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REVIEW

LCIB in the *Chlamydomonas* CO_2-concentrating mechanism

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Abstract The CO_2-concentrating mechanism confers microalgae a versatile and efficient strategy for adapting to a wide range of environmental CO_2 concentrations. LCIB, which has been demonstrated as a key player in the eukaryotic algal CO_2-concentrating mechanism (CCM), is a novel protein in *Chlamydomonas* lacking any recognizable domain or motif, and its exact function in the CCM has not been clearly defined. The unique *air-dier* growth phenotype and photosynthetic characteristics in the *LCIB* mutants, and re-localization of LCIB between different subcellular locations in response to different levels of CO_2, have indicated that the function of LCIB is closely associated with a distinct low CO_2 acclimation state. Here, we review physiological and molecular evidence linking LCIB with inorganic carbon accumulation in the CCM and discuss the proposed function of LCIB in several inorganic carbon uptake/accumulation pathways. Several new molecular characteristics of LCIB also are presented.

Keywords LCIB · CO_2-concentrating mechanism · Active inorganic carbon uptake · *Chlamydomonas*

Introduction

The CO_2-concentrating mechanism (CCM) in many microalgae has evolved as an adaptation for increased efficiency in carbon assimilation to overcome the challenge of limited CO_2 availability that often occurs in their natural habitats. Although varying in complexity, virtually all different forms of the microalgal CCM comprise three functionally interactive systems (1) inorganic carbon (Ci) uptake systems; (2) carbonic anhydrases (CAs) interconverting different Ci species; and (3) microcompartments where Rubisco is sequestered. Under the conditions where CO_2 supply becomes limited, synergistic operation of Ci uptake systems and CAs results in a large intracellular Ci pool, where the Ci concentration is substantially higher than that in the environment. The accumulated Ci is subsequently delivered into the microcompartments filled with Rubisco, elevating the CO_2 concentration for the photosynthetic carbon fixation (Badger and Price 2003; Moroney and Ynalvez 2007; Spalding 2008, 2009). In recent years, many molecular components involved in the eukaryotic microalgal CCM have been revealed by genetic/molecular studies and genome-wide gene expression analysis in the model organism *Chlamydomonas* (Spalding 2009; Wang and Spalding 2010). However, a comprehensive picture of how the CCM operates in eukaryotic microalgae is still obscure due to the lack of detailed functional analysis of many proteins involved in the CCM. Furthermore, many proteins that apparently are essential for the CCM or limiting CO_2 acclimation are novel proteins that lack known functional domains or motifs, making it challenging to delineate their functions. Understanding the functions of these novel proteins is crucial to the progress of CCM research. Here, we present a case study focusing on LCIB, a novel protein that plays a key role in limiting Ci acclimation in *Chlamydomonas*.

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LCIB mutants

The first *LCIB* mutant was identified over three decades ago. Designated as *pmp1*, the mutant showed a conditional-

lethal growth phenotype in ambient CO_2 and appeared deficient in Ci uptake or accumulation (Spalding et al. 1983a). Identification and genetic analysis of a *pmp1* allelic mutant, *ad1*, later linked the defective gene in both mutants to *LCIB* (Wang and Spalding 2006). Unlike many other mutants requiring high CO_2 to survive, *LCIB* mutants display a very unique "*air dier*" growth phenotype (Van et al. 2001; Spalding et al. 2002; Wang and Spalding 2006): growing in either a high concentration (5 %; HC) or a very low concentration (<0.02 %; VLC) of CO_2, but dying in an intermediate concentration of CO_2 (atmosphere level ~0.03–0.05 %; LC). This unusual growth phenotype implies that there are multiple acclimation states in response to varying CO_2 concentrations, which has also been demonstrated by studying physiological characteristics of a wild type *Chlamydomonas* strain in response to a range of physiologically relevant CO_2 concentrations (Vance and Spalding 2005).

The growth and physiological characteristics of *LCIB* mutants indicate that multiple Ci uptake/accumulation systems are differentially expressed or regulated in response to different Ci concentrations and that LCIB is mainly important in low CO_2 acclimation. When acclimated to low CO_2, *LCIB* mutants or *LCIB* RNAi strains show significant decreases in photosynthetic activities compared with those of wild type strains, as demonstrated by ^{14}C Ci uptake, CO_2-dependent photosynthetic O_2 evolution, and light-dependent CO_2 gas exchange activity. On the contrary, when acclimated to very low CO_2, the photosynthetic activities in these *LCIB* mutants, although still decreased, are not substantially different from those in wild type strains (Spalding et al. 1983a; Wang and Spalding 2006; Duanmu et al. 2009a; Yamano et al. 2010). The single defect in *LCIB* appears to almost entirely eliminate Ci accumulation in low CO_2, which has not been observed in other mutants with only a single CCM component either knocked out or knocked down. Therefore, it has been speculated that LCIB may function mainly in Ci uptake or accumulation under low CO_2 condition (Spalding et al. 1983a; Wang and Spalding 2006). It is evident that a Ci uptake/accumulation system responsible for very low CO_2 acclimation, which is LCIB-independent, appears still functional in *LCIB* mutants.

LCIB gene family and LCIB protein

LCIB was first identified as a novel gene among the most abundantly expressed genes under limiting CO_2 conditions (Miura et al. 2004). Besides *LCIB*, three additional *LCIB*-like genes, *LCIC*, *LCID,* and *LCIE* were found in the *Chlamydomonas* genome, and *LCIB* homologs also exist in several "green lineage" algae (e.g., green algae and diatoms) and a small number of cyanobacteria and bacteria (Wang and Spalding 2006; Spalding 2008; Yamano et al. 2010). Among the four *LCIB* homologous genes in Chlamydomonas, *LCIB* and *LCIC* show a high level of sequence similarity with each other. *LCIB*, *LCIC*, and *LCID* are constitutively expressed in high CO_2, but up-regulated by limiting CO_2. The expression level of *LCIE* is extremely low compared to other three homologs, but still shows significant up-regulation by limiting CO_2 (Miura et al. 2004; Wang and Spalding 2006; Yamano et al. 2008; Fang et al. 2011; Brueggeman et al. 2012). Surprisingly, although LCIB appears to be essential only in low CO_2, transcript levels of *LCIB* in low CO_2 and very low CO_2-acclimated liquid cultures showed no significant differences (Wang and Spalding 2006). Recent RNA-seq analyses also have revealed that none of the other limiting CO_2-induced genes was differentially expressed between low CO_2 and very low CO_2 conditions (Fang et al. 2012).

LCIB and other LCIB-like proteins appear to be soluble in nature. LCIB can be affinity-purified in a tagged-*LCIB* cDNA-complemented *ad1* strain, and LCIC was found to be co-purified with LCIB (Fig. 1a). Both N-terminal sequences of mature LCIB and LCIC, determined by N-terminal amino acid analysis, start with an alanine at the N-terminus after removing the transit peptide at an "alanine- glutamine" cleavage site (Fig. 1b). The co-existence of LCIB and LCIC in a complex has been previously demonstrated, and molecular sizes of LCIB and LCIC were determined as approximately 48 and 49 kD in vivo (Yamano et al. 2010). The deduced sizes of mature LCIB and LCIC are 42.7 and 43.6 kD, respectively, which are smaller than their apparent size on the SDS-PAGE gel, suggesting that some post-translational modifications may occur.

Subcellular localization of LCIB

LCIB has been localized in chloroplasts at two distinct areas: dispersed throughout the entire chloroplast stroma; or concentrated mainly in a discreet region surrounding the pyrenoid, (peri-pyrenoid location) appearing as a ring structure in virtual longitudinal sections under the microscope (Duanmu et al. 2009a; Yamano et al. 2010). These two chloroplast localizations appear to be correlated to different CO_2 acclimation states, although the exact location in low CO_2-acclimated cells is still somewhat controversial. Yamano et al. (2010) have reported that LCIB was only found in a peri-pyrenoid localization in both low and very low CO_2-grown cells, and no significant difference was detected under these CO_2 conditions. This contradicts an early observation that both stromal and peri-pyrenoid localizations have been detected in low CO_2-

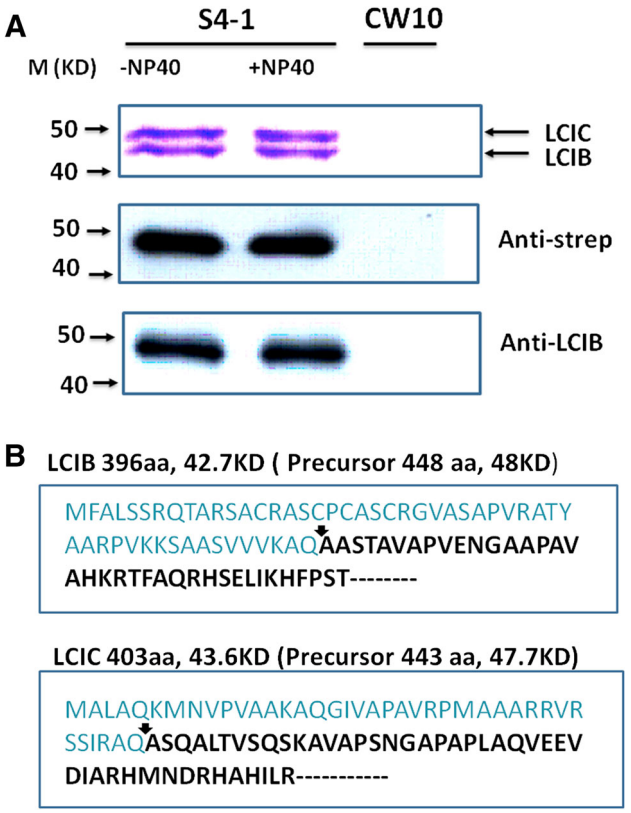

Fig. 1 **a** The LCIB–LCIC complex was affinity-purified in the presence or absence of NP-40 from *S4-1* (an *ad1* strain complemented by strep-tagged LCIB cDNA). The wild type strain *cw10* was used as a control. Proteins from the purified complex were separated by SDS-PAGE electrophoresis (*upper panel*) and analyzed by Western blot with anti-LCIB (*middle panel*) or anti-strep-tag antibodies (*lower panel*). The two protein bands evident on the SDS-PAGE gel were identified by mass-spectrometry analysis as LCIC and LCIB. **b** N-termini of mature LCIB and LCIC were determined by N-terminal amino acid analysis. The sequences of transit peptides followed by the mature proteins are shown; the cleavage sites are marked by "*arrow*"

grown cells (Duanmu et al. 2009a). Considering that a large portion of CO_2 in liquid cell cultures can be removed by photosynthesis under low CO_2 conditions (Vance and Spalding 2005), the actual CO_2 concentration in low CO_2 cultures may shift to very low CO_2 range when influx rates of gas supply cannot keep up with the active photosynthesis. Therefore, to more accurately determine the LCIB localization in response to CO_2 and to test LCIB localization under the condition demonstrated to be lethal for the LCIB mutants, we used cells grown on agar plates to avoid the depletion of CO_2 as possibly occurring in liquid culture. As shown in Fig. 2, the peri-pyrenoid localization of LCIB was only observed to be predominant in very low CO_2-acclimated cells, while LCIB in almost all low CO_2-acclimated cells showed a dispersed stromal localization. The different localizations of LCIB in low and very low CO_2 may be associated with different functions of LCIB under these conditions, although it is completely unknown what the differences in LCIB function are under these two conditions nor how the peri-pyrenoid localization may contribute to this function in very low CO_2.

In high CO_2-grown cells, LCIB is localized mainly in stromal areas. Upon switching the liquid culture from high CO_2 to low CO_2, LCIB was found to re-localize to the peri-pyrenoid area. In contrast, upon shifting low CO_2-grown cells to high CO_2 or to the dark, LCIB diffuses away from the peri-pyrenoid area to the stroma (Yamono et al. 2010). The re-localization in response to CO_2 or dark appears to occur rapidly, usually within 20 min to an hour, and can be blocked by inhibition of photosynthetic electron transfer.

LCIB–LCIC complex

LCIC shows a similar subcellular localization and CO_2 induced re-localization to that of LCIB. In *Chlamydomonas*, LCIB and LCIC appear to form a 350 KD hexameric complex (Yamano et al. 2010). It is not clear how the LCIB–LCIC complex is formed in vivo or how this complex is recruited to the peri-pyrenoid area upon transition from high CO_2 to limiting CO_2. The LCIB–LCIC interaction in complex formation appears to be mediated only by non-covalent bonds and to include no inter-molecular disulfide bonds, because in vitro LCIB–LCIC complex dissociation can be facilitated by SDS, but not by reducing reagents (Fig. 3). Furthermore, the LCIB–LCIC complexes isolated from both high CO_2- and very low CO_2-grown cells appeared to be intact, indicating that the dissociation of complex in vivo does not occur during the re-localization of the LCIB–LCIC complex in response to CO_2 concentration changes (Fig. 3c). Both LCIB and LCIC were reported to be present in phosphorylated forms (Yamano et al. 2010), although it is not demonstrated whether these phosphorylations are of functional importance. Different isoforms of LCIB and LCIC can been detected by 2D gel electrophoresis (Fig. 4), but no obvious changes in their relative positions on 2D gels were observed when cells were switched from high CO_2 to very low CO_2 or when the isolated complexes were treated with phosphatase.

Interaction of LCIB with other CCM components

Although it is likely that re-localization of the LCIB–LCIC complex in chloroplasts is mediated by their interaction with other proteins and that the functioning of LCIB may also require interactions with other components in the CCM, no protein, except LCIC, has so far been identified to physically bind to or physically interact with LCIB. No other proteins are found to be co-eluted with the affinity-purified LCIB–

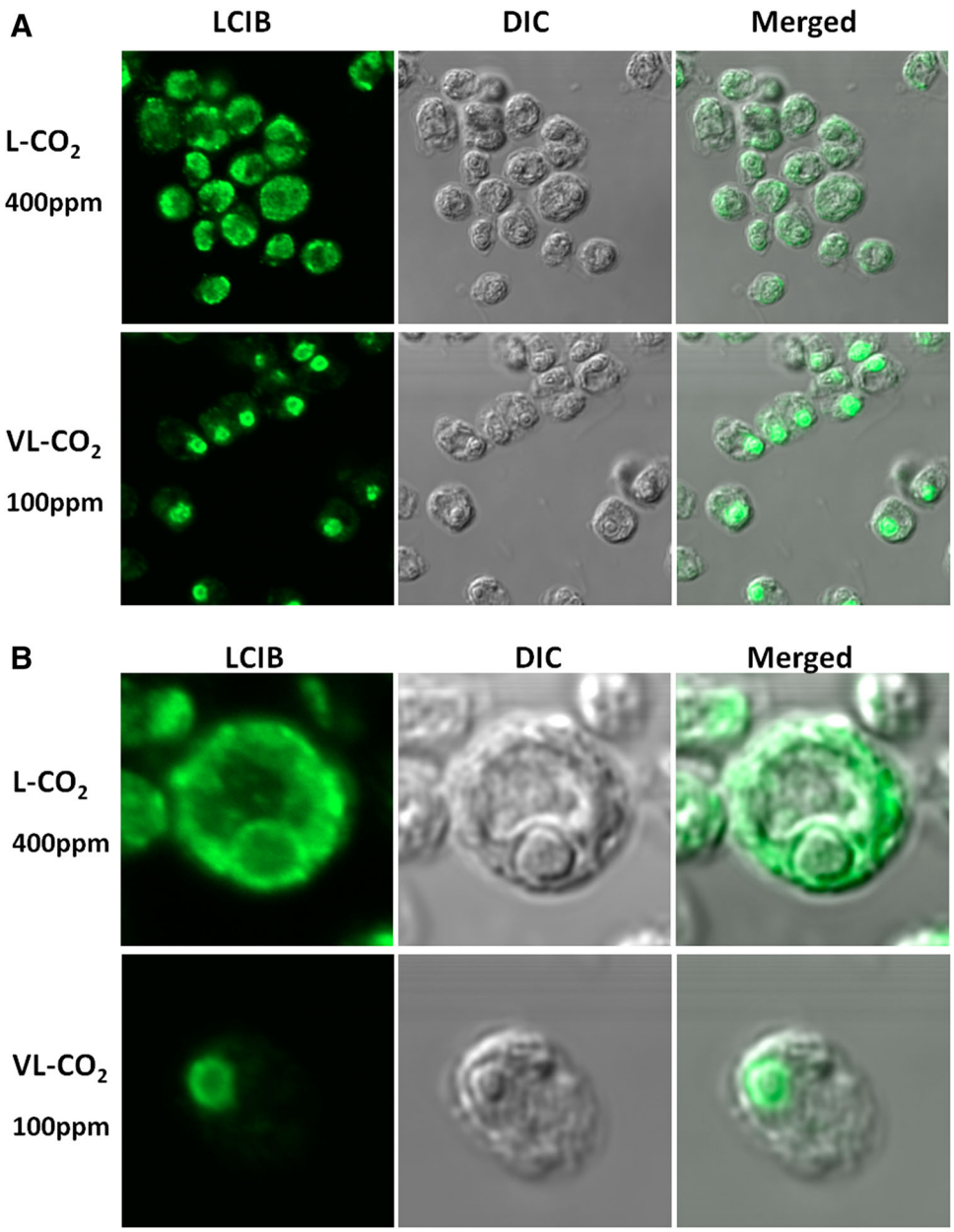

Fig. 2 **a** Immunofluorescent localization of LCIB in wild type *Chlamydomonas reinhardtii* CC125 cells acclimated in low CO_2 (LC) or very low CO_2 (VLC); **b** higher magnification view of LCIB localization in a single cell. The high CO_2-grown cells on agar plates made with the minimal medium were transferred to low CO_2 or very low CO_2 chambers for 20 h and then examined by confocal microscopy

LCIC complex (Fig. 1, also see Yamano et al. 2010). In a large scale yeast two-hybrid screening of a *Chlamydomonas* cDNA expression library using LCIB as the bait protein, 28 cDNAs were identified to encode proteins strongly interacting with LCIB. Of these 28 cDNAs, most are full-length or partial-length *LCIC* cDNAs, and all others are *LCIB* cDNAs (Table 1). It appears that LCIB can interact both with LCIC and with itself, which agrees with a similar observation reported previously (Yamano et al. 2010). One cDNA that encodes LCI24 also was identified from our initial yeast two-hybrid cDNA library screening, but the interaction appeared very weak, and so far cannot be further confirmed. Noticeably, LCI24 appears as a membrane protein and has been identified as a low CO_2-induced (LCI) protein (Miura et al. 2004), although like many "function unknown" LCI proteins, no recognizable domain or motif can be identified. It remains to be determined whether other proteins interact with the LCIB–LCIC complex and, if so, how they contribute to the function of LCIB–LCIC complex or its localization.

Aside from physical interaction, LCIB has been shown to be functionally connected to other CCM components as demonstrated by the extragenic suppressors (mutations) that suppress or overcome the *air-dier* growth phenotype of *ad1* or *pmp1* (Duanmu et al. 2009a; Duamnu and Spalding 2011). Six such second-site suppressors have been analyzed and show two distinct growth phenotypes. Four suppressors (*su1, su4, su5, and su8*) have been shown to restore wild type-like growth in both low CO_2 and very low

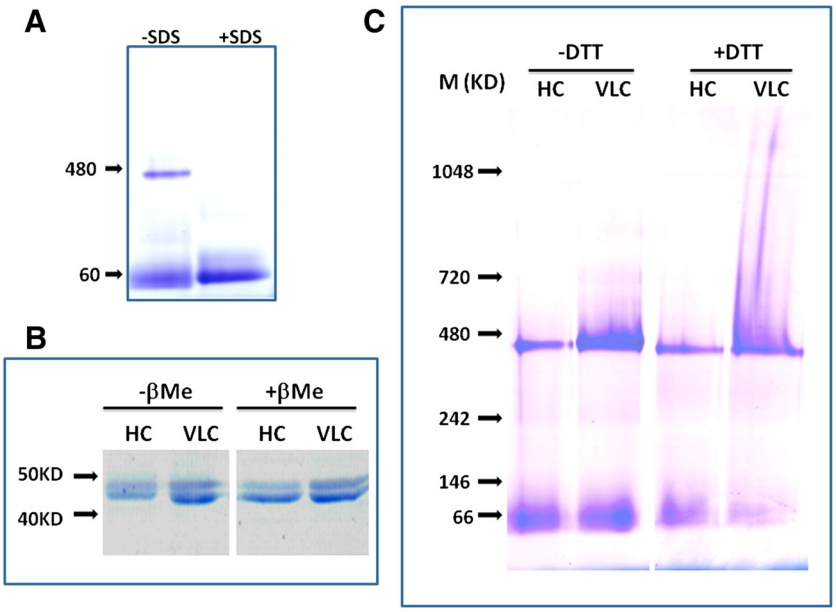

Fig. 3 **a** LCIB–LCIC complex purified from an *ad1* strain complemented with a strep-tagged LCIB gene. The complex was treated with or without 0.5 % SDS and analyzed by native (lacking SDS and reductant) protein gel electrophoresis. The upper band (between 242 and 480 kD) and the lower band (<66 kD) observed on the gel are from the complex form and the dissociated monomers, respectively. **b** LCIB–LCIC complexes from high CO_2 (HC)- or very low CO_2 (VLC)-acclimated cells, when still bound to affinity resin, were incubated with or without β-mercaptoethanol (2 %). The complex was then washed and eluted from the affinity beads and analyzed by SDS-PAGE electrophoresis. The existence of both LCIB and LCIC on the gel indicates that LCIC was still bound to LCIB after the treatment with β-mercaptoethanol. **c** LCIB–LCIC complex isolated from high CO_2 (HC) or very low CO_2 (VLC) was analyzed by native protein gel electrophoresis. Incubation with DTT (1 mM) has no detectable impact on the complex

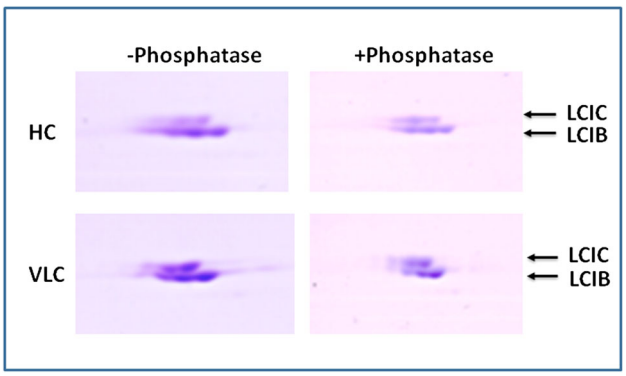

Fig. 4 2D gel electrophoresis of LCIB–LCIC complex purified from high CO_2 (HC)- or very low CO_2 (VLC)-acclimated cells. Lambda protein phosphatase (New England Biolabs) was used for dephosphorylation treatment

Table 1 LCIB-interacting proteins identified in the yeast two-hybrid screen

Protein Name	ID[a]	# of hits	Up- regulated by low CO_2	Verified[b]
LCIC	524046	23	Yes	Yes
LCIB	510298	5	Yes	Yes
LCI24	521061	1	Yes	No

Yeast two-hybrid screen was performed with HybriZAP2.1 system (Stratagene, La Jolla, CA). The bait plasmid expressing LCIB (amino acids 79–448) was co-transformed with the target plasmids containing the cDNA expression library (Wang and Spalding 2006). ~10^6 colonies were screened

[a] Augustus 5.0 gene model protein ID (http://augustus.gobics.de/predictions/chlamydomonas/)

[b] The verification includes detection of β-galactosidase activity by the filter lift assay, re-introducing target plasmids and bait plasmids into the yeast host strain and verifying the specificity of interaction with control plasmids

CO_2 conditions, but two other allelic suppressors, *su6* and *su7*, in direct contrast to the *air-dier* growth phenotype of *ad1* and *pmp1*, restore growth in low CO_2, but lose the ability to survive in very low CO_2. Genetic analysis has indicated that these 6 suppressors are distributed in four different loci. In addition to the *su6/7*allele, *su4* and *su5* also appear to be allelic to each other. Among these four loci, *su6* and *su7* have been identified as *CAH3* mutations, which also cause a high CO_2 requiring phenotype themselves (Duanmu et al. 2009a). The other 4 suppressor loci (*su1*, *su4/5*, *su8*) appear not to cause noticeable growth defects or other growth phenotypes when not combined with an *lcib* mutation (Duanmu and Spalding 2011). In all three non-*cah3* suppressors, the photosynthetic affinity for Ci and Ci uptake/accumulation are, either fully or partially, restored to the levels attained in wild type strains. The multiple suppression loci for *ad1*/*pmp1* suggest that at least

several components are required or involved in LCIB-mediated Ci uptake/accumulation or low CO_2 acclimation.

Proposed function for LCIB in the CCM

The CO_2-concentrating mechanism in eukaryotic algae is initiated by active Ci uptake systems at the plasma membrane and the chloroplast envelope (Sültemeyer et al. 1988; Amoroso et al. 1998). Although not yet fully characterized, several putative Ci transporters have been either confirmed or proposed to function in Ci uptake, including HLA3, NAR1.2(LCIA), LCI1, CCP1, and CCP2, as well as LCIB (Miura et al. 2004; Pollock et al. 2004; Mariscal et al. 2006; Spalding 2008; Duanmu et al. 2009b; Ohnishi et al. 2010; Wang et al. 2010). Once in the chloroplast, Ci is accumulated in the form of HCO_3^- at the alkaline pH in the stroma that favors HCO_3^- formation, and the equilibrium between different Ci species is suggested to be catalyzed by CA activities (Mitra et al. 2004, 2005; Moroney and Ynalvez 2007). The final step is the dehydration of accumulated HCO_3^- to provide CO_2 inside the pyrenoid for the carboxylation reaction catalyzed by Rubisco. This dehydration step is mediated by a CA, CAH3, which is localized on the luminal side of the thylakoids and inside the pyrenoid tubules (Spalding et al. 1983b; Funke et al. 1997; Karlsson et al. 1998; Hanson et al. 2003; Mitra et al. 2005; Markelova et al. 2009). As CO_2 can easily diffuse away from the pyrenoids or the chloroplasts, some CO_2 barriers or recapture systems have been proposed to prevent the CO_2 leakage (Moroney and Ynalves 2007; Duanmu et al. 2009a; Yamano et al. 2010). The primary defect of the CCM in *LCIB* mutants appears to be in Ci accumulation. Since a deficiency in Ci uptake, Ci pool maintenance, or prevention of CO_2 leakage can all potentially collapse the Ci accumulation, the functions of LCIB in these pathways have been proposed and examined.

LCIB mutants were initially thought to be defective in Ci uptake, but it is unlikely that LCIB is directly involved in Ci transport based on its molecular characteristics and subcellular location. The fact that the *LCIB* mutation, when combined with the *CAH3* mutation, has little impact on Ci over-accumulation in the *lcib/cah3* double mutants also strongly argues against the possibility of LCIB being involved directly in Ci uptake (Spalding et al. 1983c; Duanmu et al. 2009a). The *cah3* mutation appears to override the *air-dier* phenotype and the defective Ci accumulation in *LCIB* mutants, and this epistatic relationship of *cah3* and *lcib* implies that LCIB functions downstream of CAH3, and thus, is likely to capture CO_2 released by CAH3 and prevent CO_2 leakage (Duanmu et al. 2009a).

It is still not clear how LCIB is involved in this process. It has been proposed that LCIB may function as a structural barrier to prevent CO_2 diffusion from the pyrenoid (Yamano et al. 2010). This would require LCIB to be localized around the pyrenoid. Although LCIB is localized around the pyrenoid under very low CO_2 conditions, it shows stroma localization in low CO_2 conditions. The low CO_2 conditions under which LCIB is demonstrated to have a stromal localization also correspond directly to the conditions under which the *LCIB* mutation causes a lethal growth phenotype (Fig. 2), which is inconsistent with the hypothesis that LCIB acts as CO_2 barrier in low CO_2 acclimation. An alternative hypothesis that appears more appealing is one in which LCIB functions in CO_2 recapture by re-hydrating leaked CO_2 back into the HCO_3^- pool (Duanmu et al. 2009). Such a function overlaps the similar, proposed function for CAH6, a CA reportedly localized in the stroma (Mitra et al. 2005; Moroney and Ynalvez 2007). Given the fact that LCIB mutations eliminate almost all Ci pool accumulation in low CO_2-grown cells, it is not likely that CAH6 itself possesses the full capacity to maintain Ci accumulation in the chloroplast. It is possible that LCIB activates CAH6 by an unknown mechanism to fulfill the full potential of CAH6 as a CA or that LCIB may be involved in a process functionally analogous to CA activity when it interacts with other yet unidentified proteins.

In cyanobacteria, ChpX and ChpY (also named CupA and CupB) are two hydrophilic proteins that are involved in NADH dehydrogenase (NDH)-driven CO_2 uptake systems by catalyzing light-dependent CO_2 hydration reactions (Maeda et al. 2002; Price et al. 2002). The *ChpX/ChpY* double mutant showed a significant CO_2 leakage under steady state photosynthesis, suggesting that these two proteins function in intracellular Ci maintenance by re-capturing CO_2 released from carboxysomes (Price et al. 2002; Price et al. 2008). It is possible that the LCIB/LCIC complex is functionally analogous with ChpX/Y to prevent CO_2 leakage by unidirectionally hydrating intracellular CO_2. In Cyanobacteria, ChpX/ChpY (CupA/CupB)-associated CO_2 uptake systems are possibly localized at the thylakoid membranes (Price et al. 2002; Xu et al. 2008). It is not clear whether the LCIB–LCIC complex in the stroma is associated with the thylakoid membrane under low CO_2, although it was indeed reported that LCIB, when localized to the peri-pyrenoid area, is associated with thylakoid tubules (Yamano et al. 2010). It has been reported that a CA activity located on chloroplast envelope membranes is significantly up-regulated by low CO_2 (Villarejo et al. 2001), and interestingly, this CA activity was reported as missing in *pmp1*. Investigation of the molecular basis for this membrane bound CA activity may shed some light on how the Ci pool is maintained in the chloroplast and the role of LCIB in this function.

Another mystery regarding the *air-dier* phenotype in *LCIB* mutants is how acclimation to very low CO_2 is

differentiated from that to low CO_2. It was proposed that another component or system may be induced or activated in very low CO_2 to replace LCIB (Wang and Spalding 2006; Spalding 2008). It is also possible that both acclimation states share a common system for Ci uptake/accumulation and that LCIB only activates this system in the low CO_2 acclimation state. Yamano et al. (2010) have reported a very interesting multiphasic CO_2 response in low CO_2-acclimated *LCIB* mutants when photosynthetic oxygen evolution was measured. They reported that the photosynthetic activity of these cells was high in responding to addition of 100 μM Ci (corresponding to very low CO_2 concentration ~0.009 % at pH 7.8), but significantly reduced when higher Ci concentrations (500–700 μM, corresponding to low CO_2 ~0.047–0.066 %) were provided. It should be noted that these *LCIB* mutant cells were acclimated to only low CO_2, and the short period of time during the measurement may not be sufficient to allow new proteins to be synthesized. If such photosynthetic characteristics reflect the actual molecular compositions for the system responsible for CO_2 uptake/accumulation, it implies that the low CO_2-acclimated mutant cells still retain a system with high capacity for Ci uptake/accumulation in very low CO_2. Without LCIB, this system must be repressed when exposed to low CO_2. It is possible that this system functions in both low CO_2 and very low CO_2 by switching the affinity to Ci, like the dual-affinity nitrate transporter characterized previously (Liu and Tsay 2003). If this is true, it can be speculated that low CO_2 may repress the high affinity state and activate the low affinity state and that LCIB is required in such low CO_2 specific activation. The observed repression of photosynthetic oxygen evolution by low CO_2 appears rather rapid (within seconds to a few minutes) given the fact that CO_2 has to be depleted first (equivalent to very low CO_2) before external Ci was provided for the measurement. This is consistent with the observed *air dier* phenotype: the mutant appears to be locked into low CO_2 acclimation states when grown in ambient level CO_2, because otherwise the Ci uptake system responsible for very low CO_2 acclimation would function to allow the mutants to survive in low CO_2 when internal Ci is depleted. Regardless of the molecular mechanism for low or very low CO_2 acclimation, it appears that external CO_2 concentration, rather than total Ci or HCO_3^- concentration, is the sole trigger controlling the CO_2 acclimation, as external growth pH has little impact on the *air dier* phenotype in *LCIB* mutants (Fig. 5).

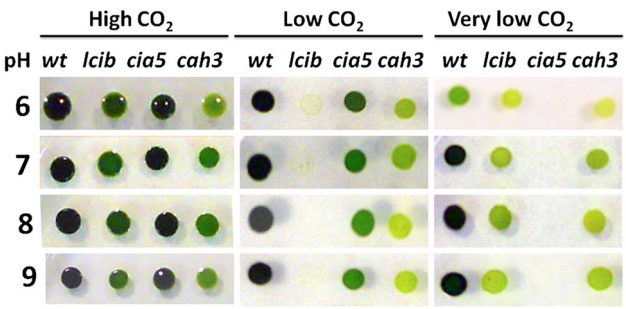

Fig. 5 Spot test for growth of *C. reinhardtii* strains in high CO_2 (5 %), low CO_2 (0.045 %), and very low CO_2 (0.015 %) under various pH conditions. The strains include wild type (*21gr*), mutants *cia5*, *pmp1*, and *ca1*(*cah3*)

Conclusion

An LCIB-mediated CO_2 uptake/accumulation system represents a unique CCM mode that occupies a central position in the *Chlamydomonas* CCM. The current hypothesis is that LCIB participates in trapping stromal CO_2 released from the thylakoid into the stromal HCO_3^- pool. If this is true, it also implies that LCIB may also function to capture the external CO_2 diffusing into chloroplasts and maintain the intracellular Ci accumulation. It is still unknown how LCIB functions in this model and how its function is differentially associated with low and very low CO_2 acclimation states. To answer these questions, further investigation are required in several key areas, including molecular and biochemical characterization of LCIB and LCIC, the relationship between the subcellular localization of the LCIB–LCIC complex and physiological acclimation states, and proteins physically or functionally interacting with LCIB or LCIC. Genetic approaches, as exemplified by study of extragenic suppressors, will also help to understand the relationship of LCIB and other CCM components and to define the position and the role of LCIB in different CCM pathways and acclimation states.

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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 Physiol 116:193–201

Badger MR, Price GD (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J Exp Bot 54:609–622

Brueggeman AJ, Gangadharaiah DS, Cserhati MF, Casero D, Weeks DP, Ladunga I (2012) Activation of the carbon-concentrating mechanism by CO_2 deprivation coincides with massive transcriptional and metabolic restructuring in *Chlamydomonas reinhardtii*. Plant Cell 24:1860–1875

Duanmu D, Spalding MH (2011) Insertional suppressors of *Chlamydomonas reinhardtii* that restore growth *of air-dier* lcib mutants in low CO_2. Photosynth Res 109:123–132

Duanmu D, Wang Y, Spalding MH (2009a) Thylakoid lumen carbonic anhydrase (CAH3) mutation suppresses air-dier phenotype of LCIB mutant in *Chlamydomonas reinhardtii*. Plant Physiol 149:929–937

Duanmu D, Miller AR, Horken KM, Weeks DP, Spalding MH (2009b) Knockdown of a limiting-CO_2-inducible gene HLA3 decreases bicarbonate transport and photosynthetic Ci-affinity in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 106:5990–5995

Fang W, Si Y, Douglass S, Casero D, Merchant SS, Pellegrini M, Ladunga I, Liu P, Spalding MH (2012) Transcriptome-wide changes in *Chlamydomonas reinhardtii* gene expression regulated by carbon dioxide and the CO_2-concentrating mechanism regulator CIA5/CCM1. Plant Cell 24:1876–1893

Funke RP, Kovar JL, Weeks DP (1997) Intracellular carbonic anhydrase is essential to photosynthesis in *Chlamydomonas reinhardtii* at atmospheric levels of CO_2. Plant Physiol 114:237–244

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 Physiol 132:2267–2275

Karlsson J, Clarke AK, Chen ZY, Hugghins SY, Youn-II P, 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. EMBO J 17:1208–1216

Liu KH, Tsay YF (2003) Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J 22:1005–1013

Maeda S, Badger MR, Price GD (2002) Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO_2 hydration in the *cyanobacterium, Synechococcus* sp. PCC7942. Mol Microbiol 43:425–435

Mariscal V, Moulin P, Orsel M, Miller AJ, Fernández E, Galván A (2006) Differential regulation of the *Chlamydomonas Nar1* gene family by carbon and nitrogen. Protist 157:421–433

Markelova A, Sinetova M, Kupriyanova E, Pronina A (2009) Distribution and functional role of carbonic anhydrase Cah3 associated with thylakoid membranes in the chloroplast and pyrenoid of *Chlamydomonas reinhardtii*. Russian J Plant Physio 56:761–768

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

Mitra M, Mason CB, Xiao Y, Ynalvez RA, Lato SM, Moroney JV (2005) The carbonic anhydrase gene families of *Chlamydomonas reinhardtii*. Can J Bot 83:780–795

Miura K et al (2004) Expression profiling-based identification of CO_2-responsive genes regulated by CCM1 controlling a Carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Physiol 135:1595–1607

Moroney JV, Ynalvez RA (2007) Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*. Eukaryot Cell 6:1251–1259

Ohnishi N, Mukherjeeb B, Tsujikawaa T, Yanasea M, Nakanoa H, Moroneyb J, Fukuzawaa H (2010) Expression of a low-CO2-inducible protein, LCI1, increases inorganic carbon uptake in the green alga *Chlamydomonas reinhardtii*. Plant Cell 9:3105–3117

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 Mol Biol 91:505–513

Price GD, Maeda SI, Omata T, Badger MR (2002) Modes of active inorganic carbon uptake in the cyanobacterium, *Synechococcus sp. PCC7942*. Funct Plant Biol 29:131–149

Price GD, Badger MR, Woodger FJ, Long BM (2008) Advances in understanding the cyanobacterial CO_2-concentrating mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. J Exp Bot 59:1441–1461

Spalding MH (2008) Microalgal carbon-dioxide-concentrating mechanisms: *Chlamydomonas* inorganic carbon transporters. J Exp Bot 59:1463–1473

Spalding MH (2009) The CO_2 concentrating mechanism and carbon assimilation. In: Harris EH, Stern DB (eds) The *Chlamydomonas* sourcebook: organellar and metabolic processes, Vol 2, 2nd edn. Academic, Oxford, pp 257–301

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

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

Spalding MH, Spreitzer RJ, Ogren WL (1983c) Genetic and physiological analysis of the CO_2-concentrating system of *Chlamydomonas reinhardtii*. Planta 159:261–266

Spalding MH, Van K, Wang Y, Nakamura Y (2002) Acclimation of *Chlamydomonas* to changing carbon availability. Funct Plant Biol 29:221–230

Sültemeyer DF, Klöck G, Kreuzberg K, Fock HP (1988) Photosynthesis and apparent affinity for dissolved inorganic carbon by cells and chloroplasts of *Chlamydomonas reinhardtii* grown at high and low CO_2 concentrations. Planta 176:256–260

Van K, Wang Y, Nakamura Y, Spalding MH (2001) Insertional mutants of *Chlamydomonas reinhardtii* that require elevated CO_2 for survival. Plant Physiol 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. Can J Bot 83:796–809

Villarejo A, Rolland N, Martínez 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

Wang Y, Spalding MH (2006) An inorganic carbon transport system responsible for acclimation specific to air levels of CO_2 in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 103:10110–10115

Wang Y, Duanmu D, Spalding MH (2011) Carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*: inorganic carbon transport and CO2 recapture. Photosynth Res 109:115–122

Xu M, Ogawa T, Pakrasi HB, Mi H (2008) Identification and localization of the CupB protein involved in constitutive CO_2 uptake in the *Cyanobacterium, Synechocystis* sp. Strain PCC 6803. Plant Cell Physiol 49:994–997

Yamano T, Miura K, Fukuzawa H (2008) Expression analysis of genes associated with the induction of the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Physiol 147:340–354

Yamano T, Tsujikawa T, Hatano K, Ozawa SI, Takahashi Y, Fukuzawa H (2010) Light and low-CO_2 dependent LCIB/LCIC complex localization in the chloroplast supports the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Cell Physiol 51:1453–1468