Thylakoid Lumen Carbonic Anhydrase (*CAH3*) Mutation Suppresses Air-Dier Phenotype of *LCIB* Mutant in *Chlamydomonas reinhardtii*[1][C][OA]

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An active CO_2-concentrating mechanism is induced when *Chlamydomonas reinhardtii* acclimates to limiting inorganic carbon (Ci), either low-CO_2 (L-CO_2; air level; approximately 0.04% CO_2) or very low-CO_2 (VL-CO_2; approximately 0.01% CO_2) conditions. A mutant, *ad1*, which is defective in the limiting-CO_2-inducible, plastid-localized LCIB, can grow in high-CO_2 or VL-CO_2 conditions but dies in L-CO_2, indicating a deficiency in a L-CO_2-specific Ci uptake and accumulation system. In this study, we identified two *ad1* suppressors that can grow in L-CO_2 but die in VL-CO_2. Molecular analyses revealed that both suppressors have mutations in the *CAH3* gene, which encodes a thylakoid lumen localized carbonic anhydrase. Photosynthetic rates of L-CO_2-acclimated suppressors under acclimation CO_2 concentrations were more than 2-fold higher than *ad1*, apparently resulting from a more than 20-fold increase in the intracellular concentration of Ci as measured by direct Ci uptake. However, photosynthetic rates of VL-CO_2-acclimated cells under acclimation CO_2 concentrations were too low to support growth in spite of a significantly elevated intracellular Ci concentration. We conclude that LCIB functions downstream of CAH3 in the CO_2-concentrating mechanism and probably plays a role in trapping CO_2 released by CAH3 dehydration of accumulated Ci. Apparently dehydration by the chloroplast stromal carbonic anhydrase CAH6 of the very high internal Ci caused by the defect in CAH3 provides Rubisco sufficient CO_2 to support growth in L-CO_2-acclimated cells, but not in VL-CO_2-acclimated cells, even in the absence of LCIB.

CO_2 serves both as the substrate for photosynthesis and as an important signal to regulate plant growth and development, so variable CO_2 concentrations can impact photosynthesis, growth, and productivity of plants. Terrestrial C_4 plants have developed a CO_2-concentrating mechanism (CCM) involving anatomical and biochemical adaptations to accumulate a higher concentration of CO_2 as substrate Rubisco and to suppress oxygenation of ribulose-1,5-bisP, a wasteful side reaction. In contrast, a different type of CCM is induced in the unicellular green microalga *Chlamydomonas reinhardtii* when the supply of dissolved inorganic carbon (Ci; CO_2 and HCO_3^-) for photosynthesis is limited (Beardall and Giordano, 2002; Giordano et al., 2005; Moroney and Ynalvez, 2007; Spalding, 2008). In response to limiting CO_2, the CCM uses active Ci transport, both at the plasma membrane and the chloroplast envelope, to accumulate a high concentration of HCO_3^- within the chloroplast (Palmqvist et al., 1988; Sültemeyer et al., 1988). The thylakoid lumen carbonic anhydrase (CAH3) plays an essential role in the rapid dehydration of the accumulated HCO_3^- to release CO_2 into the pyrenoid, a Rubisco-containing internal compartment of the chloroplast, for assimilation by Rubisco (Price et al., 2002; Spalding et al., 2002).

While a number of genes and proteins essential to the operation of the CCM in *C. reinhardtii* have been identified, our understanding of Ci uptake and its regulation, as well as other aspects of CCM function is limited. A better understanding of the similar CCM in prokaryotic organisms, specifically the cyanobacteria *Synechocystis* and *Synechococcus*, has been gained. At least five different types of Ci transporters have been identified in cyanobacteria, including three HCO_3^- transporters and two active CO_2 uptake systems (Price et al., 2002, 2004).

Recently, at least three distinct CO_2-regulated acclimation states were identified in *C. reinhardtii* based on growth, photosynthesis and gene expression characteristics, a high-CO_2 (H-CO_2) state (5%–0.5% CO_2), low-CO_2 (L-CO_2) state (air level; 0.4%–0.03% CO_2), and very low-CO_2 (VL-CO_2) state (0.01%–0.005% CO_2; Vance and Spalding, 2005). Two allelic HCR (H-CO_2-requiring) mutants, *pmp1* and *ad1*, grow as well (*pmp1*)

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or nearly as well (*ad1*) as wild-type cells in both H-CO_2 and VL-CO_2 conditions while only dying in L-CO_2, indicating a deficient Ci transport and/or accumulation system only in the L-CO_2 acclimation state (Spalding et al., 1983b, 2002). The defective gene responsible for the *pmp1/ad1* phenotype was identified as *LCIB*, a limiting CO_2-inducible gene, the product of which is predicted to be located in the chloroplast stroma and proposed to be involved with chloroplast Ci uptake in L-CO_2 conditions (Wang and Spalding, 2006). The *LCIB* gene product is a member of a small gene family so far only found in a few microalgae species (Spalding, 2008).

To investigate the roles of LCIB in eukaryotic photosynthetic organisms and identify other functional components involved in chloroplast Ci accumulation in *C. reinhardtii*, we used an insertional mutagenesis approach to select suppressors of the air-dier phenotype of the *LCIB* mutant *ad1*. In this study, we describe two *ad1* suppressors, *ad-su6* and *ad-su7*, that grow normally in L-CO_2 but, unlike *ad1*, die in VL-CO_2. This report also presents data suggesting that the air-dier phenotype of *ad1* is suppressed by increased intracellular Ci concentrations in the two suppressors, and suggesting a possible role for LCIB as a CO_2 trap rather than having any direct role in chloroplast envelope Ci transport.

RESULTS

Subcellular Localization of LCIB Protein

A putative chloroplast localization signal suggests that LCIB may target to the chloroplast. Immunofluorescent detection of LCIB with anti-LCIB antiserum was used, in combination with confocal microscopy, to visualize the subcellular localization of LCIB in cells grown under H-CO_2 (5% CO_2), L-CO_2 (approximately 0.04% CO_2), and VL-CO_2 (<0.02% CO_2). In H-CO_2-acclimated cells, LCIB was expressed only at a very low level and was barely detectable (data not shown), while in cells acclimated to L-CO_2 or VL-CO_2, LCIB protein increased dramatically in abundance, consistent with its reported mRNA accumulation in these cells (Miura et al., 2004). In L-CO_2- and VL-CO_2-acclimated cells, two different patterns of distribution were observed in the immunofluorescent detection of LCIB protein. Immunofluorescence from LCIB was either dispersed throughout the entire chloroplast stroma or concentrated mainly in a discreet region surrounding the pyrenoid, appearing as a distinct ring structure in virtual longitudinal sections inside individual chloroplasts (Fig. 1; VL-CO_2 localization data not shown).

Identification and Genetic Analysis of *ad1* Suppressors

The *C. reinhardtii* strain chosen for this study was *ad1*, containing a deletion mutation of *LCIB* (Wang and Spalding, 2006). This strain cannot grow in L-CO_2 but

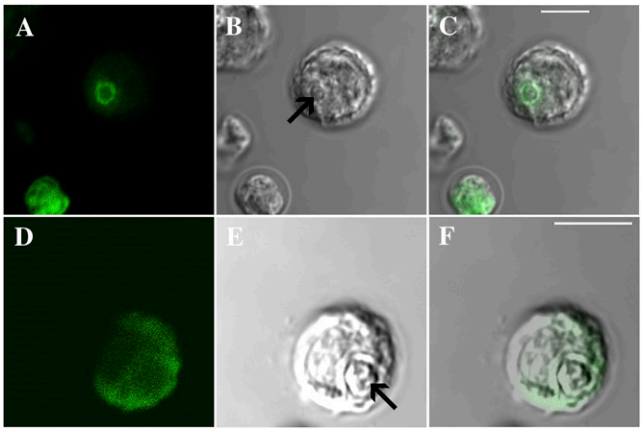

Figure 1. Immunofluorescent localization of LCIB in *C. reinhardtii* L-CO_2-acclimated CC125 cells. A and D, False color immunofluorescence images of two different cells. B and E, Confocal images of the same cells. C and F, Merged images. The white bars in C and F are 5 μm in length and the arrows in B and E indicate the location of the pyrenoid in each confocal image.

can grow either in H-CO_2 or in VL-CO_2. To isolate and identify suppressors that can grow in L-CO_2, we performed insertional mutagenesis using a Par^R-containing plasmid (pSI103) to transform *ad1* (Fig. 3A). From approximately 10^6 transformants, three displayed the suppression phenotype, and two of these, *ad-su6* and *ad-su7*, are described here. These two suppressors exhibited suboptimal growth in L-CO_2 where the parental strain *ad1* could not grow at all (Fig. 2). Unexpectedly, neither suppressor could survive in VL-CO_2, indicating that although the second site suppressor mutations could suppress the L-CO_2 lethal, air-dier phenotype of the *LCIB* mutation in *ad1*, growth of the two suppressors in VL-CO_2 was completely abolished.

The *ad-su6* and *ad-su7* strains were crossed with wild-type strain CC620 to determine whether the suppression phenotype cosegregated with the inserted Par^R gene. More than 150 Zeo^R (zeocin resistance, conferred by the Ble^R insert responsible for the *LCIB* mutation) random progeny from each cross were screened for their growth in different levels of CO_2 and their resistance to paromomycin. In the *ad-su6* cross, all 90 random progeny with the suppressor phenotype were paromomycin resistant, while all paromomycin-sensitive progeny exhibited an *ad1*-like growth phenotype in L-CO_2 and VL-CO_2, indicating cosegregation of the suppressor phenotype with the Par^R insert. Southern analysis with probe specific for the Par^R gene indicated a single insert present in *ad-su6* (Fig. 3B). Although *ad-su7* also contains only one Par^R insert, genetic analysis showed the suppressor phenotype was not linked to the insert (data not shown).

Inverse PCR was employed to identify the flanking DNA in *ad-su6*. This flanking sequence was used in a BLAST search against the *C. reinhardtii* genome

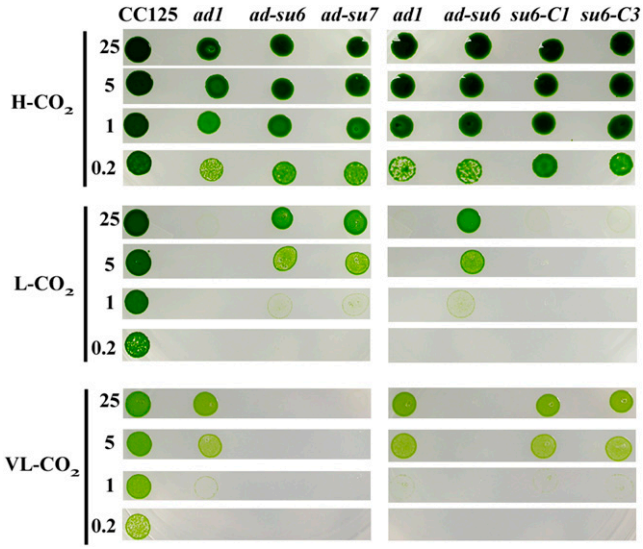

Figure 2. Growth of *ad1* suppressors and *CAH3* genomic DNA complemented *ad-su6* on minimal plates in H-CO_2, L-CO_2, and VL-CO_2 chambers. Cells grown to logarithmic phase were diluted to the indicated numbers ($\times 10^3$) per 5 μL, spotted on plates, and incubated for 9 d under dim lights. [See online article for color version of this figure.]

(http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) and the insertion site was shown to be located between exon 5 and intron 5 of *CAH3* (Fig. 3C). Further PCR and DNA gel-blot analyses revealed that only 186 nucleotides of *CAH3* (63 nt of exon 5 and 123 nt of intron 5) were deleted in *ad-su6*. Since *ad-su6* has the same growth phenotype as *ad-su7*, we PCR amplified and sequenced *CAH3* genomic DNA from *ad-su7* and found that two nucleotides were deleted downstream of the ATG translation initiation codon (ATGCGCTCAGCCGTTCTACAACGCGGCCAGGCGCGGCGAGTGTCTTGCCGGGTGAGTGAA; underline indicates deletion mutation in *ad-su7*), which predicts a premature stop codon (ATGCGCTCAGCCGCTACAACGCGGCCAGGCGCGGCGAGTGTCTTGCCGGGTGAGTGA; underline indicates stop codon).

Expression Patterns of Limiting-CO_2-Inducible Genes in *ad-su6*

Northern-blot analysis showed that *ad-su6* apparently is a hypomorphic mutant for *CAH3*. In *ad1* cells, a transient, slight increase in the *CAH3* transcript abundance was observed when cells were shifted from a H-CO_2 to either L-CO_2 or VL-CO_2 atmosphere, and, after a longer acclimation time (14 h), the *CAH3* message abundance was decreased (Fig. 4A). In the *CAH3* hypomorphic mutant *ad-su6*, *CAH3* transcript was undetectable throughout all CO_2 conditions. Western-blot analysis using polyclonal antiserum raised against CAH3 from *C. reinhardtii* also failed to detect the CAH3 protein in the suppressor mutant *ad-su6* cultures (Fig. 4B).

Message accumulations of several limiting-CO_2-inducible genes also were analyzed. Among all the genes tested, including *LCIC*, *CCP*, *LCI1*, and *LCIA*, patterns of expression in the suppressor *ad-su6* relative to the original *LCIB* mutant *ad1* were not found to be significantly different. The expression level of chloroplast stromal carbonic anhydrase (*CAH6*) was not affected by CO_2 conditions, and the transcript abundance of *CAH6* in *ad-su6* under limiting-CO_2 conditions was comparable to that in *ad1* (Fig. 4A).

Complementation of *ad-su6*

To confirm whether the *CAH3* mutation is responsible for the suppressor phenotype, we transformed a genomic DNA fragment containing a wild-type copy of *CAH3* into the suppressors *ad-su6* and *ad-su7* and selected complemented lines that could survive in VL-CO_2. Complemented *ad-su6* (*su6-C1* and *su6-C3*) and *ad-su7* lines showed the same growth phenotype as *ad1*, growth in VL-CO_2 and no growth in L-CO_2 (Fig. 2; *ad-su7* data not shown). In addition, western-blot analysis showed that complemented *ad-su6* lines recovered the expression of CAH3 protein (Fig. 4B). Complementation of the *ad-su6* VL-CO_2 lethal phenotype also was achieved by expressing *CAH3* cDNA under control of the constitutive *PsaD* promoter and terminator (Fischer and Rochaix, 2001; data not shown).

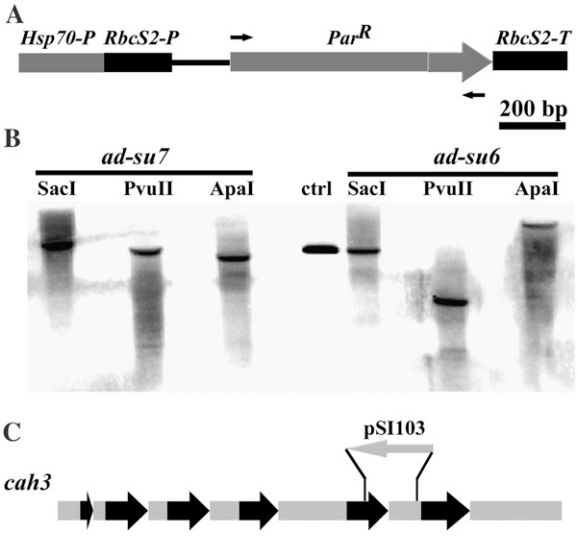

Figure 3. Southern-blot analysis of *ad-su6* and *ad-su7* probed with an 880-bp PCR fragment of the Par^R gene. A, Map of the Par^R portion of the pSI103 plasmid used for insertional mutagenesis. Arrows above and below the map indicate the primers used to make the probe. B, Southern analysis of two suppressors, with linearized plasmid as control. Genomic DNA was digested with the indicated restriction enzymes. C, Map of the *CAH3* genomic region in *ad-su6*. Black arrows, Exons; gray arrow, pSI103 insertion; gray bars, introns.

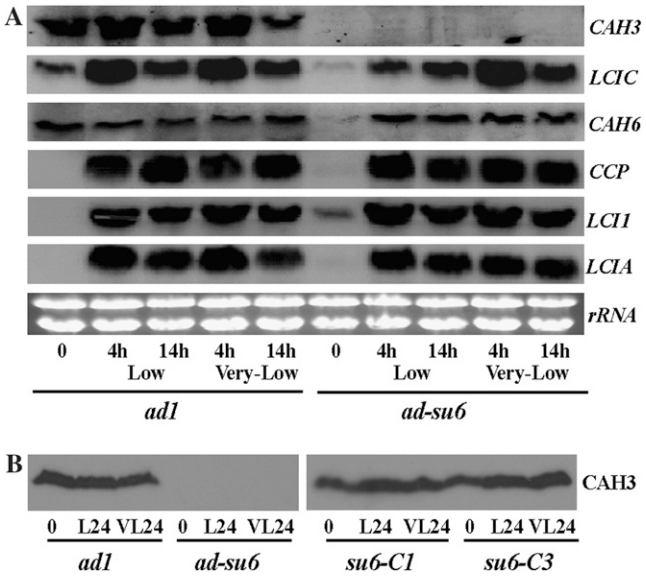

Figure 4. A, Northern-blot analysis of *ad1* and *ad-su6*. Cells were adapted to L-CO_2 or VL-CO_2 conditions for 4 and 14 h, respectively, and 10 μg of total RNA was used. B, Immunoblot analysis of whole cell fractions. Cells were either H-CO_2 grown or induced 24 h under L-CO_2 (L24) or VL-CO_2 (VL24) conditions. One hundred micrograms of total protein were analyzed with CAH3 antibody. *su6-C1* and *su6-C3* are two *CAH3* genomic DNA-complemented *ad-su6* transformants as described in Figure 2.

Photosynthetic Affinity for Ci and Direct Ci Uptake Characteristics

Photosynthetic O_2 evolution in response to Ci concentrations for L-CO_2- and VL-CO_2-acclimated wild-type and various mutant cells was compared (Table I). Walled progeny of mutants were generated for physiological measurements, including the *CAH3* mutation of *ad-su6* in a wild-type background (*wt-su6*) or in an *ad1* background (*ad-su6-1*). Consistent with the *ad1* air-dier phenotype, the *LCIB*-defective *ad1-1* mutant cells (walled progeny of *ad1*) acclimated in L-CO_2 showed dramatically decreased photosynthetic affinity for Ci compared with wild-type cells acclimated under the same conditions (approximately 11% of wild type). In contrast, when acclimated to VL-CO_2, photosynthetic affinity of *ad1-1* was increased to approximately 57% to 61% of the wild-type strain (P20 and P50, estimated at 20 and 50 μM total Ci, respectively). Photosynthetic affinity of L-CO_2-acclimated *ad-su6-1* was significantly higher than *ad1-1* at 50 μM total Ci (P50: 0.14 $\pm$ 0.02 versus 0.07 $\pm$ 0.02, P value < 0.05), while VL-CO_2-acclimated *ad-su6-1* had a lower relative affinity than *ad1-1* (P20: 0.07 $\pm$ 0.03 versus 0.27 $\pm$ 0.03, $P < 0.05$) at 20 μM total Ci, both of which are consistent with the phenotypes of these two strains. The aberrant photosynthetic affinity of *ad-su6-1* was caused by the *CAH3* mutation, since *wt-su6* showed the same pattern of photosynthetic affinity in both L-CO_2 and VL-CO_2 conditions as *ad-su6-1*.

Intracellular Ci accumulation in L-CO_2-acclimated *ad1-1* cells was similar to that of the nonacclimating mutant *cia5* (0.26 $\pm$ 0.08 mM versus 0.19 $\pm$ 0.05 mM), in which the CCM presumably does not function (Table II). An active Ci accumulation mechanism was regained in VL-CO_2-acclimated *ad1-1* cells, although not as high as the wild-type strain (0.80 $\pm$ 0.15 mM versus 1.65 $\pm$ 0.25 mM, measured with 20 μM total Ci). The measured intracellular Ci pool in the *LCIB-CAH3* double mutant *ad-su6-1* was increased more than 20-fold over that of the *LCIB* single mutant *ad1-1* when acclimated in L-CO_2 conditions (6.15 $\pm$ 1.15 mM versus 0.26 $\pm$ 0.08 mM), and the increased Ci accumulation could be attributed to the *CAH3* mutation because *CAH3* single mutant *wt-su6* accumulated the same level of intracellular [Ci] as *ad-su6-1* (7.60 $\pm$ 1.25 mM versus 6.15 $\pm$ 1.15 mM). In VL-CO_2-acclimated strains, intracellular [Ci] in *ad-su6-1* was only 2.5-fold higher than that in *ad1-1* (2.85 $\pm$ 0.32 mM versus 0.80 $\pm$ 0.15 mM, measured with 20 μM total Ci).

DISCUSSION

Rubisco, the primary enzyme for photosynthetic CO_2 assimilation, is an inefficient catalyst with a low affinity for atmospheric CO_2. For most algae, the Rubisco $K_m(CO_2)$ is greater than 25 μM, so Rubisco is functioning at <20% of capacity at 10 μM CO_2 in an air-equilibrated aquatic environment. Many photosynthetic organisms have an inducible CCM that raises the CO_2 concentration around the active site of Rubisco

Table I. *Relative affinity of photosynthetic O_2 evolution in cells acclimated to L-CO_2 or VL-CO_2 for 1 d*

Rates of O_2 evolution (μmol mg Chl^{-1} h^{-1}) were determined in pH 7.3 (MOPS-KOH) buffer at 20 μM (V20), 50 μM (V50), and 4,000 μM (V4000) $NaHCO_3$. Relative Ci affinity was calculated as ratios: P20 = V20/V4000 and P50 = V50/V4000. V_{max} = V4000.

Strains	L-CO_2			VL-CO_2		
	P20	P50	V_{max}	P20	P50	V_{max}
CC125	0.38 $\pm$ 0.04	0.60 $\pm$ 0.05	180 $\pm$ 22	0.44 $\pm$ 0.05	0.63 $\pm$ 0.07	162 $\pm$ 18
ad1-1	0.04 $\pm$ 0.02	0.07 $\pm$ 0.02	106 $\pm$ 18	0.27 $\pm$ 0.03	0.36 $\pm$ 0.04	82 $\pm$ 8
wt-su6	0.08 $\pm$ 0.01	0.15 $\pm$ 0.03	104 $\pm$ 12	0.09 $\pm$ 0.02	0.15 $\pm$ 0.02	80 $\pm$ 9
ad-su6-1	0.05 $\pm$ 0.02	0.14 $\pm$ 0.02	130 $\pm$ 21	0.07 $\pm$ 0.03	0.13 $\pm$ 0.04	83 $\pm$ 10
cia5	0.08 $\pm$ 0.02	0.12 $\pm$ 0.03	98 $\pm$ 18	0.06 $\pm$ 0.03	0.08 $\pm$ 0.02	72 $\pm$ 9

Table II. *Intracellular Ci accumulation in cells acclimated to L-CO_2 or VL-CO_2 for 1 d*

Internal Ci accumulation (mM Ci after 80 s) was determined by silicone oil centrifugation in pH 7.3 (MOPS-KOH) buffer at initial external Ci concentrations of 20 μM and 50 μM [^{14}C]$NaHCO_3$ and a Chl concentration of 25 μg/mL.

Strains	L-CO_2	VL-CO_2	
	50 μM Ci	20 μM Ci	50 μM Ci
CC125	1.80 ± 0.33	1.65 ± 0.25	1.78 ± 0.24
ad1-1	0.26 ± 0.08	0.80 ± 0.15	1.43 ± 0.22
wt-su6	7.60 ± 1.25	2.50 ± 0.33	8.10 ± 1.45
ad-su6-1	6.15 ± 1.15	2.85 ± 0.32	5.30 ± 0.58
cia5	0.19 ± 0.05	0.15 ± 0.04	0.30 ± 0.15

several-fold higher than the environmental level, thus improving the efficiency of CO_2 assimilation. In the eukaryotic microalga *C. reinhardtii*, active Ci uptake (mainly as CO_2 and HCO_3^-) at either the plasma membrane or the inner chloroplast envelope is an essential component of the CCM. Although the products of several limiting-CO_2-inducible genes have been identified as putative Ci transporters on the chloroplast envelope, including *LCIA*, *LCI1*, *CCP1/2*, and *Ycf10*, or on the plasma membrane, including *HLA3*, none of the respective gene products have been definitively determined to transport Ci species (Burow et al., 1996; Chen et al., 1997; Rolland et al., 1997; Im and Grossman, 2001; Miura et al., 2004; Pollock et al., 2004; Mariscal et al., 2006), leaving Ci transport largely a mystery.

Mutants with lesions in the limiting-CO_2-induced gene *LCIB* shed some light on the nature of Ci uptake and accumulation in chloroplasts. Two conditional lethal mutants, *pmp1* and *ad1*, with lesions in *LCIB*, are defective in Ci accumulation when acclimated in L-CO_2 but not when acclimated in VL-CO_2, indicating the existence of multiple Ci uptake and accumulation pathways in *C. reinhardtii* corresponding to multiple limiting-CO_2 acclimation states (Vance and Spalding, 2005). LCIB was proposed to be involved in active Ci transport (Spalding et al., 1983b; Wang and Spalding, 2006), although, since it is predicted to be a soluble protein with no significant transmembrane regions, it was acknowledged to be unlikely that LCIB itself is directly involved in transport. Therefore, it was argued that LCIB might be a subunit of a Ci transport complex or might have a regulatory role in Ci uptake and/or accumulation.

In this article, we identified two independent, second-site suppressors of the *LCIB* mutant air-dier phenotype that restored growth to *ad1* in L-CO_2 but, unlike *ad1* itself, were unable to grow in VL-CO_2. Both suppressors have lesions in *CAH3*, encoding a thylakoid-lumen-located, α-type carbonic anhydrase. The requirement of a thylakoidal CA and an acidic compartment in a functional CCM was first suggested by Pronina and colleagues, and later CAH3 was identified and proposed to catalyze the rapid conversion of HCO_3^- to CO_2 in the acidic thylakoid lumen, with the CO_2 then diffusing to the pyrenoid to supply elevated substrate CO_2 concentrations for Rubisco (Pronina et al., 1981; Pronina and Semenenko, 1990; Funke et al., 1997; Raven, 1997; Karlsson et al., 1998; Hanson et al., 2003; Moroney and Ynalvez, 2007). Consistent with previous reports (Spalding et al., 1983a; Moroney et al., 1987), the L-CO_2-acclimated *CAH3* single mutant *wt-su6*, generated in a cross between the suppressed line and wild type, accumulated a very high internal Ci concentration but was unable to use Ci efficiently for photosynthesis. However, the photosynthetic Ci affinity of this *CAH3* single gene mutant *wt-su6* still is significantly higher than that of the L-CO_2-acclimated *LCIB* mutant *ad1*, which accumulates only very low internal Ci levels and cannot grow at all in L-CO_2, both traits agreeing with prior reports of the characteristics of the allelic *LCIB* mutant *pmp1* and various *CAH3* single mutants (Spalding et al., 1983a, 1983b; Moroney et al., 1987; Suzuki and Spalding, 1989). The *LCIB-CAH3* double mutant suppressor *ad-su6-1* accumulated the same level of intracellular Ci as the *CAH3* single gene mutant *wt-su6*, strongly arguing against LCIB as being required for or solely responsible for Ci transport into the chloroplast. This effect of combining lesions in *CAH3* and *LCIB* has been reported previously (Spalding et al., 1983c), before the genes themselves were identified and before the full significance of the observation could be appreciated. It was recognized at the time that the lesion in *CAH3* was apparently epistatic to the lesion in *LCIB*, and that the double mutant exhibited a leaky CO_2-requiring phenotype in air, similar to the *CAH3* single mutant.

Our current work definitively demonstrates that a *CAH3* loss-of-function mutation can suppress the air-dier phenotype of *LCIB* mutants, as well as revealing a novel, VL-CO_2 lethal phenotype of the double mutant suppressors. The suppressors exhibited the same levels of photosynthetic Ci affinity and internal Ci accumulation, regardless of whether they were acclimated to L-CO_2 or VL-CO_2. No significant difference in intracellular Ci accumulation was detected between the L-CO_2-acclimated suppressor (*LCIB-CAH3* double mutant) and the *CAH3* single mutant, indicating the *LCIB* mutation had no influence on Ci accumulation in the absence of a functional CAH3, which is contrary to the proposed direct involvement of LCIB in Ci transport in L-CO-acclimated cells (Spalding et al., 1983b; Wang and Spalding, 2006), but is consistent with the reported epistatic interaction between these two mutations (Spalding et al., 1983c).

The position of LCIB in the biochemical pathway of Ci uptake and accumulation in *C. reinhardtii* is informed by the epistatic interaction between the *CAH3* and *LCIB* mutations. It is clear that CAH3 functions to dehydrate HCO_3^- accumulated in the stroma, although it is not known how HCO_3^- gains access to the thylakoid lumen. Because *CAH3* mutations are epistatic over *LCIB* mutations, LCIB must act down-

stream of CAH3, meaning it must act after the accumulated stromal HCO_3^- is dehydrated to CO_2. The localization of LCIB also is inconsistent with a direct role in Ci transport, regardless of whether it is diffusely distributed throughout the stroma or concentrated around the pyrenoid. The combination of LCIB localization and the epistatic interaction between *LCIB* and *CAH3* mutations, together with the clear demonstration that *LCIB* mutants can transport and accumulate Ci in the absence of functional CAH3, provide a very compelling argument against a direct role for LCIB in Ci transport. Since it is very unlikely LCIB is involved in Ci transport, it appears most likely that LCIB is involved in preventing the loss of CO_2 from the chloroplast (Fig. 5). One possibility is that LCIB is involved in sequestering or trapping excess CO_2 from CAH3-catalyzed dehydration of HCO_3^- that might otherwise diffuse out of the chloroplast and be lost. A functionally similar CCM exists in cyanobacteria, and Price et al. (2002) have proposed that two unique hydrophilic proteins, ChpX and ChpY (also named CupA and CupB), are critical components of a thylakoid-based NDH-1 CO_2 uptake complex in the model cyanobacterium *Synechococcus* sp. PCC7942 and aid in the recapture or recycling of CO_2 leakage from carboxysomes by CO_2 hydration activity in the light (Maeda et al., 2002; Shibata et al., 2001, 2002; Price et al., 2002). Thus LCIB may be part of a functionally analogous but novel CO_2 pump/trap and may function mainly to prevent depletion of the HCO_3^- pool by trapping CO_2 from CAH3 dehydration back into the stromal HCO_3^- pool or by providing a barrier to CO_2 diffusion back out of the pyrenoid.

Although it is clear that *CAH3* mutations are epistatic to *LCIB* mutations and thus that LCIB must function downstream of CAH3, it is more complicated to explain mechanistically how the characteristics of the individual mutants and the double mutants explain this epistatic interaction. Dehydration, either uncatalyzed or catalyzed by the stromal carbonic anhydrase CAH6, of the very high stromal HCO_3^- concentration in the *LCIB-CAH3* double mutant *ad-su6-1* may provide a direct supply of CO_2 to Rubisco sufficient to support growth and suppress the air-dier phenotype of *ad1-1* in L-CO_2-acclimated cells. However, the stromal HCO_3^- concentration of VL-CO_2-acclimated *ad-su6-1* also is fairly high, yet apparently fails to provide Rubisco sufficient CO_2 to support growth in VL-CO_2 conditions. The predicted carboxylation rates for Rubisco (64 μmol CO_2 mg^{-1} Chl h^{-1} and 41 μmol CO_2 mg^{-1} Chl h^{-1} for L-CO_2- and VL-CO_2-acclimated cells, respectively), assuming complete equilibration at pH of 8.5 between HCO_3^- and CO_2 at the observed internal Ci concentrations of 6.15 mM (50 μM external Ci) and 2.9 mM (20 μM external Ci) for L-CO_2- and VL-CO_2-acclimated cells, respectively, substantially overestimates the observed photosynthetic rates of 18 μmol CO_2 mg^{-1} Chl h^{-1} and 5.8 μmol CO_2 mg^{-1} Chl h^{-1} for L-CO_2- and VL-CO_2-acclimated cells, respectively. On the other hand, calculations assuming an uncatalyzed rate of HCO_3^- dehydration (CO_2 supply rates of 8 μmol CO_2 mg^{-1} Chl h^{-1} and 2.9 μmol CO_2 mg^{-1} Chl h^{-1} for L-CO_2 and VL-CO_2, respectively) underestimate the actual photosynthetic rates (see Spalding and Portis, 1985). Thus the observed photosynthetic rates argue for only limited CAH6-catalyzed dehydration of the accumulated HCO_3^-, which may explain why an internal Ci concentration of 2.9 mM in VL-CO_2-acclimated cells is unable to support growth under VL-CO_2 conditions.

A key question is why an observed photosynthetic rate of 18.2 μmol CO_2 mg^{-1} Chl h^{-1} in L-CO_2 allows for substantial growth of the double mutant suppressor but a rate of 5.8 μmol CO_2 mg^{-1} Chl h^{-1} in VL-CO_2 does not. A photosynthetic rate of 5.8 μmol CO_2 mg^{-1} Chl h^{-1} may simply be below the threshold for survival, a conclusion supported by the observed rates of photosynthesis of three other strains unable to grow, L-CO_2-acclimated *LCIB* mutant *ad1* at 50 μM external Ci and VL-CO_2-acclimated wild type-su6 (*CAH3* mutation alone) and *cia5* at 20 μM external Ci, which were 7.4, 7.2, and 4.3 μmol CO_2 mg^{-1} Chl h^{-1}, respectively, and by the minimum photosynthetic rate observed for any strain able to grow, which was 11.8 μmol CO_2 mg^{-1} Chl h^{-1} (L-CO_2-acclimated *cia5* at 50 μM external Ci). Based on the reported effect of photon flux density on Ci accumulation (Spalding, 1990), we also would expect substantially lower stromal HCO_3^- accumulation and thus a lower photosynthetic rate under light conditions used for growth (approximately 100 μmol photons m^{-2} s^{-1}), which is much lower than that used for photosynthesis and Ci uptake measurements (approximately 800 μmol photons m^{-2} s^{-1}).

Even though the data presented here help identify the position of LCIB in the pathway for Ci uptake and accumulation by placing its function downstream of CAH3, the actual function of LCIB remains a mystery, as does how the single mutation in *LCIB* eliminates almost all Ci accumulation to the same extent as the *cia5* mutant, in which almost no limiting-CO_2-induc-

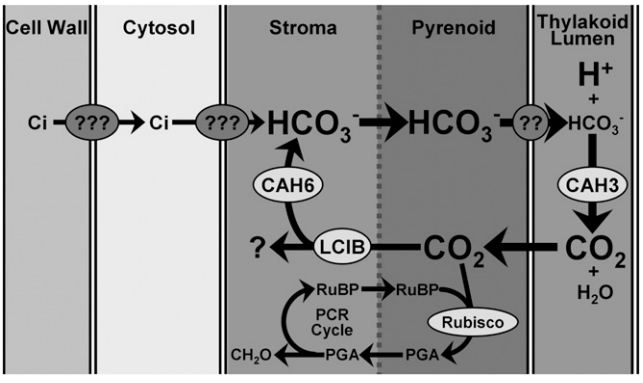

Figure 5. Simple schematic of the *C. reinhardtii* CCM, showing the relative position of CAH3 and LCIB in the pathway of Ci transport, HCO_3^- accumulation, and the subsequent dehydration of HCO_3^- to produce CO_2. PGA, 3-Phosphoglycerate.

ible genes are expressed (Fukuzawa et al., 2001; Xiang et al., 2001). LCIB is essential in L-CO_2 conditions, and we hypothesize that LCIB might be involved in somehow preventing the loss of CAH3-generated CO_2 that is not captured by Rubisco in the pyrenoid, either by preventing its diffusion from the pyrenoid or by recapturing any CO_2 escaping from the pyrenoid or from thylakoids outside the pyrenoid. The physiological observations for the single and double mutants are consistent with the role in CO_2 trapping, and the immunolocalization of LCIB in the stroma or surrounding the pyrenoid is more consistent with CO_2 trapping than with Ci transport. The constitutively expressed chloroplast stromal CAH6 might normally also help retain Ci in the stroma by trapping it as HCO_3^-. It is tempting to speculate that LCIB and CAH6 might cooperate in trapping CO_2 released from the pyrenoid and/or from thylakoids, especially since CAH6 reportedly is differentially concentrated around the pyrenoid (Mitra et al., 2004), as is LCIB, at least under some conditions. It will be interesting to investigate the potential colocalization of LCIB and CAH6, and the generation of CAH6 mutants by either an RNAi approach or by insertional mutagenesis could help to clarify the physiological roles of and any potential interactions between LCIB and CAH6 in the L-CO_2 acclimation state.

MATERIALS AND METHODS

Cell Strains and Culture Conditions

Chlamydomonas reinhardtii strains CC125 and CC620 were obtained from the Chlamydomonas Stock Center, Duke University, Durham, NC. The LCIB-defective mutant *ad1* was generated by insertional mutagenesis (Wang and Spalding, 2006) and the *cia5* mutant was a gift from Dr. Donald P. Weeks (University of Nebraska, Lincoln).

Media and growth conditions for *C. reinhardtii* strains have been previously described (Wang and Spalding, 2006). All strains were maintained on CO_2-minimal plates and kept in H-CO_2 (air enriched with 5% [v/v] CO_2) chambers at room temperature, under continuous illumination (50 μmol photons m^{-2} s^{-1}). Liquid cultures were grown on a gyratory shaker under aeration in white light (approximately 100 μmol photons m^{-2} s^{-1}). For experiments in which cells were shifted from high to limiting CO_2 (L-CO_2, 0.035%–0.04% or VL-CO_2, 0.005%–0.01%) conditions, cells were cultured in CO_2-minimal medium aerated with 5% CO_2 to a density of approximately 2×10^6 cells/mL and then shifted to aeration with the appropriate limiting CO_2 for various times. Very low CO_2 was obtained by mixing normal air with compressed, CO_2-free air.

Immunolocalization of LCIB Protein

Cells grown under different CO_2 conditions were placed on precharged microscope slides (ProbeOn Plus, FisherBiotech) for 5 to 10 min, and then rinsed briefly with CO_2 minimal medium. The immunofluorescence staining was performed as described previously (Sanders and Salisbury, 1995; Cole et al., 1998). Antiserum against LCIB, raised in rabbit, was used at a dilution of 1:500 as primary antibody, and fluorescein isothyocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used at a dilution of 1:100 as the secondary antibody for immunofluorescence. After final washing steps, the slides were mounted using a medium containing 2% *N*-propyl gallate, 30% 0.1 M Tris, pH 9 and 70% glycerol. Stained cells were viewed and digital images acquired using a Nikon C1si confocal microscope.

Isolation of Suppressors, Growth Spot Tests, and Genetic Analysis

Glass bead transformations were performed as described previously (Van and Spalding, 1999). Air-dier mutant *ad1* was transformed with linearized plasmid pSI103 (Sizova et al., 2001) and spread onto CO_2-minimal plates containing 15 μg/mL paromomycin. Plates were placed in air for 6 weeks and surviving colonies selected as putative suppressors were verified by spot tests.

For spot test of growth, actively growing cells were serially diluted to similar cell densities in minimal medium and spotted (5 μL/spot) onto minimal agar plates, and grown in various CO_2 concentrations for around 9 d. Genetic crosses and random progeny analyses were performed as described by Harris (1989).

DNA, RNA, and Protein-Blot Analysis

Southern analyses were performed as described by Van and Spalding (1999). Northern-blot hybridizations were performed by standard procedures. Total RNA was prepared by the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 2006). RNA (approximately 15 μg) were separated on 0.9% formaldehyde-agarose gels and blotted onto nylon transfer membranes (Osmonics, Inc.). PCR-amplified cDNA sequences of corresponding genes were used as templates and all probes were generated by randomly primed labeling with [α-^{32}P]dCTP following instructions of the manufacturer. Each northern blot was analyzed by phosphorimager scanning (Bio-Rad).

For total protein analyses, cells were harvested and resuspended in a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 mM dithiothreitol. Protein concentrations were measured using Bio-Rad protein assay kit (Bio-Rad catalog no. 500–0006). Proteins were separated on 12% polyacrylamide gels as described previously (Laemmli, 1970). Immunoblotting was performed as described in the protocol from Bio-Rad Laboratories. The CAH3 antiserum was a gift from James V. Moroney (Louisiana State University) and was generated using a synthetic peptide derived from an internal amino acid sequence of CAH3 (residues 213–224) as antigen.

Isolation of Sequences Flanking the Plasmid Insert in *ad-su6* by Inverse PCR

Based on information from Southern-blot analysis, *Sac*I was used to digest the genomic DNA isolated from *ad-su6* to produce a fragment with a size of approximately 4.7 kb, including part of the inserted pSI103 vector and its 3′-flanking genomic DNA. The *Sac*I-digested *ad-su6* genomic DNA (1 μg) was circularized with 1 unit of T4 DNA ligase (Invitrogen), precipitated, and the circularized product was used as a template for inverse PCR by using standard PCR procedures. Two pairs of primers were designed, with each pair complementing the pSI103 sequence in opposite orientations. Both primer pairs produced PCR products with the correct predicted sizes, and amplified DNA from one primer pair (5′-GGTCTGACGCTCAGTGGAACGA-3′ and 5′-CGCAACGCATCGTCCATGCTTC-3′) was sequenced to determine the sequences flanking the insert.

Photosynthetic O_2 Exchange and Ci Uptake Measurements

L-CO_2-induced or very low CO_2-induced cells (24-h induction) were collected by centrifugation and resuspended in 25 mM MOPS-KOH buffer to a final chlorophyll concentration of approximately 20 to 25 μg/mL for analysis of photosynthesis and Ci uptake. Photosynthetic O_2 evolution was measured at 25°C with a Clark-type oxygen electrode (Rank Brothers). Under constant illumination (800 μmol photons m^{-2} s^{-}), cells were transferred to the electrode chamber and allowed to exhaust endogenous Ci until net O_2 exchange was zero. Measurements were initiated by addition of various concentrations of $NaHCO_3$. Oxygen evolution rates were recorded as V20 or V50 when 20 or 50 μM $NaHCO_3$ were used, respectively. The maximum O_2 evolution rate, V4000, was obtained by using 4,000 μM $NaHCO_3$. Relative affinity for Ci was calculated as the ratios P20 = V20/V4000 and P50 = V50/V4000.

Ci uptake by *C. reinhardtii* cells at 20 or 50 μM total Ci was estimated by the silicone oil filtration technique (Badger et al., 1980). The cell volume was measured by using ^{14}C-sorbitol and ^3H_2O as previously described (Wirtz et al.,

1980). The intracellular Ci concentration was calculated by using cell volume and the acid labile ^{14}C data.

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