Microalgal carbon-dioxide-concentrating mechanisms: *Chlamydomonas* inorganic carbon transporters

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Abstract

Aquatic photosynthetic micro-organisms have adapted to the variable and often-limiting availability of CO$_2$ and inorganic carbon (Ci) in general, by development of inducible CO$_2$-concentrating mechanisms (CCMs) that allow them to optimize carbon acquisition. Both microalgal and cyanobacterial CCMs function to facilitate CO$_2$ assimilation when Ci is limiting via active Ci uptake systems to increase internal Ci accumulation and carbonic anhydrase activity to provide elevated internal CO$_2$ concentrations through the dehydration of accumulated bicarbonate. These CCMs have been studied over several decades, and details of the cyanobacterial CCM function have emerged over recent years. However, significant advances in understanding of the microalgal CCM have been more recent. With the aid of mutational approaches and the availability of multiple microalgal genome sequences, an integrated picture of the functional components of the microalgal CCM is emerging, together with the molecular details regarding the function and regulation of the CCM. This review will focus on the recent advances in identifying and characterizing the Ci transport components of the microalgal CCM, especially in the model organism *Chlamydomonas reinhardtii* Dangeard.

Key words: Acclimation, algae, bicarbonate, carbonic anhydrase, CCM, *Chlamydomonas reinhardtii*, photosynthesis.

Introduction

Carbon dioxide (CO$_2$) is arguably one of the most important gases on earth, even though it is present in only relatively low concentrations in the atmosphere. CO$_2$ is the major and often limiting substrate for photosynthetic carbon assimilation in plants and other photosynthetic organisms. The relatively low atmospheric CO$_2$ concentration represents a stress frequently confronted by plants, largely because of the low CO$_2$ affinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key enzyme in net carbon assimilation. Although CO$_2$ is limiting for photosynthetic rate, typically it is not limiting for growth of terrestrial plants because other factors, such as water or nitrogen availability, are more limiting than carbon. Nonetheless, strategies have evolved in some groups of plants to maintain a high photosynthetic rate despite the low atmospheric CO$_2$ concentration, all of which function by raising the CO$_2$ concentration at the site of Rubisco, including the C$_4$ carbon cycles operating in C$_4$ photosynthetic metabolism and Crassulacean acid metabolism (CAM).

The overall global atmospheric CO$_2$ concentration remains relatively constant over the short term. However, *Chlamydomonas reinhardtii* Dangeard and other aquatic or soil-borne photosynthetic organisms can be exposed to dramatic long-term and short-term variability over time and space in the supply of dissolved inorganic carbon (Ci: CO$_2$, HCO$_3^-$, and CO$_3^{2-}$), resulting from variability in sediment or soil respiration, the impact of pH on the relative distribution between dissolved CO$_2$ and bicarbonate, and the $10^4$ slower rate of diffusion of CO$_2$ in water relative to air. In the face of evolutionary selective pressure, organisms have developed mechanisms to detect changes in their environment, facilitating acclimational adjustments of their metabolism and physiology, and rapid acclimation to Ci supply enable *C. reinhardtii* and other aquatic photosynthetic organisms to survive these large Ci concentration fluctuations. Underlying this acclimation is an inducible system, often called a CO$_2$-concentrating mechanism (CCM), to concentrate CO$_2$ (Ci) internally by a Ci transport mechanism fundamentally different from the C$_4$ carbon cycles found in some terrestrial plants.
The CCM

The most extensively studied aquatic CCMs are those of cyanobacteria and microalgae (reviewed in Kaplan and Reinhold, 1999; Badger and Spalding, 2000; Giordano et al., 2005). Although, over several years, significant advances have been made in understanding the cyanobacterial CCMs and, to a lesser extent, the microalgal CCMs, many components are still unknown or uncharacterized. Recent developments in genomic and genetic approaches have brought new insights into the nature of the microalgal CCM and its regulation. This is particularly true for the CCM of *C. reinhardtii*, a unicellular green alga and robust genetic model system (Harris, 2001), since the *C. reinhardtii* genome sequence was recently completed.

The cyanobacterial and microalgal CCMs both use energy-dependent, active Ci transport to increase intracellular CO₂ concentrations at the site of Rubisco localization, facilitating high rates of photosynthetic CO₂ fixation even when the concentration of external Ci is low (Badger et al., 1980). Ci transport results in intracellular bicarbonate accumulation, so the CCMs also depend on colocalization of carbonic anhydrase (CA) activity with or near Rubisco to catalyse dehydration of bicarbonate and provide near-saturating CO₂ concentrations for carboxylation of RuBP. Thus the known essential components of the microalgal CCM (reviewed in Spalding, 1998; Kaplan and Reinhold, 1999; Badger and Spalding, 2000) include at least active Ci transport for intracellular bicarbonate accumulation and internal CA to supply CO₂ to Rubisco by dehydration of the accumulated bicarbonate (Badger et al., 1980; Spalding et al., 1983a, b; Moroney et al., 1987b).

Multiple acclimation states

As indicated above, the CCM of *C. reinhardtii* and other microalgae is activated only when the CO₂ supply is limited, and during the acclimation, rapid changes in gene expression and biochemical events occur, which are believed to be regulated by a signal transduction pathway not yet well-explored. Thus much of the research directed at understanding the CCM and the acclimation of microalgae to limiting CO₂ focused on two physiological states: elevated CO₂ (typically 1–5% CO₂ in air; no CCM induction) and limiting CO₂ (typically air levels of CO₂, 0.03%, or below; CCM induced). These two, well-characterized microalgal CO₂ assimilation states exhibit photosynthetic kinetics that mimic those of C₃ and C₄ higher plants, respectively. Although only these two CO₂-regulated physiological states are well-characterized, an additional physiological state was initially most clearly demonstrated by the characteristics of the *C. reinhardtii* pmp1 mutant, which grows in 5% CO₂, dies in air levels of CO₂, yet grows nearly as well as wild-type (WT) cells in less than 0.01% CO₂ (Van et al., 2001; Spalding et al., 2002). These unusual growth characteristics demonstrated that an additional CO₂-regulated acclimation state exists with a transition somewhere between air levels of CO₂ (~0.04%) and 0.01% CO₂, and demonstrated that a multistaged regulatory programme, controlled by CO₂ levels, is critical for the acclimation of *C. reinhardtii* to limiting CO₂ (Vance and Spalding, 2005).

Further characterizations of CO₂-concentration-dependent acclimation responses in *C. reinhardtii* using carefully controlled airlift bioreactors (Vance and Spalding, 2005) confirmed an additional CO₂-dependent acclimation state in very low CO₂ concentrations and established the approximate CO₂ concentration limits for the three CO₂ assimilation states: high CO₂ (H-CO₂), ≥0.5% CO₂; low CO₂ (L-CO₂), 0.4–0.03% CO₂; and very-low CO₂ (VL-CO₂), ≤0.01% CO₂. The defining features of these states were determined to be: H-CO₂, lack of limiting-CO₂-inducible gene expression, photosynthetic *K₅₀(CO₂)* similar to that of the *K₅₀(CO₂)* of Rubisco; L-CO₂, induction of limiting-CO₂-regulated genes and marked decrease in photosynthetic *V₅₀(CO₂)*; VL-CO₂, a decrease in the photosynthetic *V₅₀(CO₂)* and further decrease in the *K₅₀(CO₂)*. The abundance of specific transcripts associated with CO₂ limitation showed an increase in both L-CO₂ and VL-CO₂, suggesting that differences between these two states may arise from a quantitative difference in transcript levels (Vance and Spalding, 2005), although there are likely to be other, as yet unidentified genes with qualitative expression characteristics that differentiate the two states.

Carbonic anhydrases

One of the first essential components of the *C. reinhardtii* CCM identified was a thylakoid lumen CA (CAH3) that is responsible for dehydration of internally accumulated bicarbonate (Funk et al., 1997; Karlsson et al., 1998). In the absence of CAH3, internal Ci, presumably in the form of bicarbonate, accumulates to high levels but is not available for CO₂ assimilation (Spalding et al., 1983a; Hanson et al., 2003).

The genome sequence of *C. reinhardtii* (http://genome.jgi-psf.org/Chlr3/Chlr3.home.html) has revealed an unexpectedly large number of encoded CAs, but so far only the CAH3 gene product has been demonstrated to be an essential component of the CCM. Ten putative CA genes encoded on the *C. reinhardtii* genome have been identified, of which only five (CAH1–CAH5) were known prior to the development of genomics tools for *C. reinhardtii*, but many of which may contribute to CCM function in some way. Interestingly, of the ten putative CAs identified in the *C. reinhardtii* genome, all three of the major CA evolutionary lineages (Hewett-Emmett and Tashian, 1996) are represented: three (CAH1, CAH2, CAH3) are α-type CAs, six (CAH4, CAH5, CAH6, CAH7, CAH8, CAH9) are β-type CAs, and one (CAH10) is a γ-type CA.
CAH7, CAH8, CAH9) are β-type CAs, and one (CAG3) is a γ-type CA.

CAH1 encodes a periplasmic CA and was one of the first limiting CO2-induced genes identified in *C. reinhardtii* (Fukuzawa *et al.*, 1990). Although CAH1 expression is induced only in limiting CO2, the expression of a very similar, closely-linked periplasmic CA gene, CAH2, is induced only in elevated CO2 (>1% CO2; Fujiwara *et al.*, 1990; Rawat and Moroney, 1991). The roles of CAH1 and CAH2 in the CCM and in the three CO2-regulated acclimation states are not entirely clear, but CAH1, at least does not appear to be essential for CCM function (Van and Spalding, 1999). The corresponding genes are clustered with other genes possibly important for CCM (see below).

As mentioned previously, CAH3 is located in the thylakoid lumen and its importance in the function of the CCM has been well established (Hanson *et al.*, 2003). The roles of two limiting-CO2-induced mitochondrial CA isoforms, CAH4 and CAH5, are not clear, but they have been implicated in pH buffering in mitochondria and in anapleurotic reactions (Eriksson *et al.*, 1996; Giordano *et al.*, 2003).

The more recently identified CA isoforms are CAH6, CAH7, CAH8, CAH9, and CAG3 (GLP1). The chloroplast stromal location of CAH6 suggests that it may be involved in trapping Ci as bicarbonate in the alkaline stroma (Mitra *et al.*, 2004). Although CAG3 was identified as a putative γ-type CA, CA activity could not be confirmed when the gene was over-expressed (Mitra *et al.*, 2005). Also, other than the confirmation of CA activity in over-expressed CAH8, the cellular locations and functions of the other three CA isoforms have not yet been determined.

**Ci transport**

Arguably, among the most critical elements of the microalgal CCM are the Ci uptake systems responsible for accumulation of Ci internally to a level many-fold higher than that of the external medium (Spalding, 1998). For *C. reinhardtii*, the ability to take up both CO2 and HCO3− during steady-state photosynthesis has been demonstrated in whole cells. Carbon isotope disequilibrium studies have established that the major flux of Ci into *C. reinhardtii* cells occurs through direct uptake of CO2 across the plasma membrane via an active process (Marcus *et al.*, 1984; Sültemeyer *et al.*, 1989; Badger *et al.*, 1994; Palmqvist *et al.*, 1994), although the data cannot distinguish between active CO2 transport at the plasma membrane and active Ci transport at the chloroplast envelope following CO2 diffusion into the cell. There also is good evidence for direct bicarbonate transport across the plasma membrane, but generally at a lower rate than CO2 influx (Sültemeyer *et al.*, 1989; Thielmann *et al.*, 1990; Badger *et al.*, 1994; Palmqvist *et al.*, 1994; Amoroso *et al.*, 1998). Thus it appears that both of the predominant Ci species present in the aquatic environment (CO2 and HCO3−) are used by *C. reinhardtii*, although the cells exhibit a preference for CO2, at least under the conditions used in most of these studies. One key unknown with regard to conclusions about the Ci species transported is whether the microalgal cultures used in these studies were acclimated to L-CO2 or VL-CO2, since it is possible that the dominant Ci transport systems may differ between these two acclimation states.

At the level of the plasma membrane, photosynthetically-driven net uptake of Ci (mainly as CO2 and HCO3−) represents the largest nutrient flux that these cells encounter. Regardless of which form of Ci (CO2 or HCO3−) is presented to or taken up by the cell, HCO3− must be the major species accumulated internally, even though only CO2 can serve as the substrate for Rubisco. This conclusion is based on evidence from mutants with lesions in the thylakoid lumen CA, CAH3, which over-accumulate Ci but still are CO2-limited in photosynthesis (Spalding *et al.*, 1983a; Moroney *et al.*, 1986, 1987b; Suzuki and Spalding, 1989; Hansen *et al.*, 2003). Since the over-accumulated Ci in CAH3 mutants is unavailable to Rubisco, which uses CO2 as its substrate, the plastid location of CAH3 argues for active accumulation of bicarbonate in the chloroplast. However, it is unclear whether bicarbonate accumulation occurs throughout the chloroplast or is restricted to a compartment within the plastid.

Specific transporters responsible for active Ci uptake and accumulation have not been definitively identified yet, but the fact that active Ci transport occurs across both the plasma membrane and the chloroplast envelope has been confirmed (Palmqvist *et al.*, 1988; Sültemeyer *et al.*, 1989, 1998; Amoroso *et al.*, 1998). Direct bicarbonate uptake across the plasma membrane would certainly require a protein carrier, as would active CO2 transport, if it occurs. However, if CO2 enters the cells by diffusion across the plasma membrane in response to active Ci transport from the cytosol into the chloroplast, a plasma membrane transporter for CO2 would not be needed. Thus the physiological evidence suggests that *C. reinhardtii* probably has at least one and possibly two Ci transporters or transport complexes in its plasma membrane. No plasma membrane transport proteins have been unambiguously identified, although at least one potential candidate for this function has been identified (see below).

Chloroplast envelope Ci transport has been demonstrated in intact, isolated chloroplasts from *C. reinhardtii* and other green microalgae (Moroney *et al.*, 1987a; Sültemeyer *et al.*, 1988; Goyal and Tolbert, 1989; Amoroso *et al.*, 1998; van Hunnik *et al.*, 2002). Difficulties with obtaining high yields of functional chloroplasts precluded extensive investigation of the Ci species transported in many of these studies, except those of Amoroso *et al.* (1998) and van Hunnik *et al.* (2002).
Because isolated *C. reinhardtii* chloroplasts actively accumulate Ci in the chloroplast stroma as bicarbonate regardless of which species of Ci is transported from the cytosol, bicarbonate was postulated as the primary substrate for chloroplast Ci uptake (Moroney et al., 1987a; Moroney and Mason, 1991). However, apparent active transport of both CO₂ and bicarbonate by isolated chloroplasts from *C. reinhardtii*, *Dunaliella tertiolecta*, *Tetraedron minimum*, and *C. noctigama* (Amoroso et al., 1998; van Hunnik et al., 2002), suggests that a mechanism must exist for nearly quantitative conversion to bicarbonate of the CO₂ actively taken up into the chloroplast. The recently identified stromal CA isozyme, CAH₆, may provide the mechanism for this rapid and complete conversion of CO₂ to bicarbonate in the alkaline stroma.

The mechanism of transport is not understood for uptake of either bicarbonate or CO₂ across either the plasma membrane or the inner chloroplast envelope. There is evidence for the inhibition of Ci uptake by the H⁺-ATPase inhibitor vanadate both in whole cells and chloroplasts, suggesting that activity of a vanadate-sensitive H⁺-ATPase is essential for transport across both the plasma membrane and the chloroplast inner envelope (Palmeqvist et al., 1988; Goyal and Tolbert, 1989; Thielmann et al., 1990; Karlsson et al., 1994). The characteristics of the *pmp1* mutant, which appears to lack Ci transport completely (Spalding et al., 1983b), have raised the intriguing question of how this single mutation could essentially eliminate all Ci transport and accumulation in *C. reinhardtii*, in the light of substantial evidence of Ci transport, possibly of multiple Ci species, across both the plasma membrane and the chloroplast envelope. Even though the protein defective in this important mutant has been identified (Wang and Spalding, 2006), the answer to this intriguing question still is not entirely clear (see below).

**Mutant *pmp1/ad1*, LCIB, and the LCIB family**

The role played by the *C. reinhardtii* mutant *pmp1* in recognizing the multi-tiered CO₂ acclimation responses of this microalga was discussed above. However, this mutant, characterized as deficient in Ci transport, was also important in defining active Ci transport as one of the essential functional components of the CCM (Spalding et al., 1983b). The *pmp1* mutant, touted as demonstrating the Ci transport requirement in the CCM, was identified as a conditional lethal in air levels of CO₂ and was demonstrated to be deficient in Ci transport (Spalding et al., 1983b). More recent studies revealed that *pmp1* is deficient in Ci transport only in L-CO₂ but not in VL-CO₂ growth conditions, demonstrating that this mutant is defective in a specific component controlling the active accumulation of Ci in L-CO₂ but not in VL-CO₂ (Van et al., 2001; Spalding et al., 2002).

Recently, the gene defective in *pmp1* was identified by taking advantage of its novel ‘air-dier’ (*ad*) phenotype and the *C. reinhardtii* draft genome sequence (http://genome.jgi-psf.org/Chlr3/Chlr3.home.html). A tagged mutant *ad1* was found to be allelic to *pmp1*, and the defect in this mutant was shown to be in LCIB (Wang and Spalding, 2006), a gene previously identified as a limiting CO₂-induced gene (Miura et al., 2004). BLAST searches and domain searches with LCIB revealed no significant recognizable domains nor significant homologies, except a single gene each in *Ostreococcus taurii*, *O. lucimarinus*, and *Volvox carterii* (Grossman et al., 2007), as well as three additional genes in the *C. reinhardtii* genome: a similar CO₂ responsive gene, LCIC (Miura et al., 2004), and two previously unreported genes, LCID and LCIE (Wang and Spalding, 2006). Within *C. reinhardtii*, LCIB and LCIC are very similar in amino acid sequence (57% identity; 73% similarity), as are LCID and LCIE (71% identity; 78% similarity), and these two protein pairs also share substantial similarity with each other (40–44% identity; 62–65% similarity), thus constituting an LCIB protein family (Wang and Spalding, 2006). Although the paucity of genomic information from other aquatic photosynthetic eukaryotes limits conclusions, this small, unique gene family appears to be restricted to green algae, perhaps to green microalgae with CCMs.

In WT *C. reinhardtii*, LCIB and LCIC show similar CO₂ expression patterns: very low expression in H-CO₂ and high expression in both L-CO₂ and VL-CO₂ (Wang and Spalding, 2006). Low constitutive expression of LCIB and LCIC in H-CO₂ and in *cia5* is consistent with reported phenotypic penetrance of the *pmp1* lesion in H-CO₂ (Suzuki and Spalding, 1989). The LCID expression pattern in WT was similar to but much lower in mRNA abundance than LCIB and LCIC, with the mRNA being undetectable in H-CO₂. LCIE expression has not been detected by standard northern analysis, although a partial cDNA was recovered, indicating it is expressed (Wang and Spalding, 2006).

Physiological and biochemical characteristics of *pmp1* and *ad1* argue that LCIB is involved in active Ci transport, but LCIB seems an unlikely candidate for a stand-alone Ci transporter because of its predicted soluble nature and lack of any predicted transmembrane regions. Because of the strong evidence linking *pmp1* to Ci transport and accumulation, it is likely that LCIB, and perhaps other members of the LCIB family, is either a soluble subunit in an as yet unidentified Ci transport complex or acts as a regulator of Ci transport, either alone or in combination with other proteins.

On the other hand, Miura et al. (2004) suggested *pmp1* to be a regulatory mutant defective in components responsible for inducing or up-regulating expression of Ci transport genes. This suggestion was based on their observation of decreased or absent mRNA-level expression in
pmp1 of a number of Ci transporter candidates, including LCIA and HLA3/MRP1 (see below). However, in an extensive investigation of this observation, Wang and Spalding (2006) reported expression of LCIA and HLA3/MRP1 in pmp1 (and adl) to be only minimally reduced, if at all, relative to WT. Thus it appears unlikely that LCIB acts in the regulation of Ci transporters at the level of transcription or transcript stability, although its action as a post-translational regulator of Ci transport remains a viable possibility.

The air dier growth phenotype of pmp1 and adl clearly indicates that an LCIB-associated Ci transport system is essential for L-CO2 acclimation of C. reinhardtii, but that, although LCIB is expressed in VL-CO2 and even H-CO2 at a low level, as well as in L-CO2, it clearly is not essential under such conditions. In addition to understanding the role of LCIB, and other members of the LCIB family, in the L-CO2 Ci transport system(s), it also remains to be discovered what Ci transport systems are functional and essential in VL-CO2.

Additional Ci transport candidates

In addition to the role LCIB plays in Ci transport, there are clearly additional Ci transport components yet to be discovered. The draft C. reinhardtii genome sequence has facilitated identification of several candidate Ci transporter genes based on a number of criteria, including location, similarity to other transporters, and regulation of expression by Ci abundance. Some candidates, such as CCP1, CCP2, and LC11, have previously been identified as induced or up-regulated by limiting CO2 and encode putative plastid envelope proteins (Burow et al., 1996; Chen et al., 1997). Another plastid envelope protein encoded by a plastid gene, ycf10, has been functionally implicated in chloroplast Ci uptake (Rolland et al., 1997), as will be discussed below. Additional candidate Ci transporter genes include HLA3/MRP1 and LCIA, both of which are induced under limiting-CO2 conditions and which encode new members of well-known transporter families (Im and Grossman, 2001; Miura et al., 2004). The currently identified Ci transport candidates are indicated in Table 1 and will be discussed in more detail below.

Transport into the cell

As discussed earlier, substantial evidence demonstrates both the active uptake of CO2 and the active transport of bicarbonate into cells of C. reinhardtii and other microalgae. Active bicarbonate transport surely requires a protein carrier, while active CO2 uptake may reflect a carrier-mediated process or diffusion down a gradient created by active transport of cytosolic Ci into the chloroplast. Therefore, there must be at least one and possibly several plasma membrane Ci transporters, depending on redundancy level and whether L-CO2 versus VL-CO2 CCM activity involves separate carriers. So far only two plasma membrane Ci transport candidates have been identified in C. reinhardtii, HLA3/MRP1 and LCI1. HLA3 was originally identified as a high-light-induced gene but, upon closer examination, was characterized as being induced only during CO2-limited growth (Im and Grossman, 2001) and under the control of CIA5 (CCM1), the ‘master regulator’ of limiting Ci acclimation responses (Fukuzawa et al., 2001; Xiang et al., 2001). The HLA3 gene product is a putative ATP-binding cassette (ABC) type transporter, a category which includes ubiquitous membrane proteins responsible for transporting a wide variety of substrates (Rea, 2007), and falls into the multidrug-resistance-related proteins (MRP) subfamily. As such, this gene has also been named MRP1 (Miura et al., 2004; Hanikenne et al., 2005).

Although HLA3 was initially suggested to be a chloroplast-targeted protein (Im and Grossman, 2001), the deduced HLA3 sequence is predicted by both iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/) and Target-P (http://www.cbs.dtu.dk/services/TargetP/) to be targeted to the secretory pathway and possibly to be located in the plasma membrane. Because of its CIA5-regulated expression only under limiting Ci conditions, HLA3/MRP1 has been suggested as a candidate Ci transporter. One of the characterized Ci transporters in cyanobacteria, BCT1, is also a member of the ABC transporter family, although BCT1 is a bacterial type, multimeric, four subunit complex encoded by the cmpABCD operon (Badger and Price, 2003; Badger et al., 2006), rather than an MRP-type, single protein ABC transporter like HLA3/MRP1. The MRP subfamily includes members with a wide variety of confirmed or proposed substrates (Rea, 2007), so the suggested transport of Ci by HLA3/MRP1 is not unreasonable. Interestingly, MRP-associated transport is often sensitive to inhibition by vanadate, which inhibits the ATPase function responsible for the energization of transport in ABC-type transporters (Rea, 2007). This vanadate sensitivity, in combination with the reported vanadate inhibition of C. reinhardtii Ci transport (Palmqvist et al., 1988), also reinforces HLA3/MRP1 as a Ci transport candidate. As the better characterized of only two plasma membrane Ci transport candidates identified so far, HLA3/MRP1 should continue to attract substantial interest and investigation.

LCI1 was identified initially as a limiting-CO2-induced gene whose product was predicted to have four transmembrane segments (Burow et al., 1996). As part of its regulation by limiting CO2, LCI1 is regulated by CIA5 and by LCR1 (Miura et al., 2004; Yoshioka et al., 2004). Although little is known about this gene or gene product, the prediction by iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/) and Target-P (http://www.cbs.dtu.dk/services/TargetP/) that LCI1 contains a signal peptide and is
probably a plasma membrane protein makes it interesting as a possible Ci transport candidate. LCII also has no identifiable homologues or recognizable domains, so appears to represent a unique membrane protein. The function of this gene product in Ci transport, the CCM or another aspect of limiting Ci acclimation will require further investigation.

**Transport into the chloroplast**

There is also substantial evidence demonstrating active Ci transport at the chloroplast envelope, but so far no Ci transport components have been unambiguously identified or physically characterized. The substrate specificity of chloroplast Ci uptake has not been established, so it is difficult to speculate as to the number and type of Ci carriers to expect in the chloroplast envelope. The first Ci transport candidate was the product of the C. reinhardtii ycf10 plastid open reading frame, which displays sequence homology with the plastid-encoded CemA protein from plants and the cyanobacterial pxA (formerly CotA) gene product and is localized in the inner chloroplast envelope. Recognition of a possible role for the ycf10 gene product in Ci transport was based on disruption of the ycf10 open reading frame, which resulted in decreased Ci uptake in C. reinhardtii mutant plants and the cyanobacterial plastid open reading frame, which displays sequence similarity to the C. reinhardtii ycf10 gene product, which, like CemA and CotA, is a membrane protein with four transmembrane domains, is considerably larger than its higher plant homologue because of a long insertion that separates the conserved N and C termini, suggesting that the gene product may have a different function in higher plants and microalgae. The cyanobacterial pxA gene product, which is involved in light-induced Na\(^+\)-dependent proton extrusion and is altered in mutants defective in CO\(_2\) transport and proton extrusion, is thought to play an important role in proton exchange to maintain electrical and pH homeostasis during uptake of CO\(_2\), HCO\(_3^-\), and NO\(_3^-\) in cyanobacteria (Katoh et al., 1996a, b; Sonoda et al., 1998). It is not clear how the related ycf10 gene product functions in C. reinhardtii chloroplast Ci transport, although a role similar to that of the pxA gene product in cyanobacteria is likely (Rolland et al., 1997).

Other C. reinhardtii proteins considered candidates for a role in chloroplast Ci transport include the products of three limiting-CO\(_2\)-inducible-genes, all of which are predicted to be transmembrane proteins targeted to the plastid. These candidate genes include LCIA, CCP1, and CCP2. LCIA, also named NAR1.2, was identified as a limiting-CO\(_2\)-inducible gene in a large-scale analysis of gene expression profiles in C. reinhardtii (Miura et al., 2004). LCIA encodes a protein belonging to the NAR family of C. reinhardtii (Miura et al., 2004; Galvan et al., 2002; Mariscal et al., 2006). NARI genes encode members of the Formate/Nitrite Transporter (FNT) family (Rexach et al., 2000), which are found extensively in bacteria and, to a lesser extent in eukaryotic organisms such as fungi, yeast, algae, and protozoa, but so far not in plants. LCIA encodes a 366 amino acid product with a predicted chloroplast transit peptide and six transmembrane

### Table I. Candidate Ci transporter genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein location</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ycf10</td>
<td>Chloroplast inner envelope</td>
<td>Plastid-encoded gene related to pxCa of cyanobacteria and CemA of plant plastids; possible function in H(^+) extrusion from stroma</td>
<td>Rolland et al., 1997</td>
</tr>
<tr>
<td>LCIA</td>
<td>Chloroplast inner envelope</td>
<td>FNT family of transporters; limiting-Ci induced; reported to transport bicarbonate when expressed in Xenopus</td>
<td>Miura et al., 2004; Mariscal et al., 2006</td>
</tr>
<tr>
<td>LCIB</td>
<td>Chloroplast stroma</td>
<td>Unknown function; LCIB gene family unique to green microalgae; limiting-Ci induced; Ci transport dramatically decreased in LCIB mutant</td>
<td>Wang and Spalding, 2006</td>
</tr>
<tr>
<td>LCIC</td>
<td>Chloroplast stroma</td>
<td>Unknown function; LCIB gene family unique to green microalgae; limiting-Ci induced</td>
<td>Wang and Spalding, 2006</td>
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<tr>
<td>LCID</td>
<td>Chloroplast stroma</td>
<td>Unknown function; LCIB gene family unique to green microalgae; limiting-Ci induced</td>
<td>Wang and Spalding, 2006</td>
</tr>
<tr>
<td>LCIE</td>
<td>Chloroplast stroma</td>
<td>Unknown function; LCIB gene family unique to green microalgae</td>
<td>Wang and Spalding, 2006</td>
</tr>
<tr>
<td>CCP1</td>
<td>Chloroplast inner envelope</td>
<td>Mitochondrial carrier superfamily; limiting-Ci induced; nearly identical to CCP2</td>
<td>Chen et al., 1997</td>
</tr>
<tr>
<td>CCP2</td>
<td>Chloroplast inner envelope</td>
<td>Mitochondrial carrier superfamily; limiting-Ci induced; nearly identical to CCP1</td>
<td>Chen et al., 1997</td>
</tr>
<tr>
<td>MRP1 (HLA3)</td>
<td>Plasma membrane</td>
<td>MRP subfamily of ABC transporters; limiting Ci induced</td>
<td>Im and Grossman, 2001</td>
</tr>
<tr>
<td>LCII</td>
<td>Plasma membrane</td>
<td>Membrane protein with no homologs; limiting Ci induced</td>
<td>Burow et al., 1996</td>
</tr>
</tbody>
</table>

* Based on prediction from Target-P and iPSORT, except in the case of the ycf10, LCIA, CCP1, and CCP2 gene products, which have been localized experimentally.
domains, LCIA was proposed as a candidate Ci transporter rather than a nitrite transporter, as are the remaining NAR1 gene family members of C. reinhardtii, because the expression of LCIA is regulated by CO2 irrespective of the nitrogen source (Miura et al., 2004). In addition, a recent investigation reported bicarbonate transport activity associated with LCIA when expressed in Xenopus oocytes (Mariscal et al., 2006). However, LCIA-containing Xenopus oocytes displayed only low affinity bicarbonate transport activity and high affinity nitrite transport activity, making the role of LCIA in Ci transport activity uncertain.

Other NAR1 gene family members might also be considered candidates for Ci transporters, even though none other than LCIA (NAR1.2) are regulated by Ci availability. Based on physiological characteristics of NAR1.1 mutants, Mariscal et al. (2004) proposed that NAR1.1 mediates nitrogen use efficiency when Ci concentrations are low, which suggests a possible role in limiting Ci acclimation. Furthermore, a phylogenetic tree of FMT proteins shows both NAR1.1 and NAR 1.5 clustering with LCIA/NAR1.2 (Mariscal et al., 2006), suggesting that any capacity of LCIA/NAR1.2 to transport bicarbonate may be shared by NAR1.1 and NAR1.5 as well.

Two genes, CCP1 and CCP2, encode closely-related, limiting-CO2-inducible polypeptides with sequence similarity to the mitochondrial carrier protein superfamily (Chen et al., 1997). This superfamily is composed largely of small proteins catalysing the transport across the mitochondrial inner membrane of a variety of metabolites, although it also includes plastid and peroxisomal, as well as mitochondrial carrier proteins, and includes ATP/ADP translocators, uncoupling proteins, phosphate transporters, and transporters of a wide spectrum of metabolites involved in carbon and energy metabolism (Kuan and Saier, 1993; Palmieri, 1994). CCP1 and CCP2, which share 95.7% identical amino acid sequence, are predicted to contain six transmembrane domains and to be associated with the chloroplast envelope (Ramazanov et al., 1993; Chen et al., 1997), so their limiting Ci regulation in combination with their relationship to a superfamily of carriers with broad substrate specificity has made them prime candidates for involvement in chloroplast Ci transport. However, strains in which the expression of both CCP1 and CCP2 was silenced by RNA interference (RNAi) exhibited photosynthetic kinetics and Ci uptake characteristics similar to those of wild-type cells, even though they did grow slower in low CO2 conditions (Pollock et al., 2004). Although the loss of a CCP1/CCP2-associated Ci transport system could be masked by compensatory Ci transport systems, the results of the RNAi knockdown experiments raise doubts with regard to whether CCP1 and CCP2 play a role in Ci transport.

An observation that raises interest in both the CCP1/CCP2 gene pair and the uncharacterized LCID/LCIE gene pair is the clustering of these genes within a 75 kbp region of the C. reinhardtii genome (Wang and Spalding, 2006; Grossman et al., 2007). This cluster also includes the CO2-regulated periplasmic CA genes CAH1 and CAH2, in addition to CCP2, CCP1, LCID, and LCIE. In this gene cluster CCP1 and CCP2 are aligned head to head with LCIE and LCID, respectively, in an apparent inverted repeat. The inverted regions flank CAH1 and CAH2 forming a cluster of six CO2-responsive genes within this 75 kb region. This arrangement of genes suggests duplication of an ancestral CCP-LCl gene pair flanked by an ancestral periplasmic CAH, and the conserved head-to-head arrangement of CCP1-LCIE and CCP2-LCID is intriguingly suggestive of a functional relationship between the products of these gene pairs. However tempting it may be to speculate on the functional significance of this arrangement, much more information is needed before the function of these proteins is known.

Another protein of interest with regard to Ci uptake in microalgae, although not a candidate for Ci transport in limiting-CO2 conditions, is the RHP1 protein of C. reinhardtii. Two genes (RHP1 and RHP2) encoding Rhesus (Rh) proteins with similarity to the Rh proteins in the human red blood membrane are included in the C. reinhardtii genome (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). RHP1 and RHP2 are each predicted to have a chloroplast transit sequence and 12 transmembrane domains, so are expected to be chloroplast envelope proteins (Soupene et al., 2002). RHP1 is expressed at low levels in limiting CO2 and up-regulated many-fold in high CO2, and RNAi knockdown mutants with little or no RHP1 expression exhibit a growth defect in high CO2, but normal growth in limiting CO2. These two observations, in combination with the conclusion that RHP1 functions as a bidirectional CO2 gas channel, suggest that this Rh protein may allow quick equilibration of CO2 under high CO2 conditions to provide adequate CO2 for photosynthesis in the absence of a CCM (Soupene et al., 2002, 2004). The relationship between the Rh proteins and the processes of Ci uptake and CO2 assimilation in C. reinhardtii has yet to be established. Because of the close relationship between Rh in C. reinhardtii and Rh blood factors, these findings have generated considerable interest with regard to potential roles of blood Rh proteins in CO2 transfer (Kustu and Inwood, 2006; Peng and Huang, 2006).

**Speculative model of Ci transport**

A current, speculative model of Ci transport and CCM function in C. reinhardtii is illustrated in Fig. 1. One of the key features of this model is the inclusion of multiple, sometimes parallel, Ci transport systems, a feature that is based, at least in part, on the characteristics of Ci transport.
in cyanobacteria, in which our understanding of Ci uptake and CCM function is better developed, especially true for the cyanobacteria Synechocystis and Synechococcus. At least five different types of Ci transporters have been identified in cyanobacteria, including three bicarbonate transporters: (i) a high affinity, low CO\textsubscript{2}-induced, sodium-independent HCO\textsubscript{3}– transporter, BCT1, encoded by the \textit{cmpABC} operon and belonging to the ABC transporter family (Omata \textit{et al.}, 1999); (ii) a low affinity, constitutively expressed, sodium-dependent HCO\textsubscript{3}– transporter, SbtA, which is energized by an inwardly directed Na\textsuperscript{+} gradient and can be activated in less than 10 min, possibly via phosphorylation (Shibata \textit{et al.}, 2002; Price \textit{et al.}, 2002); (iii) bicA, an inducible high-affinity Na\textsuperscript{+}-dependent SulP type \textit{HCO}_{3}– transporter (Price \textit{et al.}, 2004); as well as two active CO\textsubscript{2} uptake systems: (i) a constitutive, thylakoid localized CO\textsubscript{2} uptake system, NDH-1\textsubscript{a}, based on a specialized NDH-1 complex (Maeda \textit{et al.}, 2002; Shibata \textit{et al.}, 2001; Price \textit{et al.}, 2002); and (ii) a limiting-Ci inducible CO\textsubscript{2} uptake system, NDH-1\textsubscript{b}, also based on a modified NDH-1 complex (Shibata \textit{et al.}, 2001; Maeda \textit{et al.}, 2002). By analogy then, it is possible if not probable that multiple Ci uptake/transport systems may function at each of the two membranes the Ci must cross before reaching Rubisco.

In this speculative model of the \textit{C. reinhardtii} CCM, it is assumed that bicarbonate transport occurs across the plasma membrane and the chloroplast inner envelope, with the prime transport candidates, as described above, being HLA3 and LCI1 for the plasma membrane, and LClA, a LCIB-associate transporter and possibly CCP1/2 for the chloroplast envelope. LCIB and LCIC are proposed to interact with each other and with a transporter, which could be LClA, CCP1/2 or an unidentified transporter.

On the other hand, the Ci transport model illustrated in Fig. 1 suggests that CO\textsubscript{2} uptake across both the plasma membrane and the chloroplast envelope occurs by diffusion through the membrane or a CO\textsubscript{2} channel (RHP1), rather than by an active, protein-mediated transport process. For this to be true, the reported active transport of CO\textsubscript{2} into cells and into chloroplasts would need to be explained as active processes that occur after the CO\textsubscript{2} crosses the membrane, as apparently is the case for the active CO\textsubscript{2} uptake in cyanobacteria (Badger \textit{et al.}, 2006). For example, the active CO\textsubscript{2} uptake into chloroplasts could be explained as CAH6-mediated conversion of CO\textsubscript{2} to bicarbonate driven by light-dependent alkalinization of the stroma. Apparent active transport across the plasma membrane could be explained as CO\textsubscript{2} diffusion preceding active bicarbonate transport across the chloroplast envelope or CO\textsubscript{2} diffusion across both membranes driven by the light-dependent alkalinization of the stroma. More research will be required to determine whether this view of active CO\textsubscript{2} uptake is correct or whether protein-mediated, active CO\textsubscript{2} transport does occur. In either case, CO\textsubscript{2} entering the chloroplast should be rapidly hydrated to bicarbonate by CAH6 activity under the alkaline stromal conditions in the light.

The Ci transport across the plasma membrane and chloroplast envelope should result in bicarbonate accumulation in the stroma. This accumulated bicarbonate serves as the source, after dehydration by CAH3 in the acidic thylakoid lumen, of high concentrations of substrate CO\textsubscript{2} for Rubisco, located mainly in the pyrenoid (Borkhensive \textit{et al.}, 1998). These steps (movement of bicarbonate into the thylakoid lumen, dehydration to CO\textsubscript{2} and diffusion to pyrenoid and Rubisco), although often proposed and well supported genetically, have not been unambiguously demonstrated at the biochemical level. This is especially true for the question of how bicarbonate moves from the stroma to the thylakoid lumen or how CAH3, which appears to be distributed in thylakoids throughout the chloroplast (Mitra \textit{et al.}, 2005), supplies CO\textsubscript{2} specifically to the pyrenoid to provide Rubisco with abundant substrate. This review is focused on Ci transporters of the plasma membrane and chloroplast envelope, so a detailed discussion of these issues will not be presented. However, it is clear that, in addition to further research into the identity and nature of Ci transporters, continuing research should focus on subcellular and suborganellar compartmentation of CCM processes and the roles and locations of CA isofoms is essential for achieving an understanding of the operation of the microalgal CCM.

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