>chromosome_16:1739402-1744326(+)</td>
<td>n/a</td>
<td>n/a</td>
<td>17</td>
</tr>
<tr>
<td>516273</td>
<td>NTF2</td>
<td>chromosome_16:1807672-1810726(+)</td>
<td>14</td>
<td>7.35</td>
<td>1</td>
</tr>
<tr>
<td>516290</td>
<td>Bestrophin</td>
<td>chromosome_16:1921202-1924200(-)</td>
<td>15</td>
<td>8.17</td>
<td>18</td>
</tr>
<tr>
<td>516308</td>
<td>3' part of LC11</td>
<td>chromosome_16:2022322-2026260(-)</td>
<td>n/a</td>
<td>2.23</td>
<td>~30</td>
</tr>
<tr>
<td>516309</td>
<td>5' part of LC11</td>
<td>chromosome_16:2027232-2031650(-)</td>
<td>14</td>
<td>7.25</td>
<td>~40</td>
</tr>
<tr>
<td>516770</td>
<td>PTAC17</td>
<td>chromosome_16:5072356-5074980(-)</td>
<td>15</td>
<td>7.62</td>
<td>24</td>
</tr>
<tr>
<td>517053</td>
<td>LCI31</td>
<td>chromosome_17:339215-342359(+)</td>
<td>14</td>
<td>4.09</td>
<td>17</td>
</tr>
<tr>
<td>517880</td>
<td>ORF158</td>
<td>chromosome_17:5553807-5565545(-)</td>
<td>8</td>
<td>9.33</td>
<td>0</td>
</tr>
<tr>
<td>520182</td>
<td>Na+/H+ t</td>
<td>scaffold_24:124886-139650(+)</td>
<td>8</td>
<td>4.67</td>
<td>1</td>
</tr>
<tr>
<td>522781</td>
<td>HP</td>
<td>scaffold_59:20576-24870(+)</td>
<td>15</td>
<td>11.62</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure 6.3 Position of the forward (F1, F2, F3 and F4) and reverse primers (R1, R2, R3 and R4) in a sample target gene, MITC11. The exons are shown as yellow arrows, the introns as black lines and the 5’UTR and 3’UTRs are marked as red boxes.

an annealing temperature of 60°C in a PCR reaction. This was checked by using RB1 and RB2 with either D66 or a strain with an AphVIII gene insertion as DNA templates.

Low CO₂ acclimated and phototrophically grown cells of the cell wall less strain, D66, were harvested at the mid-log phase of growth and used for transformation via electroporation. Around 100 ng of the insert DNA was used for each transformation event and resulted in the retrieval of a minimum of 100 paromomycin resistant colonies when plated on Tris-Acetate-Phosphate (TAP) medium supplemented with 5 µg/mL of paromomycin. These transformants were plated on TAP plates carrying a grid of 180 colonies and used for long-term maintenance of cultures and extraction of DNA for the PCR reactions of the reverse genetic approach.

**The process of generation and maintenance of transformants:**

Duplicates of these colony grids on Minimal medium (MIN) plates were placed in a low CO₂ chamber for detection of growth deficiencies for the forward genetic screen. Master plates of the 180 grid colonies were plated on TAP and stored at 4°C for a period of up to 6 weeks.
The reverse genetic screening via PCR:

Isolation of potential mutants: The reverse genetic screening via PCR to detect inserts of the AphVIII gene in target genes involved the following PCR steps:

Step I (preparing primer mixes): All the forward and reverse primers for each gene were mixed together in equal amounts to yield a forward and reverse primer cocktail referred to as F and R respectively. The genes were separated into three categories depending on gene length and hence the total number of forward and reverse primers and then assigned to three different researchers including the author. This approach ensured that each person had a consistent amount of primer cocktail to add to their PCR master mix easing the setup for the large number of PCR reactions that this screen entailed (Figure 6.4).

Step II (extraction and pooling of DNA): Each plate carrying a maximum of 180 transformants was used for the first round of DNA extraction leading to a single DNA pool. Five such DNA pools were used for generate one DNA superpool. The DNA from each single pool was run on an agarose gel to check for DNA yields and any large scale shearing or degeneration. The DNA from each pool was then used as a template for a PCR reaction using a set of primers for the generation of a 1Kb control fragment. In cases of failure of PCR, the DNA was re-precipitated with ethanol to clean up inhibitory residuals from the extraction process and subjected to dilution whenever needed. Once this process was completed, DNA from five individual pools was mixed in equal volumes. Once this process was completed, DNA from five individual pools was mixed in equal volumes to yield a DNA superpool (Figure 6.4).

Step III (1st round of PCR): The superpool DNA was used as a template for the first round of PCR. For each gene two PCR reactions one using the forward cocktail (F) and the reverse primer
Figure 6.4 A representation of the first 3 steps of the PCR-based mutagenesis screen. Step 1 shows the pooling of all forward primers into a single primer cocktail (F) and all the reverse primers into a single mix (R). Step II shows the extraction of DNA from each plate into single pools, 5 of which are pooled into a single superpool. These superpools were used as templates for PCR screening, using the forward and reverse primer cocktails for each target gene (Step III).

cocktail (R) in conjunction with the AphVIII specific RB1 primer were carried out (Figure 6.5).
The PCR conditions allowed for an annealing temperature of 58°C and an extension time to allow for the synthesis of roughly 3kb of DNA. When the PCR reactions for each particular gene from several different superpools were run out in parallel on an agarose gel, it was easy to differentiate the background bands (resulting out of non-specific binding of primers and other PCR artifacts) recurring in all the superpools from those that are unique to a particular superpool.
Once a unique band (with either F or R) has been identified in one or more superpools for a particular gene (Figure 6.5), those particular superpools are then subjected to another round of PCR using RB2 (another AphVIII primer upstream of RB1) to check for the recurrence of the RB1 bands (Figure 6.6A). Since RB1 and RB2 are closely placed on the 3’end of the AphVIII gene the sizes of the bands are pretty close on the agarose gel. Only in cases where the RB1 bands from a particular superpool DNA could be reproduced with RB2, were they cross-checked with the next round of PCR. In a few cases, bands that seemed unique were irreproducible (probably artifacts) with RB1 itself. In the rare cases, when some unique bands could be reproduced with RB2, the subsequent PCR follow-up was carried out.

**Step IV (2nd round of PCR):** The superpool DNA that yielded a unique band with RB1/ RB2 was then subjected to a follow-up PCR to determine which particular primer from either the forward or the reverse primer cocktail was responsible for the unique band (Figure 6.6 C). In order to do this, each individual primer is paired up with RB1 and subjected to PCR using the same superpool DNA as template. The particular gene specific primer that was identified at this point also gave a possible location of the insert within the target gene, provided the fragment generated with it was not a PCR artifact.

**Step V (3rd round of PCR):** Once the gene-specific individual primer that together with RB1/RB2, managed to reproducibly amplify a portion of the insert was identified, the next step of PCR was then undertaken. This round of PCR was geared towards the identification of the DNA pool within the larger superpool that the colony harboring the insert belonged to. In order to do this, the individual pools of DNA (5 in number) were used as a template for a PCR reaction involving the previously identified gene specific primer and RB1.
Figure 6.5 An example of the first round of PCR screening (Step III). Superpool #13, #14, and #15 were screened simultaneously using primer RB1. By comparing the signals among the three pools, as well as with previous pool PCR patterns, nine unique bands (highlighted with blue circles) for 10-R, 14-R from pool #13, 4-F, 22-R, 16-F from pool #14, 14-F, 15-R, 19-R, 25-F from pool #15 were further investigated. From this batch, the *RHP1* mutant was identified from pool #13 and the *516309* mutant was identified from pool #15. The PCR bands of *RPH1* and *516309* were highlighted with red squares.
Figure 6.6 An example of Step III (band confirmation) and Step IV (2nd round of PCR). A) RB2 confirmation of RB1 bands. The position of the internal primer RB2 (B) is shown within the paromomycin cassette. C) Step IV: 2nd round of PCR screening. Of the nine PCR bands, five could be reproduced by the RB2 primer (highlighted with the blue circles, and the RHP1 and 516309 were highlighted with the red squares). The 2nd round of PCR was performed to further identify which single primer from the primer mix was responsible for the band. In the RHP1 PCR set (consisting of the reverse primer mix (R), single reverse primer R1, R2, R3, R4, R5), the band around 400-bp using primer R5 corresponded to the original band from the primer mix R. The same situation for 516309 PRC set, in which the R5 corresponded to the band seen in the primer mix and R4 PCR product was around 1-kb bigger than the R5 PCR product.
In a successful reaction only one of the individual pools making up a DNA superpool would yield the same-sized fragment as seen in the earlier round of PCR. Since each single pool corresponds to the DNA from roughly 180 colonies on a single TAP plate, the next PCR step is the most crucial to the entire screen and might lead to the colony carrying the gene-specific insert. Figure 6.7 A and 6.7 B gives two examples of a successful 3rd round of PCR.

**Step VI (4th round of PCR):** This step involves the final identification of the individual colony that is carrying a particular gene specific insertion. In order to achieve this, the DNA from roughly 10 colonies at a time (from a 180 grid plate) are pooled to be used as a template for PCR, using the same primer pair (gene-specific primer +RB1) as used in the previous two steps (Figure 6.8 A and 6.8 B). Since a larger number of DNA extractions are involved at this stage, a cruder extraction method was used where cells are merely boiled in the presence of EDTA to release enough DNA for a PCR reaction. Once a smaller sub-section of 10 colonies on a 180-grid plate is identified as carrying the gene-specific insertion in question the final identification of the potential mutant colony becomes fairly easy. This is the last round of the bulk PCR steps and when successful results in the identification of the single colony on the plate that carries the potentially disruptive insert in a target gene.

**Determination of potential mutants**

**Step I (verification of the insert in a target gene):** Once a potential insert-carrying colony is isolated the next logical step involves the confirmation of the presence and position of the insert within the target gene. In order to confirm the presence of the insert within the gene identified through the PCR screen, DNA sequencing was followed by a BLAST alignment of the sequences. The PCR fragment generated with the help of the gene specific primer and the insert
primer RB2 was concentrated, cleaned up and sent for sequencing. If the sequences retrieved at this stage could be aligned with a portion of the target gene, the PCR fragment was considered a portion of the actual insert and not a PCR artifact. Once the presence of the insert within a target gene was confirmed, the next step involved confirming the exact position of the insert within the gene.

Figure 6.7 Identification of single pools (Step V, 3rd round of PCR). The presence of the RHP1 and 516309 bands of interest in DNA pool #13 and #15 was verified by single primers and the 3rd round of PCR (Step V) was performed to identify the exact individual plate that contains the mutants. A) The RHP1-R5 and RB1 primer pair was used to amplify the 400-bp PCR product from the 180-colony plate #109 from DNA pool #13. B) Similarly, the 516309-R5 and RB1 primer pair was used to amplify the 800-bp PCR product from the 180-colony plate #134 from DNA pool #15. The presence of the RHP1 mutation in plate #109 was further verified by the 1.4-kb PCR product using RHP1-R4 primer.
Figure 6.8 Identification of single mutant colonies (Step VI, 4th round of PCR). To determine the single mutant colony from the 180 colonies, every ten colonies were pooled together for PCR analysis. A) The RHP1 insert was found to be among the 10 colonies (#141 to #150) as evident by the identical band observed in both the test sample (highlighted by the red square) and the control. B) The 516309 insert was found to be among the 10 colonies (#91 to #100) as evident by the identical band observed in both the test sample (highlighted by the red square) and the control. For both PCRs, the amplification of the 1-kb fragment from NAR1.2 was used as a positive control.

Step II (actual position of the insert within a target gene): This step involves the determination of the exact position of the insert disrupting the target gene. It also helped in determining exactly how much of the insert was part of the disruption. Since the scrambling of sequences (including large deletions) are not unusual around the insert, the sequences of regions upstream and downstream of the insert were needed to determine not only the exact position of the insert within the gene but also confirm the 5’ and 3’ ends of the inserted DNA. To achieve this, the individual colony carrying the insert, was streaked out on a TAP+paromomycin plate to isolate 10 different colonies that were all tested for the presence of the insert. This was done to weed out any non-insert carrying cells that might be cohabitating within the initial colony and to get a genetically homogenous insert-carrying colony. Of the 10 colonies tested about 5 were maintained in a master culture until further experimentation. DNA from one such isolated single
colony was used as a template for two PCR reactions. The first PCR reaction involved a primer at the 5’ end of the insert (about 200 bp away from the 5’ end of the *Hsp70-RbcS2* promoter) and a corresponding gene-specific forward or reverse primer as applicable. The second PCR reaction involved a similar set-up, this time corresponding to the 3’ end of the insert. The retrieved sequences gave a clear indication of the position of the insert within each target gene and in most cases revealed very clean insertions into the target gene.

**Step III (determination of changes in target gene expression):** Once the position of the insert within the target gene is confirmed, the changes if any, brought to the expression of the target gene by the presence of the insert is then examined. It is this step that is the actual determinant of whether a transformant with an insert in a target gene can be dubbed as a ‘mutation’ within said gene. Using both semi-quantitative and quantitative RT-PCR, the expression levels of disrupted genes were measured using primer pairs designed within target gene sequences both upstream and downstream of the insert. The purpose of using two sets of primers was to make sure that the presence of the dual promoter that is part of the insert DNA does not influence the expression of the target gene region downstream of it, giving a false impression about the actual expression of a gene within the selected transformant. Since many of our insertions were in the UTR regions of genes, initial expectations for any of their presence affecting the expression of the genes were pretty low. However, since we have instances of insertions in both 3’ and 5’ UTR regions, a study of their effects on gene transcription if any, would provide some insight into the possible regulatory effect of these regions.

**Step IV (characterization of mutant phenotype):** Once, a mutant colony has been identified several preliminary phenotypic observations can be made. In our case this involved growth
studies at different levels of CO$_2$ and in case of the transporter genes, combining this with exposure to different pH levels (Figure 6.9). Studies are still ongoing in determination of

Figure 6.9 Growth phenotypes of some of the insertional mutants. Inserts disrupting the $RHP1$ gene ($\Delta RHP1$), a bestrophin encoding gene ($\Delta BEST$), the $MITC11$ gene ($\Delta MITC11$), the $LCI9$ gene ($\Delta LCI9$) and the three separate insertions in the $NAR1.3$ ($\Delta NAR1.3a$, $\Delta NAR1.3b$ and $\Delta NAR1.3c$). The possible mutants were studied for any growth deficiencies under different pH conditions.
the process. In order to facilitate the disruption of genes involved in the CCM, the transformation events in this screen were carried out in phototrophically growing mid-log phase cells and were coordinated to overlap with the window of most CCM gene activations. This was done with the hope that actively transcribed regions of the genome would possibly be more susceptible to insertions because of their open chromatin conformations. It was interesting to find that the PCR-based reverse genetic screening did not result in a considerable amount of mutants in the highly upregulated genes of the *C. reinhardtii* CCM. This could be the result of lethal phenotypes that affect survival rates of the transformed cells from the time of plating until the time of picking onto fresh plates which normally takes around 8-10 days. Although the transformants are plated on Tris-Acetate-Phosphate (TAP) plates that provide them with acetate as a source of carbon, depletion of acetate might occur in these plates especially with large amounts of transformants growing on them. It could also be speculated that actively transcribed areas might actually be protected against random insertions to protect the essential genes needed to acclimate to the low carbon stress experienced by the *C. reinhardtii* cells. Despite the fact that the growth conditions prior to transformation did not increase the number of mutations in key CCM genes, the pilot expression levels of all possible mutants isolates obtained thus far and the presence of growth deficiencies if any, under low CO₂ conditions and high pH. If any of the isolates thus far prove interesting for further investigations, the genetic linkage between the observed mutant phenotype (presence of insert + growth phenotype if any) and the paromomycin resistance phenotype would first be established. This is done in order to rule out other insertions contributing to the observed phenotype, since it is not uncommon to have more than one insert within the genome. After establishment of the genetic linkage of insert and mutant phenotype, further characterizations, both physiological and biochemical can be undertaken to establish the role of the mutated gene
within the *C. reinhardtii* cell, especially within its CCM. Also the complementation of the mutated gene with the wild-type copy in order to rescue the mutant phenotype is often a staple of such mutant studies.

**Summary of results**: The forward genetic screen yielded a total of 37 transformants showing a growth deficiency under low CO₂ conditions with a couple, showing an inability to grow under high CO₂ conditions (a portion of the screen is shown in Figure 6.10). The PCR-based reverse genetics approach yielded a total of 9 genes with insertions in them (Figure 6.11). Two of these 9 genes (*CAH8*, encoding a carbonic anhydrase and *NAR1.3*, encoding a putative Formate/Nitrite transporter), were found to be repeated targets for insertional disruption in different transformation events. Sometimes the insertions were in the same general vicinity throwing some light on potential insertional hotspots in the *C. reinhardtii* genome.

**Discussion**

Insertional mutagenesis in *C. reinhardtii* involves the random integration of the transformed piece of DNA into the *C. reinhardtii* genome, hopefully disrupting target genes in project did show some interesting results when it came to ‘insertional hotspots.’ It was interesting to note that of the 11 insertions detected in total (including transporter/non-transporter genes) with gene specific primers, nearly 70% were in the 5’/3’ UTR regions of the genes. The 3’UTR proved to be an insertional hotspot especially for the putative small anion transporter (belonging to the Formate/Nitrite Transporter family), *NAR1.3* that showed a total of three insertions in its 3’UTR area out of three independent transformation events. The 3’UTR region was especially large for this gene, around 2kb, which might also account for the frequency of insertion. The randomness of insertions of pseudogene constructs has not been studied in *C.*
Figure 6.10 Phenotypic screens of some transformants under high and low CO₂. Cells were grown on minimal media under high and low CO₂. Moderate growth deficiencies (marked by asterisks) and severe growth deficiencies (red circles) under low CO₂ are shown. Transformants showing growth deficiencies (circled in yellow) under high CO₂ were also identified. The wild type and mutant controls used for comparison of growth phenotypes on the plates were as follows, (a) wild-type D66 cells; (b) mutant pmp1: lacking the chloroplastic C₅ uptake facilitator, LCIB; (c) mutant cia5: lacking the CCM regulatory transcription factor, CIA5; and (d) cia3: lacking the thylakoid luminal carbonic anhydrase, CAH3.
although sometimes an insertional bias towards the endogenous promoter and terminator regions that overlap with the transforming DNA construct, have been noted in one study (Dent et al., 2005). However, there is no conclusive evidence for the existence of insertional hotspots so far. It will be interesting to see if any such bias remains in a much larger screen being carried out in the Moroney laboratory at present. The large numbers of insertions in the UTR regions often run the risk of not affecting the transcription levels of the target genes in any way unless they disrupt a transcription regulatory region within these UTRs. Most insertions

\[ \text{Figure 6.11 Summary of the PCR-based mutagenesis screen. This screen resulted in the identification of eleven insertional mutants within nine different genes. It should be noted here that only 4 of these nine genes encode proteins with transporter function out of which only RHP1 has previously been characterized. The relative locations of the mutated genes are depicted by triangles in the context of the chromosomes in } \text{C. reinhardtii}. \text{ The red triangle (▲) indicated that the insertion was found in the ORF region of the gene. The green triangle (▲) indicated a 5' UTR insertion and the blue triangle (▲) indicated a 3' UTR insertion. Three individual } NARI.3 \text{ mutants were identified, all of which had insertions in the 3' UTR region.} \]
in this pilot screen were clean insertions that did not cause any deletions close to the point of insertion or any shifts in the coding frame of the ORF. Although qualitative RT-PCR (qPCR) measurements should be carried out with primers in regions downstream and upstream of the insertions in genes (where it was possible to reverse transcribe up to those regions) to ensure that there is no misreading of transcription levels due to the insert-promoter driven increase in transcript levels downstream of insertions. In order to prevent the detection of inserts within 3′UTRs which may or may not lead to actual down regulation of transcription, the exclusion of primers that cover the 3′UTR is a suggested strategy for future screens. This will limit resources to the unearthing of possible loss-of-function mutations within the protein coding regions of genes. However, even insertions into 5′ and 3′UTRs could be used as targets for other reverse genetic approaches like RNAi mediated knockdown of expression. For instance we could possibly design a short hairpin or an amiRNA construct targeting the 5′/3′UTR embedded paromomycin sequence in a plasmid that carries a different selection marker like the bleomycin resistance gene. The loss of paromomycin resistance in the now bleomycin resistant insertional mutants could then be crosschecked for the transcriptional knockdown of the target gene. Successful RNAi knockdown mutants could hopefully be generated this way with an easier screenability. Also such mutants could be complemented by the introduction of the ORF without the previously targeted UTR region. Thus if future large scale screens fail to yield mutations in the some target genes from this pilot project an attempt at an RNAi based second reverse genetics approach should be considered.

Many of our target genes showed insertions in in their 3′ and 5′ UTRs. These insertions might be utilized in a future RNAi based reverse genetics approach. Some examples include the \textit{LCI11} gene encoding a putative bestrophin protein, \textit{MITC11} encoding a putative mitochondrial
carrier protein and NARI.3 encoding a putative formate/nitrite transporter. Bestrophin proteins are gaining interest currently as chloride channels as they show no similarity to previously characterized chloride channels and are believed to have paralogs in animals, plants and fungi (Tsunenari et al., 2003; Hartzell et al., 2008). The prokaryotic and plant bestrophins are very distantly related to their animal counterparts although there is conservation of few critical amino acid residues (Hartzell et al., 2008). Bestrophins were first identified in Caenorhabditis elegans and the absence of this chloride channel is believed to play a role in vitelliform macular dystrophy (VMD) in humans. Like most other chloride channels they are believed to play a role in ion conductance and maintenance of pH balance. In the mouse colon bestrophin channels are involved in the secretion of bicarbonate, which make them interesting in our study of this particular bestrophin protein.

This project gave us some insight into the effective generation and maintenance of insertional mutants on a large scale. The use of fungicides such as carbendazim, placement of cultures in dim light to ensure slow growth, etc are some of the ways that we could effectively maintain sterile cultures over long periods of time. Also optimization of certain factors like PCR reactions, polymerases used helped set the basis of a reliable and reproducible PCR-based screen. The knowledge accumulated from the logistics of this screen will help us carry out other successful mutagenesis screens in the future.

Endnotes

This results presented in this chapter are from a joint project carried out by the author and coworkers, Dr. Yunbing Ma and Dr. Nadine Jungnick.
CHAPTER 7
CONCLUDING CHAPTER OF THE THESIS

This thesis was the collective status reports of a few still ongoing projects that are striving to better understand the issue of the carbon acquisition process in the CCM of the eukaryotic green alga, *Chlamydomonas reinhardtii*. The ability of *C. reinhardtii* cells to acclimate successfully to sudden fluctuations of CO\(_2\) in their immediate vicinity and maintain an uncompromised photosynthetic status is largely due to the advantages bestowed by the different structural, functional and regulatory components of a functional CCM. When induced or activated under conditions of carbon stress, the key components of the CCM, mainly the carbonic anhydrases (CA) and the inorganic carbon (C\(_i\)) transporters play well coordinated roles in allowing the concentration of carbon in the gaseous form, CO\(_2\), as a substrate for Rubisco. The specific location and the possible functions of most of the active carbonic anhydrases have largely been worked out in *C. reinhardtii*. However, the investigation of the transport proteins that possibly facilitate the movement of the two different forms of inorganic carbon, the uncharged CO\(_2\) and the charged HCO\(_3^-\) ion, across the diffusive barriers posed by the plasma, chloroplast and thylakoid membranes is still ongoing. The existence of bicarbonate transporters has been reported in other well-characterized CCMs. The prokaryotic cyanobacterial CCMs have different kinds of high and low affinity bicarbonate transporters in the plasma membrane. Although the *C. reinhardtii* cell might be structurally different from cyanobacteria the need for these transport proteins are considered an integral part of its functional CCM. Although there are now a few instances of partially characterized transporters in the plasma membrane and chloroplast membrane locations, no thylakoid membrane transporter has yet been identified. Also, the synergistic roles both functionally and from a regulatory perspective are still to be
established for the multiple transport proteins so far identified on the plasma and chloroplast membranes.

Thus the issue of addressing the carbon acquisition piece of the CCM puzzle needed a two-pronged approach. Firstly, previously identified transport proteins needed to be functionally characterized and their membrane locations within the \textit{C. reinhardtii} cell determined. Secondly, newer candidates that could function as putative C\textsubscript{i} transporters and would help complete the bigger picture of the CCM carbon transport, needed identification and subsequent characterization. This thesis has summarized some of the efforts taken in these directions.

The characterization of the functional role of LCI1, a protein highly upregulated under low CO\textsubscript{2} conditions (Figure 4.4) was undertaken. LCI1 was successfully expressed under the control of an inducible promoter in a mutant background resulting in two transformants, C2 and E4 (Figure 4.6). The expression of LCI1 in C2 and E4 caused an increase in the C\textsubscript{i} uptake properties of the transformant levels even under high CO\textsubscript{2} when expression of most other CCM components is repressed (Figure 4.7). The localization of LCI1 to the plasma membrane (Figure 4.9 and Figure 4.10) further helped in elucidation of its function in the \textit{C. reinhardtii} CCM. LCI1 can now be implicated as playing a possible part in the C\textsubscript{i} uptake at the plasma membrane level of \textit{C. reinhardtii} cells under low CO\textsubscript{2} conditions. A similar approach could be taken with other transport proteins in determining their roles in the \textit{C. reinhardtii} CCM.

The study of the NAR1 family of proteins was part of the effort to characterize previous transporters of interest (NAR1.2) and newer candidates that could potentially function in the C\textsubscript{i} uptake aspect of the \textit{C. reinhardtii} CCM (the other NAR proteins). This unique family of six small anion transporters (sequence similarity to Formate/Nitrite transporters), predicted to be
directed to different membrane locations, seemed interesting candidates to investigate for their potential roles in the *C. reinhardtii* CCM. *NAR1.2* expression is repressed under high CO$_2$ conditions, when grown in the presence of ammonium as the nitrogen source in the medium (Figure 3.7). However this repression was not seen when cells were grown under high CO$_2$ with nitrate as the nitrogen source. Although *NAR1.2* expression was induced under low CO$_2$ with either nitrogen source, the extent of upregulation was much higher in ammonium than nitrate growth conditions (Figure 3.7). *NAR1.2* was localized to the chloroplast membrane (Figure 3.11) together with CCP1/CCP2. Its unique response to nitrogen sources and carbon dioxide levels in the media makes it an interesting candidate to study further. Although the other NAR proteins did not show any significant response to low CO$_2$ levels, ongoing efforts to localize them in the *C. reinhardtii* cell might identify a few other potential transporters of interest to the CCM.

The ability to generate a mutant lacking a protein and studying the resultant mutant phenotype in the context of a particular process often helps in pinpointing the functional role of the protein within the cell. Since no natural or laboratory generated mutants were available for the proteins that we aimed to study, for instance the NAR1.1 protein family and LCI1, we tried RNAi based techniques to knock down their expression. We achieved success with LCI1 using an amiRNA construct and managed to knock down protein levels in the wild-type strain. However, the lack of success with many of the other gene targets (although amiRNA constructs were not used in those cases) led us to the realization that, though efficient in some cases, the RNAi based methods cannot be solely relied on to generate mutations in all genes of our choice. However, the success of the amiRNA based knockdown of gene expression in the LCI1 RNAi mutants has given us hope that whenever necessary this technique could be tried in different combinations due to the efficacy of knock down and the stability of the mutants (resisting
endogenous silencing for more than a year). As evident from our LCI1 knockdown experiments, knocking down a single transporter at a specific membrane location often does not produce a distinct phenotype. Seeing how there are probably more than one transport protein facilitating the transport of bicarbonate anions at one membrane location, it could be speculated that the issue of redundancy in function could be the reason for the lack of a distinct mutant phenotype under CCM conditions. In Arabidopsis, the use of tandem amiRNA constructs has often been successfully used in knocking down more than one target gene. A similar approach could be undertaken in generating co-knockdown RNAi mutants of transporters located on the same membrane with the hope of generating more prominent $C_i$ uptake phenotypes. Such multiple knockdown mutants might also help understand the coordination and interactions, if any, between transporters on the same or a different membrane location. A small hairpin construct with conjoined target areas for LCI1 and NAR1.2 was tried without success. This points to the need to try out more than one construct for any particular knock-down experiment whether targeting a single gene or more than one target gene at a time. The lessons that we have learned from our various attempts at RNAi based reverse genetics approaches are that the selection of target, design of the construct, and screening for the successful knockdown mutants often have to be tailored to the particular genes being targeted. Whereas some genes are readily targeted for a knock down others might be more resistant to such attempts. When designing constructs for a singular or tandem knockdown of more than target gene, it is useful to have different regions of the mRNA targeted to identify a more effective target region. The use of 5’ and 3’ UTR regions are often useful targets for two specific reasons. In C. reinhardtii, the UTR regions often provide the most gene specificity when compared to the coding regions and are hence good targets for gene specific silencing. Also successfully generated RNAi mutants can be complemented and
hence ‘rescue of mutant phenotype’ attempted by using cDNA constructs without the target UTR regions. The success of many of the artificial knockdown constructs also depends on their active transcription and prevention of silencing within the *C. reinhardtii* cell. The use of strong promoters with enhancer regions, like the Rubisco small subunit intronic enhancer region, could be used for better transcriptional efficiencies. Also the use of inducible promoters (for example, the nitrate-inducible *NIA1* promoter) especially when targeting more than one important transporter at the same membrane location might be good to allow for ‘conditional lethality’ and thereby prevent the loss of useful mutants that may not be able to withstand the loss of essential transport functions. A frequent issue with RNAi mutant generation has been the efficacy of a large scale screening mechanism. Since the knockdown of a single protein like LCI1 fails to produce a growth phenotype under low CO₂ conditions, large scale screening of transformants in such cases often needs other techniques like Western blotting. The existence of a good antibody against LCI1 allowed us to screen as many as 52 transformants (limited by the lanes available on a SDS-PAGE gel apparatus) at one go. However in cases where a reliable antibody is absent, other strategies have to be adopted for successful screening for mutants with reduced mRNA levels. For instance, an existing Ble-GFP strain could be used as a transforming strain for RNAi construct designed for co-knockdown of GFP. Transformants that lacked GFP (detectable through fluorescence microscopy or Western blots using commercially available GFP antibodies) and bleomycin resistance could then be screened for reduction in mRNA levels of the target gene by qRT-PCR.

The determination of the membrane location of a putative transporter protein is often equally important in predicting its functional role within the cell. Peptide antibodies were raised against proteins like NAR1.2, LCI1 and CCP1 in order to localize them within the *C. reinhardtii*. 
The successful enrichment of LCI1 to the plasma membrane and NAR 1.2 and CCP1 to the chloroplast membrane was achieved. The location of the GFP-tagged LCI1 protein to the plasma membrane was also shown through confocal microscopy although misdirection of the protein to the nuclear envelope was also seen. The use of epitope tags (3xHA, GFP) to localize proteins that did not have specific antibodies, for instance the NAR family of proteins was also attempted. These tags would allow for the detection of the successfully localized proteins both microscopically and on a Western blot of membrane fractions. However, the detection and localization of these proteins was marred by the low expression of the artificial constructs. The use of a variety of stronger promoters and the replacement of endogenous introns of the target genes with RbcS2 enhancer elements are few of the approaches being taken to overcome this problem.

The unreliability and lack of reproducibility with RNAi based reverse genetics approaches to successfully generate mutant phenotypes that would help better characterize the previously identified C_i transport proteins led to the need for a different mutagenesis approach. Thus a PCR based reverse genetics approach (already used successfully by other C. reinhardtii laboratories) was undertaken with the dual purpose of unearthing novel C_i transport proteins and better characterize previously identified ones for their involvement in the CCM (Figure 6.1). A number of insertional mutants were identified mostly in transport proteins whose functions remain uncharacterized within the C. reinhardtii cell (Figure 6.11). Although most of these insertions were outside of the coding regions they provide the motivation for further screening specifically targeting coding regions in the future.

While focused on generating artificial mutants to better study the role of transport proteins in the C. reinhardtii CCM we accidentally chanced upon an existing mutant strain for the
periplasmic carbonic anhydrase CAH1. The wild-type wall-less strain CC-503 was used for the sequencing of the *C. reinhardtii* genome. This strain was being used in the laboratory as a cell wall-less strain for efficient transformations. In course of knock-down and knock-out mutation generations using this strain it was soon revealed that the protein CAH1 could not be detected with a specific CAH1 antibody (Figure 5.1). This periplasmic CA is often lost in cell-wall less strains following a round of washing with media. However, this protein could not be detected even in unwashed CC-503 cells. Although the expression of the gene seems to be at the same level as any other cell-wall less strain (Figure 5.3), the protein could not be detected in Western blots. A few issues still need to be investigated with regards to this finding for instance the possible reasons for either the lack of translation or the instability of the CAH1 protein in CC-503. Also, the compensation if any by other CA proteins in this mutant when compared to an artificially generated *cah1* mutant needs to be studied further.

This thesis brings to the fore and establishes the location and roles of few known *C*<sub>i</sub> transporters, mainly LCI1. Together with HLA3, this transporter can now be considered as part of the plasma membrane localized carbon acquisition system of the CCM. The chloroplast membrane locations of NAR1.2 and CCP1 also point to another internal bicarbonate transport system. However, a lot of questions regarding the *C*<sub>i</sub> transport mechanism of the CCM still remain unresolved. The transporter/transporters located on the thylakoid membrane that would bring in the bicarbonate to be converted to CO<sub>2</sub> by CAH3 still remains to be identified. The successful localization of the small anion transporters of the Nar family and of any of the newer transporters being identified through further mutagenesis attempts might help zero in on the thylakoid membrane *C*<sub>i</sub> transporter. As in the past the identification and possible implication of a particular transporter in *C*<sub>i</sub> transport is often just the beginning of a complex series of follow up
investigations. For instance the membrane topology of each of these membrane transporters, their interaction with each other and the complexities of their regulation will still need to be studied. Also there might be soluble protein complexes (LCIB family) that might be playing a role in facilitating the movement of $\text{C}_i$ into the thylakoid lumen that need to be closely investigated. For the purpose of generation of mutants that lack multiple transport proteins in one membrane location, efforts to generate double or in rare cases triple mutants via either crossing single mutants or tandem targets in RNAi experiments will still need to be attempted. Thus this thesis can be considered as only one of the numerous ongoing attempts by researchers devoted to understanding the $C. \text{reinhardtii}$ CCM and the path that inorganic carbon takes from the outside of the cell to final fixation by Rubisco.

Intricate knowledge of the functioning of these $C_i$ transporters singularly and the process of carbon acquisition as a whole, might benefit not only those interested in studying eukaryotic CCMs, but the increasing number of genetic engineers who wish to utilize this knowledge for the betterment of commercial crops or generation of newer bio-energy sources. Juxtaposition of the ability of transport proteins within $C. \text{reinhardtii}$ to successfully acquire $C_i$ even in carbon-limited conditions onto higher plant systems, creates the possibility of improving the productivity and photosynthetic efficiency of commercial crop plants. This has large-scale ramifications in meeting the nutritional and energy needs of an ever-increasing world population.
REFERENCES


Chlamydomonas reinhardtii by sensing CO₂ availability. Proceedings of the National Academy of Sciences 98: 5347-5352


Funke R, Kovar J, Weeks D (1997) Intracellular carbonic anhydrase is essential to photosynthesis in Chlamydomonas reinhardtii at atmospheric levels of CO₂ (demonstration via genomic complementation of the high-CO₂-Requiring Mutant ca-1). Plant Physiology 114: 237-244


Hiroyuki S, Susumu K (1981) New restriction endonucleases from Flavobacterium okeanokoites (FokI) and Micrococcus luteus (Mlu I). Gene 16: 73-78


Hyams J, Davies D (1972) The induction and characterisation of cell wall mutants of Chlamydomonas reinhardii. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 14: 381-389


Kucho K, Ohyama K, Fukuzawa H (1999) CO₂-responsive transcriptional regulation of CAH1 encoding carbonic anhydrase is mediated by enhancer and silencer regions in *Chlamydomonas reinhardtii*. Plant Physiology 121: 1329-1337


Kustu S, Inwood W (2006) Biological gas channels for NH₃ and CO₂: evidence that Rh (Rhesus) proteins are CO₂ channels. Transfusion Clinique et Biologique 13: 103-110


Ludwig M, Sülttemeyer D, Price G (2000) Isolation of ccmKLMN genes from the marine cyanobacterium, Synechococcus sp. PCC7002 (Cyanophyceae), and evidence that CcmM is essential for carboxysome assembly. Journal of Phycology 36: 1109-1119


OrÚs MI, Rodríguez M, Martinez F, Marco E (1995) Biogenesis and ultrastructure of carboxysomes from wild type and mutants of Synechococcus sp. strain PCC 7942. Plant Physiology 107: 1159-1166


Park W, Zhai J, Lee J (2009) Highly efficient gene silencing using perfect complementary artificial miRNA targeting AP1 or heteromeric artificial miRNA targeting AP1 and CAL genes. Plant cell reports 28: 469-480


Ramazanov Z, Rawat M, Henk M, Mason C, Matthews S, Moroney J (1994) The induction of the CO\textsubscript{2}-concentrating mechanism is correlated with the formation of the starch sheath around the pyrenoid of \textit{Chlamydomonas reinhardtii}. Planta 195: 210-216


Wang Y, Spalding M (2006) An inorganic carbon transport system responsible for acclimation specific to air levels of CO$_2$ in *Chlamydomonas reinhardtii*. Proceedings of the National Academy of Sciences 103: 10110-10115


### APPENDIX I

*C. REINHARDTII* STRAINS USED IN THIS DISSERTATION

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>D66 (Wild-type)</td>
<td><em>nit1</em>, <em>nit2</em>, <em>cw15</em>, mt⁺</td>
</tr>
<tr>
<td>C9 (Wild-type)</td>
<td>mt⁺</td>
</tr>
<tr>
<td>CC-503 (Wild-type)</td>
<td><em>cw92</em>, mt⁺</td>
</tr>
<tr>
<td>CC407 (Wild-type)</td>
<td><em>nit1⁺</em>, <em>nit2⁺</em>, mt⁺</td>
</tr>
<tr>
<td>CC-400 (Wild-type)</td>
<td><em>cw15</em>, mt⁺</td>
</tr>
<tr>
<td><em>cia5</em></td>
<td><em>nit1</em>, <em>nit2</em>, mt⁺</td>
</tr>
</tbody>
</table>
## APPENDIX II
LIST OF SOME PRIMERS USED IN CHAPTERS 3, 4 AND 5

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAR 1.2</td>
<td>CTGTCCGCGCTCCATCCTGCTG (F) CGCGAGCTTCAACCTGCCTAAA (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>NAR1.3</td>
<td>GACGGTCACTACAGAAGGTTGGAATG (F) AGGGTAGCTGGGTCAC (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>NAR1.4</td>
<td>ACTAATGACGGCGGGCAGGGTTGTA (F) CAGTGTACGGGGAATGAGGTTGTC (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>NAR1.5</td>
<td>GGCGCGACGGGCTTTGTTTG (F) CAGGCGGGGTTTGGGTGGAC (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>NAR1.6</td>
<td>CGAGGGGAAGACCAAGATGACAGC (F) AGGGGTAGTGGAATGTCGAC (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>LCI1</td>
<td>AGGTCTTCACGGTGACCACCTACA (F) CAGGGCTGATGAGTGTGCTCC (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>CAH1</td>
<td>ACCCGGGTACCTCACCTAGC (F) CTTGGGAATGAGTGCCTGCT (R)</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>LCI1</td>
<td>ctagCGTGGCTACGTTCACCTGTActecgtgatccacgtgggtggtgcagcgctTACACGGTCAACGTAGCCACGg (F)</td>
<td>RNAi knockdown</td>
</tr>
<tr>
<td>NAR1.2</td>
<td>CCGGAATTGCTACACCTATGACTCG (F) CCGGATTTGAGCCACTGCTG (R)</td>
<td>E. coli expression</td>
</tr>
</tbody>
</table>
VITA

Bratati Mukherjee was born in India and obtained her Bachelor’s and Master’s degree in Botany, from Calcutta University. She graduated with a PhD degree in Biological Sciences from Louisiana State University in August 2013, and will be pursuing her postdoctoral study immediately afterward, in the laboratory of Dr. James V Moroney.