Functional Plant Science and Biotechnology ©2007 Global Science Books

CO_2 Concentrating Mechanisms in Eukaryotic Microalgae

Yingjun Wang • Martin H. Spalding*

Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa 50011, USA

Corresponding author: * **mspaldin@iastate.edu**

ABSTRACT

Many aquatic photosynthetic microorganisms possess inducible CO_2 concentrating mechanisms (CCMs) that allow them to optimize carbon acquisition in environments with frequently changing and often limiting CO_2 concentrations. The CCMs function by accumulation of a large quantity of intracellular inorganic carbon (Ci) through concerted Ci uptake systems and enzymes catalyzing the interconversion between different species of Ci. In addition, an array of regulatory devices appears present to facilitate the sensing of CO_2 availability and the regulation of metabolic pathways. Over the past several decades, significant advances have been made in understanding the CCM and its regulation. With the aid of mutant studies and the availability of several cyanobacterial and eukaryotic algal genomes, an integrated picture is emerging to reveal many of the molecular details in the microalgal CCMs. This review will focus on the recent advances in identifying and characterizing the major components involved in the CCM, including Ci uptake systems and regulatory pathways in eukaryotic microalgae, especially in the model organism, *Chlamydomonas reinhardtii*.

Keywords: acclimation, algae, carbonic anhydrase, *Chlamydomonas reinhardtii*, inorganic carbon, signal transduction pathway, transport systems

Abbreviations: CA, carbonic anhydrase; **CCM**, CO_2 concentrating mechanism; **Ci**, inorganic carbon

CONTENTS

INTRODUCTION

As the primary machinery for carbon fixation on the earth, photosynthetic organisms constantly confront changing ambient CO_2 concentrations and must adjust themselves accordingly. Although rising levels of CO_2 in the atmosphere have lately been a serious concern, a shortage in CO_2 supply, paradoxically, is often a major stress for photosynthetic organisms, not only due to low ambient CO_2 concentrations for photosynthesis, but also due to the inherent inefficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a key enzyme in carbon fixation. Several strategies have evolved in photosynthetic organisms to accommodate the CO_2 limitation by raising the CO_2 concentration at the site of Rubisco, including the well-known C4 and CAM photosynthetic pathways adopted in certain higher plants and a unique CO_2 concentration mechanism (CCM) adopted in many aquatic microalgae. While CO_2 enrichment in C4 or CAM pathways is achieved by spatial or temporal separation of ambient CO_2 fixation into C4 acids, and re-release of CO_2 into the C3 pathway at the site of Rubisco, the microalgal CCM relies on active inorganic carbon (C_i)

Received: 28 February, 2007. Accepted: 1 May, 2007.

Invited Review

transport by which a large intracellular Ci pool is created. Because of its ubiquitous existence in photosynthetic microorganisms and its simple and efficient nature, the microalgal CCM has gained significant attentions since it was discovered. First, since microalgal photosynthesis accounts for a significant portion of the carbon capture and sequestration on the earth, the operation of microalgal CCMs has a profound influence on global atmospheric CO_2 concentrations. Therefore, understanding the microalgal CCMs has been of great interest because of their potential influences on global environments and biomass production. Moreover, as microalgal C_i transport systems exhibit a great efficiency in concentrating CO_2, they appear to be excellent candidates for engineering many traditional C3 crop plants and other economically important C3 plants whose photosynthetic performances might be elevated by introducing a microalgal-like CCM. Last, but not the least, because the microalgal CCM represents an exquisite system by which an organism responds and interacts with environmental stresses, understanding the microalgal CCMs helps us to comprehend the acclimation of an organism to changing environments.

Since the discovery of the microalgal CCMs, significant effort has been devoted in understanding the mechanism of their operation and regulation. Knowledge on the CCMs is largely based on studies in some model organisms, including several cyanobacterial species and eukaryotic algal species. Considerable progress has been made thus far with regard to understanding cyanobacterial CCMs. With the aid of genetic and biochemical studies and availability of genome sequences from many cyanobacteria species, an integrated picture is merging, which reveals many fundamental aspects of cyanobacterial CCM functions, including diverse genes and proteins involved in Ci transport systems and carboxysome micro-compartments (Badger and Price 2003; Badger *et al*. 2006). For the CCM in eukaryotic algae, however, the molecular basis of the CCM still remains largely unknown. *Chlamydomonas reinhardtii*, a unicellular green alga with a photosynthetic apparatus similar to that of higher plants, is often used as a model system to study the microalgal CCM, and most of our knowledge of the eukaryotic algal CCM has been derived from physiological and biochemical studies on this organism. *C. reinhardtii* has a well characterized genetic background and many unique physiological and biochemical characteristics which make it an attractive model system for studying the interaction of photosynthetic eukaryotes with their environments. The recent completion of the genome sequences of *C. reinhardtii* and several other eukaryotic microalgae, in combination with increasingly available genetic and molecular tools, is facilitating research that will finally reveal many functional components involved in the eukaryotic CCM. This review will focus on the recent advances in our understanding of the eukaryotic microalgal CCMs, especially in *C. reinhardtii*.

ACCLIMATION TO LIMITING CO_2

The CCM of microalgae was originally recognized as distinguishable physiological states induced by limiting CO_2 conditions (Spalding 1998; Kaplan and Reinhold 1999). One significant acclimation to limiting CO_2 appears as a much higher efficiency in photosynthetic carbon assimilation and the apparent suppression of photorespiration. As well demonstrated, such high photosynthetic efficiency in limiting CO_2 acclimated cells can be attributed to their capacity for accumulating a large intracellular Ci pool, which is resulted from induction of the CCM. The high internal Ci level maintains a saturated or near saturated CO_2 concentration at the site of Rubisco, favoring its carboxylation activity over its oxygenation activity and thus efficiently promoting photosynthesis and suppressing photorespiration. Transport systems for active Ci uptake and enzymatic systems catalyzing rapid interconversion between different Ci species are generally considered to be two essential components of the CCM. In a simplified working model, the CCM comprises at least the following elements: active Ci transport and Ci accumulation, interconversion among different Ci species, and final dehydration of accumulated bicarbonate (HCO_3^-) to release CO_2 at the sites of Rubisco (Kaplan and Reinhold 1999; Badger and Spalding 2000; Giordano *et al*. 2005).

Ci CONCENTRATING SYSTEMS

Diversity in Ci transport systems

Active Ci transport plays a vital role and exhibits a great diversity in microalgal CCMs. Five different Ci transport modes have so far been identified in various cyanobacteria species (Badger *et al*. 2006). Eukaryotic Ci transport systems are expected to be of greater complexity than those in cyanobacteria, not only due to the highly compartmented structures in eukaryotic cells, but also due to the heterogeneity of Ci species being transported and the existence of multiple CO_2-level-dependent acclimation states in these organisms.

In eukaryotic microalgae, active uptake of Ci appears to take place in at least two locations: chloroplast envelope and plasma membrane. In *C. reinhardtii*, Ci species must penetrate these two barriers to reach the pyrenoid, a specialized protein structure in chloroplasts where Rubisco is localized. It has been demonstrated that the induction of an active Ci transport system occurs at both the plasma membrane and the chloroplast envelope (Goyal and Tolbert 1989; Sültemeyer *et al*. 1989, 1991; Palmqvist *et al*. 1994). There is still some debate as to whether the plasma membrane or chloroplast envelope is the primary site for Ci transport. However, given the fact that all photosynthetic enzymes are located in the chloroplast, it is commonly accepted that chloroplasts play an irreplaceable role in Ci concentrating (Moroney *et al*. 1987; Amoroso *et al*. 1998; Spalding 1998; Kaplan and Reinhold 1999). The *C. reinhardtii pmp1* mutant was identified as conditional lethal in air levels of CO_2, and was demonstrated to be deficient in Ci transport (Spalding *et al*. 1983a). A plastid localization of the *PMP1* gene product has been predicted (Miura *et al*. 2004; Wang and Spalding 2006), and been recently confirmed by immunolocalization (Wang and Spalding, unpublished). The lethal phenotype caused by a single lesion in *pmp1* supports the essentiality of plastid Ci transport. However, this does not necessarily exclude a requirement for any potential plasma membrane transporters. If the plasma membrane Ci transport functions concomitantly with the plastid transport system, the phenotype of *pmp1* implies no functional redundancy between the plastid transport system and the one on plasma membrane. Nevertheless, it supports a central role played by the chloroplast in active Ci transport in *C. reinhardtii*.

Heterogeneity of Ci species is another possible reason for the requirement of diverse Ci transport systems. HCO_3^- and CO_2 are the two principal Ci species over the physiological pH range, although CO_3^{-2} and other Ci species can not be excluded as potential substrates. The distribution of the individual Ci species vary depending on the pH of the extracellular environments or the intracellular compartments. Different Ci transporters may display distinct substrate specificity. HCO_3^- has been postulated as the primary substrate for chloroplast Ci uptake (Moroney *et al*. 1987; Moroney and Mason 1991), and CO_2 as the species predominantly transported across the plasma membrane, possibly by passive diffusion (Spalding 1998; Kaplan and Reinhold 1999). Mass-spectrometric disequilibrium analyses have apparently demonstrated the active transport of CO_2 by both cells and isolated chloroplasts of *C. reinhardtii* (Amoroso *et al*. 1998). Uptake of both HCO_3^- and CO_2 proceeded at similar rates in isolated chloroplasts from *C. reinhardtii*, although HCO_3^- was the dominant Ci species transported in *Dunaliella tetiolecta*. Similar patterns of active Ci uptake by chloroplasts also were demonstrated in *Tetraedron minimum* and *C. noc-*

tigama (van Hunnik *et al*. 2002). It is not clear whether an individual transport system always displays preferences for certain Ci species, but it appears that existence of substrate specificity is common for many Ci transport systems (Giordano *et al*. 2005).

Diversity of Ci transport systems also might be associated with multiple acclimation states. In nature, the ambient CO_2 concentrations for photosynthetic organisms can vary across orders of magnitude due to the diversity of habitats and seasonal or daily changes in environmental CO_2. Accordingly, distinct acclimation states are induced to cope with various levels of Ci supply. Natural habitats for *Chlamydomonas* species include metabolite-rich environments (such as sewage or rich soil) where the levels of Ci or acetate can be considerably high and freshwater aquatic systems (such as freshwater pond) where CO_2 levels are low and diffusion of Ci species are highly restricted. Moreover, extreme low levels of CO_2 or depleted Ci can be resulted in a rapidly growing population with active photosynthesis. In the past, two physiological states corresponding to enriched CO_2 concentrations (1-5% CO_2 in air, high CO_2) and low CO_2 concentrations (air, ~0.03% CO_2) have been well documented. Another physiological state corresponding to very low CO_2 (<0.02% CO_2), perhaps neglected previously, was recently recognized based on an unusual growth phenotype of *pmp1* (Van *et al*. 2001; Spalding *et al*. 2002; Wang and Spalding 2006). The *pmp1* mutant, as well as its allelic mutant *air dier1* (*ad1*), displays an "air-dieing" phenotype: it grows well in either high or very low CO_2, but dies in low (air-level) CO_2. This conspicuous phenotype indicates that at least three distinct CO_2 acclimation states exist in *C. reinhardtii*, associated with high, low, or very low CO_2, respectively. The three distinct CO_2 acclimation states were confirmed by comparisons of physiological and photosynthetic characteristics among high CO_2-, low CO_2- and very low CO_2-acclimated cells (Vance and Spalding 2005). The cells acclimated to very low CO_2 exhibited a much lower V_{max}, a longer cell-doubling time and a smaller cell size when compared with those acclimated to low CO_2 and high CO_2.

Despite the common assumption that the CCM is induced only in limiting CO_2, there is evidence indicating that active uptake of Ci may take place also in high CO_2 grown cells. Active transport of HCO_3^- has been reported in high CO_2 grown cells from *C. reinhardtii* and *D. tertiolecta*, although with a much lower apparent affinity than that of low CO_2 cells (Palmqvist *et al*. 1994; Amoroso *et al*. 1998). The main reported differences between the transporters from high CO_2 grown cells and low CO_2 grown cells appear to be their substrate affinities (Amoroso *et al*. 1989), since no significant differences in maximal Ci uptake activities were observed. This would be consistent with the observation that high CO_2 cells and low CO_2 cells displayed similar photosynthetic V_{max} (Vance and Spalding 2005).

As discussed in a recent paper, requirement for transport systems with different Ci affinities and transport capacities in *C. reinhardtii* may represent a common survival strategy in eukaryotic microalgae (Wang and Spalding 2006). When grown in very low CO_2, transporters with high Ci affinities may favor uptake of Ci present at a very low concentration, and the low capacities for Ci influx may allow *C. reinhardtii* to maintain some growth without quickly depleting all available Ci. On the other hand, in low CO_2 (air level), a system with low Ci affinity would be sufficient to accommodate the available Ci concentration, and the high Ci transport capacity would maintain a growth rate comparable to that of high CO_2 cells. The ability to acclimate to multiple CO_2 levels in eukaryotes apparently requires a network of transport systems which is elaborately orchestrated.

Candidate Ci transporter genes in *Chlamydomonas*

Although there is substantial evidence demonstrating the existence of active transport systems in eukaryotic algae, only very few candidate Ci transport genes have been identified, and the functions of their gene products in the CCM are far from being fully characterized.

PMP1/AD1/LCIB and LCIB family

The *pmp1* mutant is one of only a small number of mutants with a lesion directly affecting the CCM, and it has been touted as demonstrating a requirement for active Ci transport in the CCM (Spalding *et al*. 1983a). Recently the gene defective in *pmp1* was identified by genetic characterization of its allelic insertional mutant, *ad1* (Wang and Spalding 2006). PMP1/AD1 protein is encoded by *LCIB*, a gene previously identified as a CO_2 responsive gene (Miura *et al*. 2004). Blast searches and domain searches with *LCIB* revealed no significant recognizable domains or homologies, except for three additional genes in the *C. reinhardtii* genome, *LCIC*, *LCID* and *LCIE* (Wang and Spalding 2006). *LCIB* and *LCIC* show similar expression patterns: very low expression in high CO_2 (5%) and substantial increased expression induced by a wide range of limiting CO_2 (0.15%-0.01%). *LCID* shows a similar CO_2 expression pattern but at a much lower apparent mRNA abundance, while expression of *LCIE* so far has been confirmed only by its existence in a cDNA library.

Although physiological and biochemical characterization of *pmp1* and *ad1* suggests that LCIB is involved in active Ci transport, LCIB seems unlikely to perform as a Ci transporter by itself, because it is predicted to be a soluble protein lacking any obvious transmembrane regions. It is possible that LCIB is an integral component of a multi-subunit transport complex and that its Ci transport function relies on its interaction with other proteins. Plastid localization was predicted for both LCIB and LCIC. There is evidence indicating a possible physical interaction between LCIB and LCIC (Wang and Spalding, unpublished). The *air dier* growth phenotype of *pmp1* and *ad1* clearly indicates that the LCIB associated Ci transport system is essential for the low CO_2 (air) acclimation state in *C. reinhardtii*. Even though *LCIB* also is expressed in very low CO_2 and high CO_2, obviously it is not essential under such conditions, if it functions at all at those CO_2 concentrations.

YCF10 (Cem A) dependent system

Disruption of the plastid *ycf10* open reading frame was found to cause light sensitivity and decreased Ci uptake in *C. reinhardtii* (Rolland *et al*. 1997). The product of the chloroplast *ycf10* gene is localized in the inner chloroplast envelope and displays sequence homology with the chloroplast CemA protein from plants and cyanobacterial *CotA* product. Ycf10, like CemA and CotA, is a membrane protein with four transmembrane domains. The cyanobacteria *cotA* mutants exhibited a slow growth in limiting CO_2 and decreased CO_2 transport, but it was suggested that the decreased CO_2 transport in the mutants was a result from the impaired proton extrusion (Katoh *et al*. 1996a, 1996b). Even though the *ycf10* mutants display defective CO_2 uptake, their sensitivity to high light in both high CO_2 and low CO_2 argues against the direct involvement of *ycf10* gene product in CO_2 uptake (Kaplan and Reinhold 1999). Whether and how *ycf10* participates in Ci uptake still remains to be determined.

CCP1 and CCP2

CCP1 and CCP2 (Lip36) were first identified as two low CO_2 induced polypeptides in *C. reinhardtii* (Spalding and Jeffery 1989; Ramazanov *et al*. 1993). These proteins are associated with chloroplast envelope and have been suggested as candidates for the involvement in chloroplast Ci transport. *CCP1* and *CCP2* were demonstrated to encode two similar proteins sharing 95.7% identical amino acid sequence (Chen *et al*. 1996). CCP1 and CCP2 are predicted

to be membrane proteins with six transmembrane domains, and are similar to the mitochondrial carrier protein super family. Mitochondrial carrier proteins are small proteins catalyzing the translocation of metabolites across the mitochondrial inner membrane, which include ATP/ADP translocators, uncoupling proteins (proton carriers), phosphate carriers and carriers for a variety of metabolites in the mitochondrial carbon/energy metabolism (Kuan and Saier Jr. 1993; Palmieri 1994). It was reported that strains with silenced *CCP1/2* expression by RNA interference (RNAi) grew slower in low CO_2, but exhibited photosynthetic kinetics and Ci affinity similar to those in wild type cells (Pollock *et al*. 2005). The lack of a clear phenotypic link between the *CCP1/2* RNAi knockdowns and CCM function raises questions regarding any essential role for CCP1 and CCP2 in the CCM, although it is possible that their functional importance was masked by the compensating function of other Ci transport systems under the conditions employed, as has been observed for Ci transport in cyanobacteria (Shibata *et al*. 2002).

LCIA/NAR1.2

LCIA, also named as *NAR1.2*, was identified as a low-CO_2 inducible gene that encodes a protein belonging to the *NAR1* multigene family from *C. reinhardtii* (Galvan *et al*. 2002; Miura *et al*. 2004; Mariscal *et al*. 2006). The members of NAR1 family are related to the formate/nitrite transporter (FNT) family (Miura *et al*. 2004; Mariscal *et al*. 2006). Proteins in FNT family have been found in various bacteria and some eukaryotic organisms such as fungi, yeast, algae and protozoa, but not in plants (Galvan *et al*. 2002; Mariscal *et al*. 2006). LCIA is a polypeptide of 336 amino acids with a predicted chloroplast transit peptide and 6 transmembrane domains. LCIA has been postulated as a Ci transporter because the expression of *LCIA* is regulated by CO_2 irrespective of the nitrogen sources (Miura *et al*. 2004). LCIA also has recently been reported to exhibit transport activities for both bicarbonate and nitrite when expressed in *Xenopus* oocytes (Mariscal *et al*. 2006). However, in this research, LCIA exhibited a rather low affinity for HCO_3^-, but a high affinity for nitrite, leaving the question of its importance as a major Ci transporter open.

HLA3/MRP1

Im and Grossman (2001) identified a limiting CO_2-induced gene, *HLA3*, encoding a putative ATP-binding-cassette (ABC)-transporter. *HLA3* also has been named *MRP1*, and its gene product suggested as a prime candidate for a Ci transporter (Miura *et al*. 2004). ABC transporters are ubiquitous membrane proteins that transport a large variety of molecules across membranes, and represent one of the largest protein families (Rea 2007). In cyanobacteria, the best-known Ci transporter belonging to the ABC transporter family is BCT1, an inducible high affinity HCO_3^- transporter encoded by the *cmpABCD* operon (Badger and Price 2003; Badger *et al*. 2006). Unlike BCT1, which is a bacterial type, multimeric, four subunit complex, HLA3 is a whole-molecule ABC transporter with significant homology to members of the multidrug-resistance-related protein (MRP) subfamily of ABC transporters (Im and Grossman 2001). MRP-related transport is often inhibited by vanadate, an inhibitor of the plasma membrane bound ATPase, because ABC-dependent transport is energized by ATPase (Rea 2007). The inhibition of Ci uptake in *C. reinhardtii* by vanadate was reported (Palmqvist *et al*. 1988), which may reflect the sensitivity of HLA3 associated Ci transport to the inhibitor. Although HLA3 was first suggested as being a plastid-localized protein, based on protein targeting prediction (TargetP, http://www.cbs.dtu.dk/services/TargetP/; or PSORT, http://psort.nibb.ac.jp/), the *HLA3* gene product was predicted to enter the endomembrane system, and thus may be targeted to the plasma membrane. HLA3/MRP1 is the only clear candidate for a plasma membrane Ci transporter identified so far in eukaryotic systems.

LCI1

LCI1 was originally identified as a limiting CO_2 inducible gene encoding a putative protein of 192 amino acids (Burow *et al*. 1996). The expression of *LCI1* was regulated by CIA5, a master regulator in the CCM (Im *et al*. 2003; Miura *et al*. 2004), and by LCR1, a transcriptional factor associated with CO_2 signal transduction pathways (Yoshioka et al. 2004). LCI1 appears to be a membrane protein with three predicted transmembrane domains and a signal peptide (TMHMM, http://www.cbs.dtu.dk/services/TMHMM/; TargetP and PSORT), suggesting that LCI1 is perhaps targeted to the plasma membrane. No information is currently available with regard to the possible function of LCI1 in the CCM.

RHP1

C. reinhardtii has two Rhesus (Rh) proteins (RHP1 and RHP2 or Rh1 and Rh2) which are similar to the Rh proteins in the human red blood membrane (Soupene *et al*. 2002). Both RHP1 and RHP2 are predicted to have 12 transmembrane domains. RHP1 has a potential chloroplast transit sequence, and may be associated with the chloroplast membranes (Soupene *et al*. 2002; Kustu and Inwood 2006). The expression of *RHP1* is highly inducible by high CO_2 (3%) with very low expression in low CO_2 (air). RNAi mutants that had no *RHP1* expression exhibited a growth defect in high CO_2, but normal growth in air (Soupene *et al*. 2004). RHP1 apparently functions as bidirectional CO_2 gas channels, which allow quick equilibration of CO_2 under high CO_2 conditions.

Carbonic anhydrase in the CCM

Carbonic anhydrase, being present in three independent evolutionary lines (α-, β- and γ-type), is ubiquitously present over a wide spectrum of living organisms (Hewett-Emmett and Tashian 1996). In the CCM, CA plays a critical role by catalyzing the interconversion between different Ci species. Diverse forms of CAs appear to be involved in various fundamental aspects of the eukaryotic CCM (Spalding 1998; Moroney and Somanchi 1999; Mitra *et al*. 2004; Giordano *et al*. 2005; Mitra *et al*. 2005). In cyanobacteria, one prominent feature regarding the function of CA is the co-localization of CA with Rubisco in the carboxysomes, which is thought to be necessary for dehydrating accumulated intracellular HCO_3^- and generating an elevated CO_2 concentration in close proximity to Rubisco. No Rubisco associated CA in pyrenoid has been demonstrated in eukaryotic algae, but the similar role has been proposed for a thylakoid lumenal CA in *C. reinhardtii*. CAs are also necessary to facilitate the uptake of Ci across different membranes (Moroney *et al*. 1985; Spalding 1998; Giordano *et al*. 2005), and perhaps function in concert with various Ci transport systems. Six CAs have by far been characterized in *C. reinhardtii*, including three α-CAs (CAH1, CAH2, and CAH3) and three β-CAs (CAH4, CAH5 and CAH6).

CAH3

Despite multiple CAs present in *C. reinhardtii*, CAH3 is so far the only one that has been confirmed as essential for limiting CO_2 acclimation. Mutants defective in *CAH3* (*ca1-1-12-1C*, *ca1-2-18-6A*, *ca1-3-18-7C*, and *cia3*) cannot survive under limiting CO_2 conditions (Spreitzer and Mets 1981; Spalding *et al*. 1983b; Moroney *et al*. 1986; Suzuki and Spalding 1989; Karlsson *et al*. 1995; Funke *et al*. 1997; Karlsson *et al*. 1998). Even though over-accumulation of internal Ci has been demonstrated in mutant cells with defects in *CAH3*, photosynthesis is still limited by CO_2 in these mutants (Spalding *et al*. 1983b; Hanson *et al*. 2003). CAH3 is targeted to the thylakoid lumen and may be asso-

ciated with PSII (Karlsson *et al*. 1998; Park *et al*. 1999). This supports a hypothesis with regard to the role played by CAH3 in the CCM; in this model, HCO_3^- is delivered into the thylakoid lumen from the stroma, and then dehydrated by CAH3 upon lumen acidification in the light; the released CO_2 thus diffuses back into stroma and becomes available to Rubisco, which prefers CO_2, rather than HCO_3^-, as the substrate (Pronina and Semenenko 1992; Raven 1997). This model requires a low CA activity in the stroma, where the alkaline pH would favor formation of HCO_3^-, unless the CO_2 released from lumen can directly reach Rubisco in the pyrenoid without traversing much stromal space. In any case, it requires the absence of CA activity in the pyrenoid. Some researchers also have proposed that CAH3 is involved in PSII function, or in the formation of a proton gradient cross the thylakoid membranes (Villarejo *et al*. 2002; van Hunnik and Sültemeyer 2002). However, a recent re-examination of CAH3 still supports the role of CAH3 as providing CO_2 to Rubisco, because PSII electron transport was not affected by the *cia3* mutation (Hanson *et al*. 2003).

CAH1 and CAH2

Under limiting CO_2 conditions, the majority of the CA activity in *C. reinhardtii* is found in the periplasmic space. The products of two genes, *CAH1* and *CAH2*, were identified as α-type CA isoforms associated with the periplasmic CA activity (Fujiwara *et al*. 1990; Fukuzawa *et al*. 1990; Rawat and Moroney 1991). The regulation of *CAH1* and *CAH2* expression by Ci availability shows opposite trends: whereas the less abundant *CAH2* gene product (pCA2) is expressed at high Ci concentrations and repressed by low Ci concentrations, the *CAH1* gene product (pCA1), which accounts for the majority of the extracellular CA activity, is expressed only in limiting Ci concentrations. The regulation of the *CAH1* expression has been closely scrutinized, and different roles for the periplasmic CA in the CCM have been proposed (Spalding 1998; Giordano *et al*. 2005). It appears that the periplasmic CA activity could facilitate the uptake of CO_2 at the plasma membrane (Moroney *et al*. 1985; Spalding 1998; Giordano *et al*. 2005). However, a null-mutant with a disrupted *CAH1* gene did not show any discernable phenotype differing from the wild type cells, questioning the essentiality of pCA1 in the CCM (Van and Spalding 1999).

CAH4 and CAH5

Two mitochondrial CAs encoded by very similar genes, *CAH4* and *CAH5* (*βCa1* and *βCa2*), were identified as members of the β-type CA family (Eriksson *et al*. 1996). The expression of *CAH4* and *CAH5* is regulated by CO_2 at the transcriptional level, with the abundant *CAH4* and *CAH5* transcripts induced by low CO_2 (Eriksson *et al*. 1998). Although CAH4 and CAH5 are among the most abundant low CO_2 inducible proteins, their functions in the CCM have not been clearly defined. It has been suggested that they are required for buffering pH in mitochondria to neutralize ammonia released during photorespiration (Eriksson *et al*. 1996). Giordano *et al*. (2003) proposed that CAH4 and CAH5 might be involved in anaplerotic carbon recycling in *C. reinhardtii* to balance carbon and nitrogen assimilation. β-type CAs have an ancient evolutionary origin and are ubiquitously distributed in almost all living organisms (Smith *et al*.1999). A study has showed that a β-type CA is essential for the growth of *Corynebacterium glutamicum* under atmospheric conditions (Mitsuhashi *et al*. 2004), as demonstrated by a high CO_2 requiring phenotype caused by a deficiency in the CA. It is possible that the functions of CAH4 and CAH5 are related to mitochondrial carbon metabolism, but not directly related to the CCM.

CAH6

CAH6 is another β-type CA recently identified as a probable chloroplast stromal CA (Mitra *et al*. 2004). The presence of CA activity in the stroma indicates that the CCM in eukaryotic microalgae must be different from the cyanobacterial CCMs, which are largely dependent on the absence of CA activity in the cytosol. As demonstrated in the cyanobacterium *Synechococcus* PCC7942, heterologous expression of human CA, HCAII, in the cyanobacteria cytosol caused dramatic decreases in photosynthetic Ci affinity and Ci accumulation, and resulted in a high CO_2 requiring phenotype (Price and Badger 1989). As discussed earlier, a stromal CA does not favor the entry of stromal CO_2 into pyrenoids. It would require very close contact between the thylakoid membranes and the pyrenoid so that lumenal CO_2 can be directly released into the pyrenoid. Indeed, it has been reported that CAH3, although distributed throughout the chloroplast, is enriched in the thylakoids around the pyrenoid, and also is present in the thylakoid tubules penetrating the pyrenoid (Mitra *et al*. 2005). The function of CAH6 has been proposed to be trapping of CO_2 that diffuses out from the pyrenoid as the more easily confined HCO_3^-, the formation of which would be facilitated by the alkaline stromal pH (Mitra *et al*. 2004, 2005).

Other CAs in C. reinhardtii

The recent completion of the *C. reinhardtii* genome sequence has revealed a large number of CAs. Besides of the six CAs mentioned above, at least three additional putative β-type CAs (*CAH7*, *CAH8*, *CAH9*) are present in the *C. reinhardtii* genome, although none of these has yet been fully characterized (Mitra *et al*. 2005; http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). Moreover, a γ-CA-like gene, *CAG3* (*GLP1*), also was identified in the genome, but no CA activity was detected in CAG3 when over-expressed (Mitra *et al*. 2005). Except that CAH8 was confirmed to exhibit CA activity, no information is available with regard to the biochemical characteristics or intracellular locations of these CAs or the CA-like protein. Villarejo *et al*. (2001) have demonstrated a CA activity associated with the chloroplast envelope, but it is not clear if there is any relationship between this envelope CA and CAH6 or any other identified putative CAs. Further investigation of the CA distribution in chloroplasts is critical to elucidate the CCM model in eukaryotic algae.

REGULATION OF THE CCM AND SIGNAL TRANSDUCTION

Like many other acclimation responses, the CCM is highly regulated to allow the cells to respond the variability of CO_2 supply in an efficient and economical way. During acclimation, rapid changes in gene expression and biochemical events occur, which are believed to be regulated by an array of regulatory devices apparently present to facilitate the sensing of CO_2 availability and monitor carbon metabolism. Many questions with regard to CO_2 sensing and signaling are still unsolved, largely due to lack of knowledge of the molecular components associated with these functions.

Perception of CO_2 and signals controlling the CCM expression

In cyanobacteria, either the external HCO_3^- or the internal Ci pool has been postulated as the signal controlling the expression of the CCM (Mayo *et al*. 1986; Woodger *et al*. 2005). It appears that at least certain types of the eukaryotic microalgal CCMs are not controlled by the internal Ci pool. This can be demonstrated by the *C. reinhardtii pmp1* mutant (Spalding *et al*. 1983a; Wang and Spalding 2006). The functional CCM associated with very low CO_2 cannot be induced by low CO_2 in *pmp1*, even though a very low inter-

nal Ci is expected under such conditions because of the defective *LCIB*. However, a very low external Ci is enough to induce the CCM in the same mutant, indicating that the induction of the CCM is not correlated with the internal Ci, at least not in the very low CO_2 acclimation state. It is not clear whether different signals, or different levels of the same signal, triggers the different CO_2-level-dependant acclimation states. In several Chlorophyta species, it has been proposed that external CO_2 is the primary signal being directly perceived (Matsuda and Colman 1995, 1996; Bozzo and Colman 2000; Bozzo *et al*. 2000; Giordano *et al*. 2005). The conclusion of CO_2 being the signal in these Chlorophyta is based on the absolute correlation between the induction of the CCM by low CO_2 and the external CO_2 concentration over different pH levels, regardless of total Ci or HCO_3^- concentration in the medium. In these cases, the low CO_2 signal appears to be perceived at plasma membrane by a mechanism that has not yet been identified.

Although a change in Ci availability is the primary signal initiating changes in gene expression, some intermediate metabolites in carbon metabolism and some environmental signals also appear to be involved in the regulation of the CCM. Light and O_2 or CO_2/O_2 ratio have been reported to affect the induction of the CCM and the expression of carbonic anhydrase (Spalding and Ogren 1982; Ramazanov and Semenenko 1984; Dionisio-Sese *et al*. 1990; Villarejo *et al*. 1996; Spalding 1998). It has been suggested that the regulation by light or O_2 is associated with photosynthesis and photorespiration (Ramazanov and Cardenas 1992; Spalding 1998; Giordano *et al*. 2005). However, contradictory evidence has also been shown to question the light or O_2 involvements in the regulation of the CCM. Induction of *Cah1* has been reported to be light-independent (Rawat and Moroney 1995). Recent re-examination of O_2 effects on acclimation failed to support any role of O_2 or of photorespiration in regulating gene expression, at least that of *CAH1* and *GDH1*, or the CCM function, as determined by photosynthetic characteristics (Vance and Spalding 2005).

Considering the complexity of interaction among different metabolic pathways and signaling systems in eukaryotes, it is not surprising that the regulation of the CCM is often entangled with other signal pathways. It has been demonstrated that blue light signaling, cell cycle and circadian rhythm are involved in the regulation of gene expression associated with the CCM (Dionisio *et al*. 1989a, 1989b; Rawat and Moroney 1995; Spalding 1998). Dionisio *et al*. (1989a) has proposed two light-requiring-steps, a photosynthesis-dependent step and a photosynthesis-independent (blue light) step, controlling *CAH1* induction during the limiting CO_2 acclimation. The induction of the *CAH1* gene product, pCA1, also was inhibited by potassium iodide, a flavin quencher, indicating that a flavoprotein may be the blue light sensor (Dionisio *et al*. 1989b). Induction of the CCM and the regulation of CO_2 responsive genes also are complicated by the cell cycle, which itself is regulated by circadian rhythm (Marcus *et al*. 1986; Rawat and Moroney 1995; Eriksson *et al*. 1998; Spalding 1998). The expression of *CAH1* and *CAH4/CAH5* has been reported to exhibit a circadian rhythm in synchronously grown cells (Rawat and Moroney 1995; Eriksson *et al*. 1998). This circadian pattern of gene expression may reflect the complex gene regulation by both Ci and the cell cycle in *C. reinhardtii* (Spalding 1998).

Gene expression in the CCM

Many *de novo*- induced genes in the CCM appear to be involved in the fundamental functions in limiting CO_2 acclimation, including Ci transport systems, CA and photorespiration. Moreover, various *C. reinhardtii* genes involved in a wide spectrum of metabolic reactions and acclimation responses have been identified as quantitatively up- or down-regulated by changes in Ci supply.

The most abundant proteins induced by limiting CO_2 include three CAs (CAH1, CAH4 and CAH5; Fujiwara *et al*. 1990; Fukuzawa *et al*. 1990; Eriksson *et al*. 1996) and several Ci transporter candidates (CCP1, CCP2, LCIB, LCIC, LCID, HLA3/MRP1 and LCIA/NAR1.2; Chen *et al*. 1997; Im and Grossman 2001; Miura *et al*. 2004; Wang and Spalding 2006). Some apparent constitutively expressed genes also exhibit CO_2 responses, such as the thylakoid lumenal CA (*CAH3*) which shows 2-fold increases in transcript abundance induced by low CO_2 (Karlsson *et al*. 1998). Several genes involved in photorespiration also have been identified as being either induced or up-regulated by limiting CO_2, including *AAT1* (alanine:α-KG aminotransferase), *PGP1* (phosphoglycolate phosphatase), *GDH1* (glycolate dehydrogenase), *SHMT1* (serine hydroxymethyltransferase) and several other photorespiration-related genes (Chen *et al*. 1996; Mamedov *et al*. 2001; Im and Grossman 2001; Im *et al*. 2003; Miura *et al*. 2004; Nakamura *et al*. 2005).

Not only is the induction of gene expression associated with limiting CO_2, the expression of many genes also is induced or up-regulated by high CO_2. *CAH2* and *RHP1* are two genes which are induced by high CO_2, and their functions appear to be associated with CO_2 uptake in high CO_2 conditions (Fujiwara *et al*. 1990; Fukuzawa *et al*. 1990; Rawat and Moroney 1991; Soupene *et al*. 1998, 2002; Kustu and Inwood 2006). Other high CO_2 induced genes include H43, coding a periplasmic protein of unknown function (Shiraiwa and Kobayashi 1999; Hanawa *et al*. 2007) and many additional photosynthesis-related and nutrient-related genes (Miura *et al*. 2004).

Translational regulation and post-translational regulation have also been demonstrated to control the expression of limiting Ci acclimation-related and CCM-related genes. Transient translational down-regulation of the large and small subunits of Rubisco is induced by limiting CO_2 (Winder *et al*. 1992), and a posttranslational modification of CIA5 has been postulated to control the CCM (Fukuzawa *et al*. 2001; Xiang *et al*. 2001). Recently, the phosphorylation of some thylakoid proteins was reported in association with limiting CO_2 acclimation (Turkina *et al*. 2006). Interestingly, in this latter research, the authors reported multiple phosphorylations of LCI5, the gene product of a previously identified, low CO_2 inducible gene, *LCI5* (Im and Grossman 2003; Miura *et al*. 2004). LCI5 was found to be peripherally associated to the stroma side of chloroplast thylakoids. It is not clear whether this protein plays any specific role in the CCM or in other aspects of limiting CO_2 acclimation.

The recent applications of genome-wide analyses have revealed a large number of CO_2-regulated-genes. The functions of these gene products fall into several broad categories which include many metabolic pathways and stress responses (Im and Grossman 2001; Im *et al*. 2003; Miura *et al*. 2004). This is not surprising since, in essence, utilization of Ci affects many fundamental aspects of metabolism, growth and development of photosynthetic organisms. It has been noticed that many CO_2-responsive-genes also are regulated by light (Im and Grossman 2001; Im *et al*. 2003), although the genes associated with the CCM can be distinct from non-CO_2-responsive-genes by their sensitivity to CO_2 and are controlled by the Cia5-associated signal transduction pathway. Several stress-related genes have been identified to be responsive to Ci change, but their relationship to limiting CO_2 acclimation has not been proven (Im *et al*. 2003; Miura *et al*. 2004).

Signal transduction pathways associated with the CCM

The mechanism underlying the gene regulation associated with the CCM is largely unknown. The major impediment to understanding the signal transduction pathway(s) is inadequate information about the components in the pathway(s). So far only a very few elements associated with the CCM signaling have been identified.

The *cia5* mutant has played an unparalleled role so far in furthering our understanding the signal transduction in the microalgal CCM. This mutant lacks all known limiting CO_2 inducible characteristics, including Ci transport activity, gene regulation, and biochemical and structural changes associated with the limiting CO_2 acclimation (Moroney *et al*. 1989; Marek and Spalding 1991; Spalding *et al*.1991). Since CIA5 appears to be a master regulator in the CCM, identification of CIA5 must be regarded as the most important advance to date in studying the signal transduction associated with limiting Ci acclimation and the CCM.

Two research groups simultaneously identified the *CIA5* (or *CCM1*) gene, which encodes a putative transcription factor (Fukuzawa *et al*. 2001; Xiang *et al*. 2001). The deduced gene product of *CIA5* is a 699-aa hydrophilic polypeptide with a putative zinc-finger motif in its N-terminal region and a glycine repeat region characteristic of transcriptional activators. Near the C-terminus of CIA5 several putative phosphorylation sites are found, and it was speculated that the C-terminus of CIA5 might be post-translationally modified in response to a limiting CO_2 signal. CIA5 is constitutively expressed irrespective of Ci conditions, and it is still not clear how CIA5 regulates gene expression or how this protein interacts with other members in the signal transduction pathway(s). It seems likely that a limiting CO_2 signal causes modification of the CIA5 protein, and that the modified CIA5 then either directly regulates CO_2 responsive genes or regulates downstream signal transduction components.

CO_2 responsive promoters have been identified in *CAH1* and *CAH4/CAH5* by using the arylsulfatase (*ARS1*) gene as a reporter (Villand *et al*. 1997; Kucho *et al*. 1999, 2003). Two regulatory regions were found to be present in the *CAH1* promoter: a silencer region responsible for repressing transcription in high CO_2, and an enhancer region for activating transcription in low CO_2 (Kucho *et al*. 1999). Present in the enhancer region of *CAH1* are two *cis*-acting elements, EE-1 and EE2, which were found to interact with some nuclear proteins (Kucho *et al*. 2003). Since many CO_2 responsive genes appear to be coordinately regulated, they must share some regulatory elements or be controlled by the same pathway. In deed, the core sequence motif, EEC, in *CAH1* EE-1 and EE-2 elements is also present in the *CAH4* promoter. Recently, Yoshioka *et al*. (2004) identified and characterized LCR1, a Myb transcription factor which binds to the *CAH1* promoter. *LCR1* is involved in the regulation of several low-CO_2-inducible genes, including *CAH1*, and its expression is induced by low CO_2 under the control of CIA5. LCR1 appears to be associated with the CIA5 signaling cascades, but it is not clear whether its expression is regulated directly by CIA5, or through other signal mediator(s).

Several lines of evidence have indicated that many low CO_2 inducible genes are differentially regulated. Even though expression of *CAH1*, *CCP1*/*CCP2* and *CAH4*/*CAH5* appear to be correlated, they do not always follow the same expression pattern (Villarejo *et al*. 1996, 1997). Whereas *CAH1* could be induced in darkness, *CAH4*/*CAH5* and *CCP1*/*CCP2* apparently were induced only in the light. Inhibition of the glycolate pathway appeared to repress the expression of *CAH1* and *CAH4/CAH5*, but had no effect on the expression of *CCP1*/*CCP2*. Even being highly homologous, the *CCP1* and *CCP2* genes exhibited different timing in their expression upon limiting CO_2 induction (Chen *et al*. 1997). It seems likely that, even sharing some regulatory elements, these CO_2 responsive genes are regulated by divergent pathways. The differential regulation may reflect an association of these genes with different CO_2 acclimation states, or with functions in distinct aspects of limiting CO_2 acclimation.

PROSPECTS

As lack of detailed molecular composition is becoming a bottleneck for our understanding of the CCM in eukaryotic microalgae and its regulation, it is expected that more future work will continue to focus on identification of the functional components in the CCM. This task can be achieved by identification and characterization of non-acclimating mutants, global analysis of gene expression profiles, genome mining, and identification of proteins interacting with known CCM components. The recent availability of the genome sequences from *C. reinhardtii* and other microalgae, and molecular tools increasingly available for *C. reinhardtii* genetics, provide an immense step forward. Once candidate genes are available, their functions can be characterized by a reverse genetic approach, because RNA interference has been proven successful in silencing *C. reinhardtii* genes. Advances in the understanding of the CCM and its regulatory pathways in *C. reinhardtii* will give us better insight into the integrated network associated with carbon acquisition and utilization in photosynthetic organisms.

REFERENCES

Amoroso G, Sültemeyer D, Thyssen C, Fock HP (1998) Uptake of HCO_3^- and CO_2 in cells and chloroplasts from the microalgae *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta*. *Plant Physiology* **116**, 193-201

Badger MR, Spalding MH (2000) CO_2 acquisition, concentration and fixation in cyanobacteria and algae. In: Leegood RC, Sharkey TD, von Caemmerer S (Eds) *Photosynthesis: Physiology and Metabolism*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 369-397

Badger MR, Price GD (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *Journal of Experimental Botany* **54**, 609-662

Badger RM, Price GD, Long BM, Woodger FJ (2006) The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. *Journal of Experimental Botany* **57**, 249-265

Bozzo GG, Colman B, Matsuda Y (2000) Active transport of CO_2 and bicarbonate is induced in response to external CO_2 concentration in the green alga *Chlorella kessleri*. *Journal of Experimental Botany* **51**, 1341-1348

Bozzo GG, Colman B (2000) The induction of inorganic carbon transport and external carbonic anhydrase in *Chlamydomonas reinhardtii* is regulated by external CO_2 concentration. *Plant Cell and Environment* **23**, 1137-1144

Burow MD, Chen ZY, Mouton TM, Moroney JV (1996) Isolation of cDNA clones of genes induced upon transfer of *Chlamydomonas reinhardtii* cells to low CO_2. *Plant Molecular Biology* **31**, 443-448

Chen ZY, Burow MD, Mason CB, Moroney JV (1996) A low-CO_2-inducible gene encoding an alanine:α-ketoglutarate aminotransferase in *Chlamydomonas reinhardtii*. *Plant Physiol*ogy **112**, 677-684

Chen ZY, Lavigne LL, Mason CB, Moroney JV (1997) Cloning and overexpression of two cDNAs encoding the low-CO_2-inducible chloroplast envelope protein LIP-36 from *Chlamydomonas reinhardtii*. *Plant Physiol*ogy **114**, 265-273

Dionisio ML, Tsuzuki M, Miyachi S (1989a) Light requirement for carbonic anhydrase induction in *Chlamydomonas reinhardtii*. *Plant Cell Physiology* **30**, 207-213

Dionisio ML, Tsuzuki M, Miyachi S (1989b) Blue light induction of carbonic anhydrase activity in *Chlamydomonas reinhardtii*. *Plant Cell Physiology* **30**, 215-219

Dionisio-Sese ML, Fukuzawa H, Miyachi S (1990) Light-induced carbonic anhydrase expression in *Chlamydomonas reinhardtii*. *Plant Physiology* **94**, 1103-1110

Eriksson M, Karlsson J, Ramazanov Z, Gardeström P, Samuelsson G (1996) Discovery of an algal mitochondrial carbonic anhydrase: molecular cloning and characterization of a low-CO_2-induced polypeptide in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences USA* **93**, 12031-12034

Eriksson M, Villand P, Gardestrom P, Samuelsson G (1998) Induction and regulation of expression of a low-CO_2-induced mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiology* **116**, 637-641

Fujiwara S, Fukuzawa H, Tachiki A, Miyachi S (1990) Structure and differential expression of two genes encoding carbonic anhydrase in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences USA* **87**, 9779-9783

Fukuzawa H, Fujiwara S, Yamamoyo Y, Dionisio-Sese ML, Miyachi S (1990) cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*: regulation by environmental CO_2 concentration. *Proceedings of the National Academy of Sciences USA* **87**, 4383-4387

Fukuzawa H, Miura K, Ishizaki K, Kucho K, Saito T, Kohinata T, Ohyama K (2001) *Ccm1*, a regulatory gene controlling the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2. *Proceedings of the National Academy of Sciences USA* **98**, 5347-5352

Funke RP, Kovar JL, Weeks DP (1997) Intracellular carbonic anhydrase is essential to photosynthesis in *Chlamydomonas reinhardtii* at atmospheric le-

vels of CO_2. *Plant Physiology* **114**, 237-244

Galván A, Rexach J, Mariscal V, Fernández E (2002) Nitrite transport to the chloroplast in *Chlamydomonas reinhardtii*: molecular evidence for a regulated process. *Journal of Experimental Botany* **53**, 845-853

Giordano M, Norici A, Forssen M, Eriksson M, Raven JA (2003) An anaplerotic role for mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiology* **132**, 2126-2134

Giordano M, Beardall J, Raven JA (2005) CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *The Annual Review of Plant Biology* **56**, 99-131

Goyal A, Tolbert NE (1989) Uptake of inorganic carbon by isolated chloroplasts from air-adapted *Dunaliella*. *Plant Physiology* **89**, 1264-1269

Hanawa Y, Watanabe M, Karatsu Y, Fukuzawa H, Shiraiwa Y (2007) Induction of a high-CO_2-inducible, periplasmic protein, H43, and its application as a high-CO_2-responsive marker for study of the high-CO_2-sensing mechanism in *Chlamydomonas reinhardtii*. *Plant Cell Physiology* **48**, 299-309

Hanson DT, Franklin LA, Samuelsson G, Badger MR (2003) The *Chlamydomonas reinhardtii cia3* mutant lacking a thylakoid lumen-localized carbonic anhydrase is limited by CO_2 supply to Rubisco and not photosystem II function *in vivo*. *Plant Physiology* **132**, 2267-2275

Hewett-Emmett D, Tashian RE (1996) Functional diversity, conservation, and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. *Molecular Phylogenetics and Evolution* **5**, 50-77

Im CS, Grossman AR (2001) Identification and regulation of high light-induced genes in *Chlamydomonas reinhardtii*. *Plant Journal* **30**, 301-313

Im CS, Zhang Z, Shrager J, Chang CW, Grossman AR (2003) Analysis of light and CO_2 regulation in *Chlamydomonas reinhardtii* using genome-wide approaches. *Photosynthesis Research* **75**, 111-125

Kaplan A, Reinhold L (1999) The CO_2-concentrating mechanism of photosynthetic microorganisms. *The Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 539-570

Karlsson J, Hiltonen T, Husic HD, Ramazanov Z, Samuelsson G (1995) Intracellular carbonic anhydrase of *Chlamydomonas reinhardtii*. *Plant Physiology* **109**, 533-539

Karlsson J, Clarke AK, Chen ZY, Hugghins SY, Park YI, Husic HD, Moroney JV, Samuelsson G (1998) A novel α-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. *The EMBO Journal* **10**, 1208-1216

Katoh A, Lee KS, Fukuzawa H, Ohyama K, Ogawa T (1996a) *cemA* homologue essential to CO_2 transport in the cyanobacterium in *Synechocystis* PCC 6803. *Proceedings of the National Academy of Sciences USA* **93**, 4006-4010

Katoh A, Sonoda M, Katoh H, Ogawa T (1996b) Absence of light-induced proton extrusion in a *cotA*-less mutant of *Synechocystis sp*. strain PCC 6803. *Journal of Bacteriology* **178**, 5452-5455

Kuan J, Saier MH Jr. (1993) Expansion of the mitochondrial carrier family. *Research in Microbiology* **144**, 671-672

Kucho K, Ohyama K, Fukuzawa H (1999) CO_2-responsive transcriptional regulation of *CAH1* encoding carbonic anhydrase is mediated by enhancer and silencer regions in *Chlamydomonas reinhardtii*. *Plant Physiology* **121**, 1329-1338

Kucho K, Yoshioka S, Taniguchi F, Ohyama K, Fukuzawa H (2003) *Cis*-acting elements and DNA-binding proteins involved in CO_2-responsive transcriptional activation of *Cah1* encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiology* **133**, 783-793

Kustu S, Inwood W (2006) Biological gas channels for NH_3 and CO_2: evidence that Rh (Rhesus) proteins are CO_2 channels. *Transfusion Cliqinue et Biologique* **13**, 103-110

Mamedov TG, Suzuki K, Miura K, Kucho Ki K, Fukuzawa H (2001) Characteristics and sequence of phosphoglycolate phosphatase from a eukaryotic green alga *Chlamydomonas reinhardtii*. *The Journal of Biological Chemistry* **276**, 45573-45579

Marcus Y, Schuster G, Michaels A, Kaplan A (1986) Adaptation to CO_2 level and changes in the phosphorylation of thylakoid proteins during the cell cycle of *Chlamydomonas reinhardtii*. *Plant Physiology* **80**, 604-607

Marek LF, Spalding MH (1991) Changes in photorespiratory enzyme activity in response to limiting CO_2 in *Chlamydomonas reinhardtii*. *Plant Physiology* **97**, 420-425

Mariscal V, Moulin P, Orsel M, Miller AJ, Fernandez E, Galvan A (2006) Differential regulation of the Chlamydomonas *Nar1* gene family by carbon and nitrogen. *Protist* **157**, 421-33

Matsuda Y, Colman B (1995) Induction of CO_2 and bicarbonate transport in the green alga *Chlorella ellipsoidea*. II. Evidence for induction in response to external CO_2 concentration. *Plant Physiology* **108**, 253-260

Matsuda Y, Colman B (1996) Active uptake of inorganic carbon by *Chlorella saccharophila* is not repressed by growth in high CO_2. *Journal of Experimental Botany* **47**, 1951-1956

Mayo WP, Williams TG, Birch DG, Turpin DH (1986) Photosynthetic adaptation by *Synechococcus leopoliensis* in response to exogenous dissolved inorganic carbon. *Plant Physiology* **80**, 1038-1040

Mitra M, Lato SM, Ynalvez RA, Xiao Y, Moroney JV (2004) Identification of a new chloroplast carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiology* **135**, 173-182

Mitra M, Mason CB, Xiao Y, Ynalvez RA, Lato SM, Moroney JV (2005) The carbonic anhydrase gene families of *Chlamydomonas reinhardtii*. *Canadian Journal of Botany* **83**, 780-795

Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M (2004) A gene homologous to beta-type carbonic anhydrase is essential for the growth of *Corynebacterium glutamicum* under atmospheric conditions. *Applied Microbiology and Biotechnology* **63**, 592-601

Miura K, Yamano T, Yoshioka S, Kohinata T, Inoue Y, Taniguchi F, Asamizu E, Nakamura Y, Tabata S, Yamato KT, Ohyama K, Fukuzawa H (2004) Expression profiling-based identification of CO_2-responsive genes regulated by CCM1 controlling a carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiology* **135**, 1595-1607

Moroney JV, Husic HD, Tolbert NE (1985) Effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*. *Plant Physiology* **79**, 177-183

Moroney JV, Tolbert NE, Sears BB (1986) Complementation analysis of the inorganic carbon concentrating mechanism of *Chlamydomonas reinhardtii*. *Molecular and General Genetics* **204**, 199-203

Moroney JV, Kitayama M, Togasaki RK, Tolbert NE (1987) Evidence for inorganic carbon transport by intact chloroplasts of *Chlamydomonas reinhardtii*. *Plant Physiology* **83**, 460-463

Moroney JV, Husic HD, Tolbert NE, Kitayama M, Manuel LJ, Togasaki RK (1989) Isolation and characterization of a mutant of *Chlamydomonas reinhardtii* deficient in the CO_2 concentration mechanism. *Plant Physiology* **89**, 897-903

Moroney JV, Mason CB (1991) The role of the chloroplast in inorganic carbon acquisition by *Chlamydomonas reinhardtii*. *Canadian Journal of Botany* **69**, 1017-1024

Moroney JV, Somanchi A (1999) How do algae concentrate CO_2 to increase the efficiency of photosynthetic carbon fixation? *Plant Physiology* **119**, 9-16

NakamuraY, Kanakagiri S, Van K, He W, Spalding MH (2005) Disruption of a glycolate dehydrogenase gene in a high-CO_2-requiring mutant of *Chlamydomonas reinhardtii*. *Canadian Journal of Botany* **83**, 796-809

Palmieri F (1994) Mitochondrial carrier proteins. *FEBS Letters* **346**, 48-54

Palmqvist K, Sjoberg S, Samuelsson G (1988) Induction of inorganic carbon accumulation in the unicellular green algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*. *Plant Physiology* **87**, 437-442

Palmqvist K, Yu J-W, Badger MR (1994) Carbonic anhydrase activity and inorganic carbon fluxes in low and high Ci of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. *Physiologia Plantarum* **90**, 537-547

Park YI, Karlsson J, Rojdestvenski I, Pronina N, Klimov V, Oquist G, Samuelsson G (1999) Role of a novel photosystem II-associated carbonic anhydrase in photosynthetic carbon assimilation in *Chlamydomonas reinhardtii*. *FEBS Letters* **444**, 102-105

Price GD, Badger MR (1989) Expression of human carbonic anhydrase in the cyanobacterium *Synechococcus* PCC7942 creates a high CO_2-requiring phenotype. Evidence for a central role for carboxysomes in the CO_2 concentrating mechanism. *Plant Physiology* **91**, 505-513

Pollock SV, Prout DL, Godfrey AC, Lemaire SD, Moroney JV (2004) The *Chlamydomonas reinhardtii* proteins Ccp1 and Ccp2 are required for long-term growth, but are not necessary for efficient photosynthesis, in a low-CO_2 environment. *Plant Molecular Biology* **56**, 125-132

Pronina NA, Semenenko VE (1992) Role of the pyrenoid in concentration, generation and fixation of CO_2 in the chloroplast of microalgae. *Soviet Plant Physiology* **39**, 470-476

Ramazanov Z, Semenenko VE (1984) Oxygen-dependent induction of synthesis of the CO_2-dependent soluble form of carbonic anhydrase in *Chlorella* cells. *Soviet Plant Physiology* **31**, 344-349

Ramazanov Z, Cardenas J (1992) Involvement of photorespiration and glycolate pathway in carbonic anhydrase induction and inorganic carbon concentration in *Chlamydomonas reinhardtii*. *Physiologia Plantarum* **84**, 502-508

Ramazanov Z, Mason CB, Geraghty AM, Spalding MH, Moroney JV (1993) The low CO_2-inducible 36 kDa protein is localized to the chloroplast envelope of *Chlamydomonas reinhardtii*. *Plant Physiology* **101**, 1195-1199

Raven JA (1997) CO_2 concentrating mechanisms: a role for thylakoid lumen acidification? *Plant Cell and Environment* **20**, 147-154

Rawat M, Moroney JV (1991) Partial characterization of a new isozyme of carbonic anhydrase isolated from *Chlamydomonas reinhardtii*. *The Journal of Biological Chemistry* **266**, 9719-9723

Rawat M, Moroney JV (1995) The regulation of carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase activase by light and CO_2 in *Chlamydomonas reinhardtii*. *Plant Physiology* **109**, 937-944

Rea PA (2007) Plant ATP-binding cassette transporters. *The Annual Review of Plant Biology* **58**, 347-375

Rolland N, Dorne AJ, Amoroso G, Sültemeyer DF, Joyard J, Rochaix JD (1997) Disruption of the plastid *ycf10* open reading frame affects uptake of inorganic carbon in the chloroplast of *Chlamydomonas*. *The EMBO Journal* **16**, 6713-6726

Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kaplan A, Ogawa T (2002) Genes essential to sodium-dependent bicarbonate transport in cyanobacteria. Function and phylogenetic analysis. *The Journal of Biological Chemistry* **277**, 18658-18664

Shiraiwa Y, Kobayashi H (1999) Characterization of a high-CO_2-inducible 43-KD-polypeptide in *Chlamydomonas reinhardtii*. *Plant Cell Physiology* **40**,

s123

Smith KS, Jakubzick C, Whittam TS, Ferry JG (1999) Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. *Proceedings of the National Academy of Sciences USA* **96**, 15184-15189

Soupene E, King N, Feild E, Liu P, Niyogi KK, Huang CH, Kustu S (2002) Rhesus expression in a green alga is regulated by CO_2. *Proceedings of the National Academy of Sciences USA* **99**, 7769-7773

Soupene E, Inwood W, Kustu S (2004) Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO_2. *Proceedings of the National Academy of Sciences USA* **101**, 7787-7792

Spalding MH, Ogren WL (1982) Photosynthesis is required for induction of the CO_2-concentrating system in *Chlamydomonas reinhardtii*. *FEBS Letters* **145**, 41-44

Spalding MH, Spreitzer RJ, Ogren WL (1983a) Reduced inorganic carbon transport in a CO_2-requiring mutant of *Chlamydomonas reinhardtii*. *Plant Physiology* **73**, 273-276

Spalding MH, Spreitzer RJ, Ogren WL (1983b) Carbonic anhydrase deficient mutant of *Chlamydomonas* requires elevated carbon dioxide concentration for photoautotrophic growth. *Plant Physiology* **73**, 268-272

Spalding MH, Jeffrey M (1989) Membrane-associated polypeptides induced in *Chlamydomonas* by limiting CO_2 concentrations. *Plant Physiology* **89**, 133-137

Spalding MH (1998) CO_2 acquisition: acclimation to changing carbon availability. In: Rochaix J-D, Goldschmidt-Clermont M, Merchant S (Ed) *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 529-547

Spalding MH, Van K, Wang Y, Nakamura Y (2002) Acclimation of *Chlamydomonas* to changing carbon availability. *Functional Plant Biology* **29**, 221-230

Spreitzer RJ, Mets L (1981) Photosynthesis-deficient mutants of *Chlamydomonas reinhardtii* with associated light-sensitive phenotypes. *Plant Physiology* **67**, 565-569

Sültemeyer DF, Miller AG, Espie GS, Fock HP, Canvin DT (1989) Active CO_2 transport by the green alga *Chlamydomonas reinhardtii*. *Plant Physiology* **89**, 1213-1219

Sültemeyer DF, Fock HP, Canvin DT (1991) Active uptake of inorganic carbon by *Chlamydomonas reinhardtii*: evidence for simultaneous transport of HCO_3^- and CO_2 and characterization of active CO_2 transport. *Canadian Journal of Botany* **69**, 995-1002

Suzuki K, Spalding MH (1989) Adaptation of *Chlamydomonas reinhardtii* high-CO_2-requiring mutants to limiting-CO_2. *Plant Physiology* **90**, 1195-1200

Turkina MV, Blanco-Rivero A, Vainonen JP, Vener AV, Villarejo A (2006) CO_2 limitation induces specific redox-dependent protein phosphorylation in *Chlamydomonas reinhardtii*. *Proteomics* **6**, 2693-2704

van Hunnik E, **Amoroso G**, **Sultemeyer D** (2002) Uptake of CO_2 and bicarbonate by intact cells and chloroplasts of *Tetraedron minimum* and *Chlamydomonas noctigama*. *Planta* **215**, 763-769

van Hunnik E, Sultemeyer D (2002) A possible role for carbonic anhydrase in the lumen of chloroplast thylakoids in green algae. *Functional Plant Biology* **29**, 243-249

Van K, Spalding MH (1999) Periplasmic carbonic anhydrase (*Cah1*) structural gene mutant in *Chlamydomonas reinhardtii*. *Plant Physiology* **120**, 757-764

Van K, Wang Y, Nakamura Y, Spalding MH (2001) Insertional mutants of *Chlamydomonas reinhardtii* that require elevated CO_2 for survival. *Plant Physiology* **127**, 607-614

Vance P, Spalding MH (2005) Growth, photosynthesis and gene expression in *Chlamydomonas* over a range of CO_2 concentrations and CO_2/O_2 ratios: CO_2 regulates multiple acclimation states. *Canadian Journal of Botany* **83**, 820-833

Villand P, Eriksson M, Samuelsson G (1997) Carbon dioxide and light regulation of promoters controlling the expression of mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. *Biochemistry Journal* **327**, 51-57

Villarejo A, Garcia Reina G, Ramazanov Z (1996) Regulation of the low-CO_2-inducible polypeptides in *Chlamydomonas reinhardtii*. *Planta* **199**, 481-485

Villarejo A, Martinez F, Ramazanov Z (1997) Effect of aminooxyacetate, an inhibitor blocking the glycolate pathway, on the induction of a CO_2-concentrating mechanism and low-CO_2-inducible polypeptides in *Chlamydomonas reinhardtii* (Chlorophyta). *Europen Journal of Phycology* **32**, 141-145

Villarejo A, Rolland N, Martinez F, Sültemeyer DF (2001) A new chloroplast envelope carbonic anhydrase activity is induced during acclimation to low inorganic carbon concentrations in *Chlamydomonas reinhardtii*. *Planta* **213**, 286-295

Villarejo A, Shutova T, Moskvin O, Forssen M, Klimov VV, Samuelsson G (2002) A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution. *The EMBO Journal* **15**, 1930-1938

Wang Y, Spalding MH (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 USA* **103**, 10110-10115

Winder TL, Anderson JC, Spalding MH (1992) Translational regulation of the large and small subunits of ribulose bisphosphate carboxylase/oxygenase during induction of the CO_2 concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiology* **98**, 1409-1414

Woodger FJ, Badger MR, Price GD (2005) Sensing of inorganic carbon limitation in *Synechococcus* PCC7942 is correlated with the size of the internal inorganic carbon pool and involves oxygen. *Plant physiology* **139**, 1959-1969

Xiang YB, Zhang J, Weeks DP (2001) The *Cia5* gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences USA* **98**, 5341-5346

Yoshioka S, Taniguchi F, Miura K, Inoue T, Yamano T, Fukuzawa H (2004) The novel Myb transcription factor LCR1 regulates the CO_2-responsive gene *Cah1*, encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *The Plant Cell* **16**, 1466-1477