CO₂-dependent migration and relocation of LCIB, a pyrenoid-peripheral protein in *Chlamydomonas reinhardtii*

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Abstract

Most microalgae overcome the difficulty of acquiring inorganic carbon (Ci) in aquatic environments by inducing a CO₂-concentrating mechanism (CCM). In the green alga *Chlamydomonas reinhardtii*, two distinct photosynthetic acclimation states have been described under CO₂-limiting conditions (low-CO₂ [LC] and very low-CO₂ [VLC]). LC-inducible protein B (LCIB), structurally characterized as carbonic anhydrase, localizes in the chloroplast stroma under CO₂-supplied and LC conditions. In VLC conditions, it migrates to aggregate around the pyrenoid, where the CO₂-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase is enriched. Although the physiological importance of LCIB localization changes in the chloroplast has been shown, factors necessary for the localization changes remain uncertain. Here, we examined the effect of pH, light availability, photosynthetic electron flow, and protein synthesis on the localization changes, along with measuring Ci concentrations. LCIB dispersed or localized in the basal region of the chloroplast stroma at 8.3–15 μM CO₂, whereas LCIB migrated toward the pyrenoid at 6.5 μM CO₂. Furthermore, LCIB relocated toward the pyrenoid at 2.6–3.4 μM CO₂, even in cells in the dark or treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea and cycloheximide in light. In contrast, in the mutant lacking CCM1, a master regulator of CCM, LCIB remained dispersed even at 4.3 μM CO₂. Meanwhile, a simultaneous expression of LCIC, an interacting protein of LCIB, induced the localization of several speckled structures at the pyrenoid periphery. These results suggest that the localization changes of LCIB require LCIC and are controlled by CO₂ concentration with ~7 μM as the boundary.

Introduction

CO₂ fixation by photosynthesis in aquatic organisms is limited by several factors, including the slow diffusion rate of CO₂ in the aquatic environment, the low catalytic activity of the CO₂-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), and the oxygenase activity of Rubisco (Raven et al., 2011; Fukuzawa et al., 2012). To maintain CO₂ fixation rates in such CO₂-limited environments,
most aquatic algae induce a CO₂-concentrating mechanism (CCM) that actively takes up inorganic carbon (Ci; CO₂ and HCO₃⁻) into the cell and increases the CO₂ concentration in the pyrenoid, a chloroplast microcompartment containing most of the cell’s Rubisco (Hennacy and Jonikas, 2020).

Pyrenoids are one of the central features of eukaryotic algal CCMs, increasing the CO₂/O₂ ratio at the active site of Rubisco, decreasing its oxygenase activity, and leading to maximal carboxylase activity. The molecular and biochemical aspects of pyrenoids have been well studied using the green alga *Chlamydomonas reinhardtii* (Meyer et al., 2017; Mackinder, 2018; Barrett et al., 2021). The *Chlamydomonas* pyrenoid is composed of a spherical Rubisco matrix that is traversed by pyrenoid tubules, a network of membranes continuous with stromal thylakoids (Ohad et al., 1967; Engel et al., 2015). Rubisco molecules are linked by essential pyrene component 1 (EPYC1; Mackinder et al., 2016). The Rubisco-EPYC1 system is dynamic and can undergo liquid–liquid phase separation, notably in response to changes in CO₂ concentration (Freeman Rosenzweig et al., 2017; Wunder et al., 2018). A starch sheath, consisting of multiple starch plates, encapsulates the Rubisco-EPYC1 matrix to complete the pyrenoid structure (Sager and Palade, 1957).

Another essential feature of the CCM is its active Ci transport system that concentrates extracellular Ci in the form of CO₂ into the pyrenoid. *Chlamydomonas* has at least two types of Ci transport systems depending on the CO₂ concentration (Wang and Spalding, 2014a) and can adapt to at least two types of CO₂-limiting conditions (Vance and Spalding, 2005) called very low-CO₂ (VLC) and low-CO₂ (LC).

In VLC conditions, HCO₃⁻ is actively transported from the extracellular medium to the chloroplast stroma by the cooperative function of high-light activated 3 (HLA3), an ABC-type transporter in the plasma membrane, and LC-inducible protein A (LCIA), an anion channel in the chloroplast envelope (Duanmu et al., 2009; Miura et al., 2004; Gao et al., 2015; Yamano et al., 2015). Furthermore, bestrophin-like proteins (BST1–3), anion channels in the thylakoid membrane, are thought to transport HCO₃⁻ from the stroma to the thylakoid lumen (Mukherjee et al. 2019). Once in the lumen, carbonic anhydrase 3 (CAH3) conjugates with photosynthetic proton transport across the thylakoid membrane to convert HCO₃⁻ to CO₂, which diffuses into the pyrenoid matrix and is fixed by Rubisco (Karlsson et al., 1998).

In LC conditions, Ci-transport switches from the HCO₃⁻ uptake system to a CO₂ uptake system, and it has been proposed that the HLA3/LCIA-mediated HCO₃⁻ uptake system is inhibited by an unknown mechanism as the CO₂ concentration increases to LC levels (Wang and Spalding, 2014a). LCIB is an essential factor in the CO₂ uptake system (Miura et al. 2004; Wang and Spalding, 2006); LCIB interacts with its homologous protein LCIC (Yamano et al., 2010), and the crystalline structures of these proteins resemble that of β-type carbonic anhydrase (CA), which possesses an active CA site that coordinates with a zinc ion (Jin et al., 2016). Based on these findings, the LCIB/LCIC complex is assumed to convert CO₂ to HCO₃⁻ to maintain the Ci pool in the chloroplast stroma.

LCIB accumulates slightly in high-CO₂ (HC) conditions but is strongly accumulated in LC and VLC conditions (Yamano et al., 2010). The lcib mutant ad1 cannot grow in LC conditions (air-dier phenotype; Wang and Spalding, 2006) but can survive in HC and VLC conditions. This unique phenotype is supported by a biphasic curve of photosynthetic O₂-evolving activity of several lcib mutants. In VLC conditions, the O₂-evolving activity of lcib mutants is comparable to that of wild-type (WT) cells, but it decreases in LC conditions, suggesting that LCIB is indispensable for survival in LC conditions (Yamano et al., 2010; Wang and Spalding, 2014a).

Of note, the localization of LCIB in the chloroplast changes in response to changes in CO₂ concentration. In HC and LC conditions, LCIB is dispersed within the chloroplast stroma (Wang and Spalding, 2014a). In contrast, in VLC conditions, LCIB moves close to the pyrenoid to form the ring-like structure (referred to as “migration” in this study; Yamano et al., 2010; Wang and Spalding, 2014b). Furthermore, when the CO₂ concentration is changed from VLC to HC or LC, LCIB disperses within the chloroplast stroma, but it again moves to around the pyrenoid when switching the CO₂ concentrations from HC or LC to VLC (referred to as “relocation” in this study; Yamano et al., 2010). Recently, we showed that the structure of the starch sheath itself is required for LCIB localization around the pyrenoid and the maintenance of increased Ci-affinity in VLC conditions (Toyokawa et al., 2020). Thus, the physiological importance of LCIB function has been elucidated; however, the factors necessary and sufficient for LCIB migration and relocation are not fully understood.

In this study, to clarify the factors required for LCIB migration and relocation in the chloroplast, we traced the high-resolution localization of LCIB and examined the effect of pH, light availability, photosynthetic electron flow, and protein synthesis on the localization changes along with measuring Ci concentrations of the medium. We showed a series of evidence to support that the reversible localization changes of LCIB in the chloroplast was switched at an external CO₂ concentration boundary of ~7 µM without any requirement for light, photosynthetic electron flow, and de novo protein synthesis other than LCIC. Because LCIB is one of the key factors for driving the CCM, and homologs of LCIB are highly conserved in algal species harboring a CCM (Yamano et al., 2010), elucidating part of the mechanism of LCIB localization change will provide essential insights for CCM research.

**Results**

**Isolation of a transgenic line expressing LCIB-Clover**

In our previous study (Toyokawa et al., 2020), we used lcib-insertion mutant B1 obtained from the *Chlamydomonas* Library Project (CLiP; Li et al., 2016). However, B1 cells had a lower amount of chlorophyll per cell, smaller cell size,
adhered to the test tube surface more than the WT strain C9, making it unsuitable for physiological experiments. Thus, to isolate an lcib-insertion mutant with a C9 background, we first crossed B1 with WT strain CC-1690 (LCIB, mt⁺) and obtained progeny strain F₁, line 73-4 (lcib, mt⁺; B2 hereafter; Figure 1A). Next, we crossed B2 with C9 (LCIB, mt⁻) and obtained five progenies. Among these, strain F₂ 99-4 (lcib, mt⁺; B3 hereafter) showed a comparable amount of chlorophyll per cell and cell size to C9 cells. Finally, to visualize the localization of LCIB in vivo, we introduced an LCIB-Clover expression plasmid (pCT1; Nitta et al., 2018), in which the target gene is driven by a constitutive HSP70A-RBCS2 promoter and terminated by RBCS2 3’-UTR, into B3 cells and obtained strain MBC-3 (LCIB-Clover, mt⁺). In the MBC-3 cells, the fluorescence signal of Clover (a green fluorescence protein variant) fused with LCIB was observed around the pyrenoid in VLC conditions. In B3 cells, LCIC (49 kDa), the interacting protein of LCIB (48 kDa), was also hardly detected as with the previously reported LCIB RNAi strains (Yamano et al., 2010); however, the LCIC accumulation was recovered in MBC-3 cells along with the accumulation of LCIB-Clover (68 kDa; Figure 1B).

Figure 1 Isolation and characterization of LCIB-Clover expressing strain. A, Strategy to obtain the LCIB-Clover-expressing strain, MBC-3, by the genetic crossing of lcib-insertion mutant with WT strains (CC-1690 and C9) and introduction of the expression plasmid pCT1. mt⁻, mating type minus; mt⁺, mating type plus. B, Accumulation of LCIB, LCIC, and LCIB-Clover fusion proteins in C9, B3, and MBC-3 cells. Cells were grown in liquid culture aerated with 5% CO₂ or 0.04% CO₂ for 12 h. Histone H3 was used as a loading control. The asterisk indicates non-specific bands from C9 and B3 cells, which appeared at the same molecular weight as LCIB-Clover. kDa, kilo Dalton. C, Spot tests for the growth of C9, B3, and MBC-3 cells. Cell suspensions were diluted to the indicated optical density at 730 nm, and 3 μL of each diluted cell suspension was spotted onto MOPS-P agar plates and incubated in HC (5% CO₂) conditions for 4 d, LC (0.04% CO₂) conditions for 5 d, or VLC (0.01% CO₂) conditions for 8 d. D, Typical responses of net O₂-evolving activities of C9 (closed circles), B3 (open circles), and MBC-3 cells (closed squares) against calculated CO₂ concentrations at pH 7.8 for the ranges of 0–400 μM CO₂ and 0–27.5 μM CO₂ (inset). Shaded areas represent different physiological CO₂-acclimation states of Chlamydomonas: VLC (7 μM CO₂; dark gray), LC (7–70 μM; medium gray), and HC (70 μM CO₂; light gray). CO₂ concentrations were calculated from the concentration of Ci added, assuming that CO₂ and HCO⁻ are in equilibrium due to the activity of CA localized in the periplasmic space. Before measurements, cells were grown in the liquid culture aerated with 0.04% CO₂ for 12 h. E, Vₘₐₓ and K₅₀ (CO₂) values. The CO₂ concentration required for the half-maximal rate of Vₘₐₓ of C9, B3, and MBC-3 cells was calculated from the O₂-evolving activities in D. Data in all experiments are mean values ± standard deviation from three or four biological replicates. *P-value < 0.01, Student’s t-test.
To examine whether LCIB-Clover in MBC-3 cells was functional in vivo, we compared the growth rates of C9, B3, and MBC-3 cells in different CO₂ conditions and evaluated their photosynthetic characteristics by measuring their Ci-dependent O₂-evolving activity. The growth of B3 cells was inhibited in LC conditions but not in HC and VLC conditions (Figure 1C). Ci-depleted B3 cells grown in medium aerated with 0.04% CO₂ showed a biphasic curve of photosynthetic O₂-evolving activity with the gradual addition of NaHCO₃ from lower to higher concentrations (Figure 1D); the activity increased at calculated CO₂ concentrations <7 μM, decreased between 7 and 70 μM CO₂, and again increased above a concentration of ~70 μM CO₂. The CO₂ concentration of 7 μM, at which the O₂-evolving activity of B3 cells switched from increasing to decreasing, corresponded to the threshold CO₂ concentrations in VLC and LC conditions as reported previously (Wang and Spalding, 2014a). Considering that B3 cells’ phenotypes with retarded growth rates and decreased O₂-evolving activity under LC conditions were complemented with MBC-3 cells, we concluded that LCIB-Clover in MBC-3 cells was functional in vivo.

Additionally, we also found that the maximum rate of O₂-evolving activity (Vₘαx) of B3 cells decreased to 66% and 78% of that of C9 cells when grown in medium aerated with 5% CO₂ or with 0.04% CO₂ (Figure 1E). Moreover, the K₅₀ (CO₂) value, the CO₂ concentrations required for half of Vₘαx of B3 was 3.8-fold higher than that of the C9 cells when grown with aeration with 5% CO₂ (Figure 1E). These results suggested that LCIB is necessary for maintaining photosynthetic activity in HC conditions as well as LC conditions.

### LCIB migration to the pyrenoid depends on CO₂, not on total Ci and HCO₃⁻ concentrations in liquid culture medium

So far, changes in LCIB localization in liquid culture conditions were observed by varying the CO₂ concentration (e.g., 5% or 0.04% CO₂) aerated into the liquid medium. However, because CO₂ dissolves in water to form dissolved Ci, it was not clear whether LCIB migration depended on CO₂ or HCO₃⁻. To clarify whether LCIB migration to around the pyrenoid depended on either CO₂ or HCO₃⁻ in total Ci in the culture medium, we observed the fluorescence signals of LCIB-Clover in MBC-3 cells with different ratios of HCO₃⁻ to CO₂ concentrations by varying the pH of the medium (Figure 2A and B). To this end, we measured the actual Ci concentrations ([Ci]) in the culture medium using gas chromatography along with observation and calculated pH-dependent dissolved CO₂ concentrations ([CO₂]) and HCO₃⁻ concentrations ([HCO₃⁻]). We also calculated the coefficient of variation (CV) values of LCIB-Clover fluorescence signals to quantify the subcellular localization pattern in the chloroplast (Figure 2C). As shown in previous reports (Nitta et al., 2018; Toyokawa et al., 2020), high- and low-CV values corresponding to aggregation and dispersion patterns of LCIB-Clover in the chloroplast, respectively.

When we cultured MBC-3 cells in medium aerated with 0.04% CO₂ at pH 7.0 for 24 h, actual [Ci], calculated [CO₂], and calculated [HCO₃⁻] were 16, 2.9, and 13 μM, respectively. In this condition, [CO₂] was in the range of VLC, and LCIB-Clover localized around the pyrenoid as a “ring-like” structure in two-dimensional projection (median CV value 1.86; I in Figure 2B). In three-dimensional reconstructions from Z-stacked images, LCIB-Clover was clustered as several puncta around the pyrenoid (Supplemental Figure S1, A and B and Supplemental Movie S1). Next, when we replaced the medium with those at pH 7.0, 7.4, or 7.9 and cultured with aeration with 0.12% CO₂ for 3 h, [CO₂] increased to 8.3–15 μM, which was in the LC range. The CO₂ concentration of 0.12% was chosen after examining that at which LCIB-Clover dispersed completely at pH 7.0. LCIB-Clover dispersed in the chloroplast at pH 7.0 (median CV value 0.99; II in Figure 2B) or localized in the basal region of the chloroplast at pH 7.4 (median CV value 1.38; III in Figure 2B) and pH 7.9 (median CV value 1.56; IV in Figure 2B). In contrast, when we replaced the medium with that at pH 8.4 with aeration with 0.12% CO₂ for 3 h, [CO₂] increased slightly to 65 μM but remained in the range of VLC conditions, and LCIB-Clover localized around the pyrenoid (median CV value 2.05; V in Figure 2B) despite the increase in [HCO₃⁻] from 13 to 730 μM. A CO₂ concentration of ~7 μM, at which the LCIB-Clover localization switched from the basal region or dispersed in the chloroplast to the periphery of the pyrenoid, corresponded to the threshold between LC (7–70 μM CO₂) and VLC (<7 μM CO₂) estimated from the physiological function of LCIB (Figure 1D). Moreover, considering that LCIB-Clover dispersed in the chloroplast (median CV value 1.03; VI in Figure 2B) when cultured in medium at pH 8.4 and aerated with 5% CO₂, LCIB migration to around the pyrenoid was not due to the effect of the pH increase itself but the decrease in [CO₂] in the culture medium to VLC.

### Light, de novo protein synthesis, and photosynthetic electron flow are not required for LCIB relocation

In our previous studies, we discussed that LCIB relocation to the pyrenoid might require light, de novo protein synthesis, or photosynthetic electron flow because LCIB remained dispersed in the chloroplast in the dark or after the addition of cycloheximide (CHX) or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; Yamano et al., 2010, 2014). However, because we did not evaluate [CO₂] in the medium while observing LCIB localization, we could not distinguish whether the dispersion of LCIB was due to the treatments of inhibitors or caused by increased [CO₂] in the medium. To re-examine the effect of light, de novo protein synthesis, and the photosynthetic electron flow on LCIB relocation, we observed LCIB-Clover in light-to-dark conditions or after treatment with CHX or DCMU along with the measurement of total Ci (Figures 3 and 4).

First, we cultured MBC-3 cells in the light with aeration with 0.04% CO₂ for 24 h, where CCM was fully induced and
Figure 2 Subcellular localization of LCIB-Clover in different CO$_2$ and pH conditions. A, Schematic of the liquid culture conditions. MBC-3 cells were cultured in medium aerated with 0.04% CO$_2$ at pH 7.0 for 24 h (indicated as I to the left of the white box), which was replaced with fresh medium aerated with 0.12% CO$_2$ at pH 7.0 (II), 0.12% CO$_2$ at pH 7.4 (III), 0.12% CO$_2$ at pH 7.9 (IV), 0.12% CO$_2$ at pH 8.4 (V), or 5.0% CO$_2$ at pH 8.4 (VI), and cultured for 3 h. Red boxes indicate the time of observation. B, Representative LCIB-Clover fluorescence images of MBC-3 cells. Roman numerals correspond to the culture conditions indicated in A. Ci concentrations in the culture medium were measured using gas chromatography, and the calculated CO$_2$ and HCO$_3^-$ concentrations are shown below the images. CO$_2$ concentrations shown in red indicate VLC (<7 μM CO$_2$) conditions. DIC, differential interference contrast image. Scale bars: 2 μm. C, Quantification of localization patterns of LCIB-Clover. Roman numerals correspond to the culture conditions indicated in A. The CV value of the fluorescence intensity was calculated in each cell to quantify LCIB-Clover localization. The median values derived from the analysis of MBC-3 cells are represented with error bars depicting the interquartile range ($n = 20–24$). Dunn’s multiple comparisons test was used to assess the statistical significance of LCIB-Clover localization between the different conditions. **P-value < 0.01; ****P-value < 0.0001, Kruskal–Wallis test with Dunn’s multiple comparison.
[CO2] was in the range of VLC (2.9 μM; I in Figure 2B) and transferred them to the dark. After 2 or 5 h in the dark (I and II in Figure 3A), LCIB-Clover dispersed in the chloroplast (median CV value 0.98 and 1.00; I and II in Figure 3B and C), as shown previously (Yamano et al., 2010). [CO2] was increased from 2.9 μM to the range of LC conditions (12–18 μM) due to the cessation of photosynthesis and the activation of respiration in dark conditions. In contrast, when we reduced CO2 aeration from 0.04% to 0.001% in the dark (III and IV in Figure 3A), [CO2] decreased to the range of VLC conditions (2.6–3.0 μM), and LCIB-Clover relocated to around the pyrenoid even in cells treated with CHX (median CV value 1.76 and 1.89; III and IV in Figure 3B and C).

Next, we cultured MBC-3 cells in the light with aeration with 0.04% CO2 for 24 h, switched the aeration to 5% CO2 for 1 h, where LCIB-Clover dispersed in the chloroplast (median CV value 1.76 and 1.89; I in Figure 4B and C), and then we once again switched aeration from 5% to 0.04% (Figure 4A). At 3 h after transfer to 0.04% CO2, LCIB-Clover relocated to around the pyrenoid (median CV value 2.05; II in Figure 4B and C), as shown previously (Yamano et al., 2010), but adding DCMU inhibited the relocation with the increase in [CO2] to the range of LC conditions (24 μM), and LCIB-Clover dispersed in the chloroplast (median CV value 1.00; III in Figure 4B and C). However, when we switched aeration from 5% to 0.001%, [CO2] decreased to the range of VLC conditions (3.4 μM), and LCIB-Clover relocated to around the pyrenoid even in cells treated with DCMU (median CV value 1.99; IV in Figure 4B and C).

Considering these results, light, de novo protein synthesis, and photosynthetic electron flow were not required for LCIB relocation from dispersed in the chloroplast to around the pyrenoid, and the decrease in [CO2] in the culture medium was enough for the relocation.

Illumination of light in VLC conditions is required for LCIB migration to the pyrenoid

To examine the effect of light on LCIB migration, we cultured MBC-3 cells in the light in medium aerated with 5% CO2 for 24 h, where CCM was not induced, switched the aeration to 0.001% CO2 in light or dark conditions, and cultured them for 3 or 6 h (Figure 5A). While LCIB-Clover

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**Figure 3** Effect of light and de novo protein synthesis on the relocation of LCIB-Clover. **A**, Schematic of the liquid culture conditions. MBC-3 cells were cultured in medium aerated with 0.04% CO2 illuminated at 120 μmol photons m−2 s−1 (white box) for 24 h were shifted to the dark (black box) for 2 h (indicated as I to the left of the white box) or 5 h (II). After incubation in the dark for 2 h, the concentrations of CO2 aeration were switched to 0.001% for 3 h without adding CHX (III) or with the addition of CHX (IV). Red and yellow boxes indicate the time of observation and the addition of CHX, respectively. For all culture conditions, the pH of the medium was 7.0. **B**, Representative LCIB-Clover fluorescence images of MBC-3 cells. Roman numerals correspond to the culture conditions indicated in A. Ci concentrations in the culture medium were measured using gas chromatography, and the calculated CO2 and HCO3− concentrations are shown below the images. CO2 concentrations shown in red indicate VLC (<7 μM CO2) conditions. DIC, differential interference contrast image. Scale bars: 2 μm. **C**, Quantification of localization patterns of LCIB-Clover. Roman numerals correspond to the culture conditions indicated in A. The CV value of the fluorescence intensity was calculated in each cell to quantify LCIB-Clover localization. The median values derived from the analysis of MBC-3 cells are represented with error bars depicting the interquartile range (n = 5–7). Dunn’s multiple comparisons test was used to assess the statistical significance of LCIB-Clover localization between the different conditions. P-value < 0.05, Kruskal–Wallis test with Dunn’s multiple comparison.
migrated to around the pyrenoid so that it was no longer dispersed in the chloroplast within 6 h in light conditions (median CV value from 0.84 to 1.96; I–III in Figure 5, B and C), LCIB-Clover remained dispersed in the dark (median CV value 0.71 for 3 h and 0.65 for 6 h; IV and V in Figure 5, B and C) despite the decrease in [CO₂] to other range of VLC conditions (2.1–2.5 μM; IV and V in Figure 5B). These results suggested that light as well as VLC conditions are required for LCIB migration, and other factors induced during CCM induction could be involved in the migration.

Accumulation of LCIB and LCIC in VLC conditions is required for LCIB migration to the pyrenoid

Because the accumulation of LCIC increases with CCM induction and LCIC interacts with LCIB (Miura et al., 2004; Yamano et al., 2010), we assumed that LCIC is a strong candidate as a factor required for LCIB migration. Thus, to examine the effect of LCIC on LCIB migration, we introduced LCIC and/or LCIB-Clover driven by a constitutive promoter into strain C16 (Figure 6A), in which the CCM was not induced due to a mutation in CCM1, a master regulator of the CCM (Fukuzawa et al., 2001). In strain C16-B, where only LCIB-Clover was expressed in strain C16, LCIB-Clover dispersed in the chloroplast even in VLC conditions (I in Figure 6B). On the other hand, in strain C16-BC, where LCIB-Clover and LCIC were overexpressed simultaneously, several speckled structures were observed in the chloroplast in HC conditions (median CV value 2.1–2.5 μM; IV and V in Figure 5B). These results suggested that light as well as VLC conditions are required for LCIB migration, and other factors induced during CCM induction could be involved in the migration.

Figure 4  Effect of photosynthetic electron flow on the relocation of LCIB-Clover. A, Schematic of the liquid culture conditions. MBC-3 cells were cultured in medium aerated with 0.04% CO₂ for 24 h, which was replaced with the fresh medium aerated with 5% CO₂ for 1 h (indicated as I to the left of the white box). After aerating with 5% CO₂ for 1 h, MBC-3 cells were transferred to fresh medium aerated with 0.04% for 3 h without the addition of DCMU (II), with the addition of DCMU (III), or medium aerated with 0.001% CO₂ with DCMU (IV). Red and green boxes indicate the time of observation and addition of DCMU, respectively. For all culture conditions, the pH of the medium was 7.0. B, Representative LCIB-Clover fluorescence images of MBC-3 cells. Roman numerals correspond to the culture conditions indicated in A. Ci concentrations in the culture medium were measured using gas chromatography, and the calculated CO₂ and HCO₃⁻ concentrations are shown below the images. CO₂ concentrations shown in red indicate VLC (<7 μM CO₂) conditions. DIC, differential interference contrast image. Scale bars: 2 μm. C, Quantification of localization patterns of LCIB-Clover. Roman numerals correspond to the culture conditions indicated in A. The CV value of the fluorescence intensity was calculated in each cell to quantify LCIB-Clover localization. The median values derived from the analysis of MBC-3 cells are represented with error bars depicting the interquartile range (n = 5–7). Dunn’s multiple comparisons test was used to assess the statistical significance of LCIB-Clover localization between the different conditions. *P-value < 0.05; **P-value < 0.005, Kruskal–Wallis test with Dunn’s multiple comparison.
Although the speckled structures migrated toward the pyrenoid in medium aerated with 0.001% CO₂ even in the dark (median CV value 1.61 for 3 h and 1.60 for 6 h; VI and VII in Figure 6, B and C), the number of speckles with no fluorescence at their cores were decreased significantly from 4–7 to 1–2 (Supplemental Figure S2, A and B and Supplemental Movie S3). These results suggest that the accumulation of LCIC in VLC conditions is sufficient for LCIB migration, but other factors could be required for the LCIB/LCIC to localize around the pyrenoid uniformly.

**Discussion**

In this study, we examined the effects of pH, light availability, photosynthetic electron flow, and protein synthesis on localization changes of LCIB in the algal chloroplast. LCIB migration to around the pyrenoid required the accumulation of LCIC in the *Chlamydomonas* chloroplast, and once LCIB localized around the pyrenoid, reversible changes in LCIB localization were tightly regulated by the external [CO₂] without any requirement for light, photosynthetic electron flow, or protein accumulation other than LCIC. We also found that LCIB could be responsible for maintaining photosynthetic Ci-affinity even in HC conditions. These results will help to strengthen the current CCM model further.

**Strengthening the current CCM model**

The current model of CCM suggests that LCIB is involved in CO₂ recapturing around the pyrenoid in VLC conditions as well as CO₂ uptake in LC conditions (Wang and Spalding, Figure 5).
Figure 6 The requirement of LCIC for the LCIB-Clover migration and relocation. A, Schematic of the liquid culture conditions. C16-B cells were cultured in medium aerated with 5% CO$_2$ illuminated at 120 μmol photons m$^{-2}$ s$^{-1}$ (white box) for 24 h, which was replaced with the fresh medium aerated with 0.001% CO$_2$ for 6 h (indicated as I to the left of the white box). C16-BC cells were cultured in medium aerated with 5% CO$_2$ for 24 h (II), which was replaced with the fresh medium aerated with 0.001% CO$_2$ for 3 h (III) or 6 h (IV) in the light or for 3 h (VI) or 6 h (VII) in the dark (black box). After aeration with 0.001% CO$_2$ for 6 h, the concentration of aerated CO$_2$ was switched to 5% for 2 h (V). Red boxes indicate the time of observation. For all culture conditions, the pH of the medium is 7.0. B, Representative LCIB-Clover fluorescence images of C16-B and C16-BC cells. Roman numerals correspond to the culture conditions indicated in A. Ci concentrations in the culture medium were measured using gas chromatography, and calculated CO$_2$ and HCO$_3^-$ concentrations are shown below the images. CO$_2$ concentrations shown in red indicate VLC ($<7$ μM CO$_2$) conditions. DIC, differential interference contrast image. Scale bars: 2 μm. C, Quantification of localization patterns of LCIB-Clover. Roman numerals correspond to the culture conditions indicated in A. The CV value of the fluorescence intensity was calculated in each cell to quantify LCIB-Clover localization. The median values derived from the analysis of C16-BC cells are represented with error bars depicting the interquartile range (n = 11–16). Dunn’s multiple comparisons test was used to assess the statistical significance of LCIB-Clover localization between the different conditions. * P-value < 0.05; ** P-value < 0.01; *** P-value < 0.001; **** P-value < 0.0001, Kruskal–Wallis test with Dunn’s multiple comparison.
LCIA-mediated HCO\textsuperscript{–} mechanisms to switch the Ci-uptake system between HLA3/C24 activity and Ci transport activity (Bozzo and Colman, 2000). It was also reported that light is not essential for inducing CA patterns in the chloroplast found in this study. Moreover, it is close to that (~7 \mu M) required to switch LCIB localization patterns in the chloroplast found in this study. Furthermore, it was also reported that light is not essential for inducing Ca activity and Ci transport activity (Bozzo and Colman, 2000).

The different localization patterns of LCIB depending on extracellular CO\textsubscript{2} levels should reflect multistep CO\textsubscript{2}-regulated acclimation to overcome the difficulty of acquiring Ci in aquatic environments. Such dynamic localization changes are difficult to capture in conventional experiments that deal with cells grown in fixed CO\textsubscript{2} concentrations, indicating the need to estimate the acclimation state (HC, LC, or VLC) of the cells by constantly measuring and/or calculating the Ci and CO\textsubscript{2} concentration of the culture medium. Considering that CCM studies need to be done from the ecological and physiological point of view, our finding will strengthen the current CCM model and indicates a more flexible survival strategy for algal cells than previously thought, especially under fluctuating CO\textsubscript{2} environment.

**CO\textsubscript{2} acclimation systems in a fluctuating CO\textsubscript{2} environment**

A previous study also reported that [CO\textsubscript{2}] in the medium, but not [HCO\textsubscript{3}\textsuperscript{–}], are a critical factor for inducing the activity of HCO\textsubscript{3}\textsuperscript{–} transport in *Chlamydomonas* and that HCO\textsubscript{3}\textsuperscript{–} transport activity was fully induced at ~10 \mu M of CO\textsubscript{2} (Bozzo and Colman, 2000). This CO\textsubscript{2} concentration was close to that (~7 \mu M) required to switch LCIB localization patterns in the chloroplast found in this study. Moreover, it was also reported that light is not essential for inducing CA activity and Ci transport activity (Bozzo and Colman, 2000). Considering these results, there are unknown molecular mechanisms to switch the Ci-uptake system between HLA3/LCIA-mediated HCO\textsubscript{3}\textsuperscript{–} transport along with LCIB-driven CO\textsubscript{2} recapturing around the pyrenoid in VLC conditions and LCIB-driven CO\textsubscript{2} uptake in the entire chloroplast in LC and LC conditions to acclimate to fluctuating CO\textsubscript{2} conditions. Under VLC conditions with a high HCO\textsubscript{3}\textsuperscript{–}/CO\textsubscript{2} ratio in the chloroplast stroma, it would be important to deactivate the putative Ca activity of the LCIB/LCIC complex to prevent CO\textsubscript{2} leakage due to HCO\textsubscript{3}\textsuperscript{–} dehydration activity. Recombinant LCIB, LCIC, LCIB-LCIC complex, and native LCIB-LCIC complex do not have Ca activity (Jin et al., 2016). Although the stoichiometry of the LCIB/LCIC complex is not yet clear, it cannot yet be ruled out the possibility that the putative Ca activity is regulated by the complex formation and dissociation of LCIB and LCIC in response to CO\textsubscript{2} concentration.

It is not clear to what extent the local CO\textsubscript{2} concentration fluctuates over short periods in aquatic environments. A previous study using the large diatom *Odontella sinensis* showed that rapid and substantial changes in pH and CO\textsubscript{2} concentrations occurred at the cell surface due to external Ca activity and played an important role in photosynthetic Ci-uptake (Cherchi et al., 2018). The CO\textsubscript{2} concentration at the cell surface may also constantly fluctuate due to respiration and photosynthetic activity in surrounding bacteria and microalgae. Rapid localization changes of LCIB in response to external [CO\textsubscript{2}] without transcription and translation could play an essential role in the flexible acclimation to fluctuating CO\textsubscript{2} conditions in the aquatic environment. Moreover, because changes in LCIB localization in the chloroplast are easy to observe and are strictly regulated by external [CO\textsubscript{2}], LCIB-Clover used in this study can be used as a molecular marker to estimate the LC and VLC states of the culture medium.

**Mechanism of LCIB migration and relocation**

When LCIB-Clover was expressed alone in the *ccm1*-deficient mutant C16, LCIB-Clover was uniformly dispersed throughout the entire stroma. In contrast, when the accumulation of LCIC and LCIB-Clover were overexpressed simultaneously, several speckled structures were observed in the chloroplast. These structures were localized to the pyrenoid periphery in VLC conditions, suggesting that VLC conditions and the interaction between LCIB and LCIC are necessary for the complex to migrate to the pyrenoid. This is consistent with the results that the accumulation of LCIC was also missing in the *lcib* mutant (Figure 1B). So far, there is no solid explanation for why LCIB accumulation could not be detected (or remarkably reduced) in the *lcib* background; there could be negative feedback on LCIB synthesis, or LCIB could be destabilized/degraded due to the loss of LCIB. We previously showed that mRNA expression of LCIC was normally induced, but the protein accumulation was markedly reduced in the LCIB RNAi lines (Yamano et al., 2010), so the missing of LCIC could occur at the posttranscriptional level. So far, it has been discussed that CCM1 is a master regulator of the CCM in green algae and that CCM1 itself could be a sensor to sense the change in environmental Ci concentrations. However, even in the *ccm1* background, LCIB can respond to the decrease in [CO\textsubscript{2}] by interacting with LCIC and migrate to the pyrenoid periphery, suggesting another CO\textsubscript{2}-sensing mechanism besides CCM1.

The speckle structures of LCIB-Clover located around the pyrenoid had no fluorescence visible at their core and did
not localize uniformly around the pyrenoid. These results indicate that other factor(s), such as protein expression and/or posttranslational modification, are necessary to form the ring-like structure around the pyrenoid. Although we previously reported that immunoprecipitated LCIB could be phosphorylated (Yamano et al., 2010), mass spectrometry profile to determine the posttranslational modification under HC, LC, and VLC conditions have not been examined. Considering that LCIB and LCIC are soluble proteins, they could require some form of anchoring to relocalize from the stroma to the pyrenoid periphery. This could be mediated by the pyrenoid tubules, thylakoid membranes penetrating pyrenoid, because the pyrenoid tubules are radically arranged and limited in number, which is consistent with the discontinuous labeling of LCIB. Previous results also showed that LCIB aggregates near the gap in the starch sheath where the thylakoid membranes penetrate the pyrenoid (Yamano et al., 2010).

Recently, it has been examined that three BST1–3 localize to the thylakoid membrane around the pyrenoid and may be involved in HCO$_3^-$ transport to the lumen of the thylakoid membrane (Mukherjee et al., 2019). Considering that both LCIB and LCIC interact with BST3 (Cre16.g663450), LCIC also interacts with BST1 (Cre16.g662600; Mackinder et al., 2017), and genes encoding BST1–3 are regulated by CCM1 (Yamano et al., 2008), one of BST1–3 may be necessary for LCIB/LCIC to be correctly positioned around the pyrenoid as a ring-like structure. It would be of interest to examine the localization of LCIB and LCIC in mutants missing BST1–3. Another possibility is that the pyrenoid structure, including pyrenoid tubules and starch sheaths, did not develop normally in the background of ccm1 mutants, resulting in abnormal LCIB localization. Because the previous study showed the reduced size of pyrenoid in the mutant strain ccm1 (Fukuzawa et al., 2001), observation of TEM images of the ccm1 and C16-BC lines would explain the placement of the speckles around the pyrenoid.

**Toward reconstitution of algal CCMs**

There has been research aimed at enhancing the CO$_2$-fixing capacity of Rubisco by introducing algal CCMs into the chloroplasts of terrestrial plants. Because the interaction between Rubisco and the Rubisco linker EPYC1 is sufficient to form phase-separated structures and the motif sequences of proteins that bind to Rubisco have been identified (He et al., 2020; Meyer et al., 2020), it should be possible to reconstitute functional pyrenoids by heterologous expression of algal proteins (Atkinson et al., 2020). Furthermore, HCO$_3^-$ and CO$_2$ transporters/channels associated with Ci-uptake have also been identified, and it would also be possible to express and localize them to the correct positions in land plant cells. However, to fully operate the algal CCM in the chloroplasts of terrestrial plants, the starch sheath formed around the pyrenoid and the CAs responsible for CO$_2$-uptake and CO$_2$-recapturing must also be stably expressed. Notably, the starch sheath morphology itself is essential for the correct localization of LCIB around the pyrenoid (Toyokawa et al., 2020) as well as for the regulation of the pyrenoid number in the chloroplast (Itakura et al., 2019). In terrestrial plants, CO$_2$ is taken up into the leaves through the stomata and it diffuses into the chloroplast stroma; therefore, it is essential to properly position CAs in the chloroplast to form HCO$_3^-$ pools with little CO$_2$ leakage. The findings of this study will contribute to understating of the regulation of heterologous expression and localization of algal CAs in the chloroplasts of terrestrial plants.

**Materials and methods**

**Strains and culture conditions**

The *C. reinhardtii* WT strain C9 was provided from IAM Culture Collection at the University of Tokyo and is now available from Microbial Culture Collection at the National Institute for Environmental Studies, Japan, as strain NIES-2235 (alternatively named as CC-5098 in the *Chlamydomonas* Resource Center). *Chlamydomonas* WT strain CC-1690 was obtained from the *Chlamydomonas* Resource Center. The *lcib*-insertion mutant LM1.RY0402.173287, designated as B1 (Toyokawa et al., 2020), was obtained from the mutant resources of the CLiP (Li et al., 2016).

For the short maintenance of strains, cells were cultured on an agar plate with Tris-acetate-phosphate (TAP) medium and kept at 20°C in dim light (~1 μmol photons m$^{-2}$ s$^{-1}$). For physiological and biochemical experiments, cells were precultured in 5 mL of liquid TAP medium by vigorous shaking with ~50 μmol photons m$^{-2}$ s$^{-1}$ and diluted with ~50 mL of MOPS-P medium, which contained phosphate (620 μM of K$_2$HPO$_4$ and 412 μM of KH$_2$PO$_4$), Hutner’s trace elements, and 20 mM MOPS (pH 7.0). Then, cells were cultured by aerating with air containing 5% (v/v) CO$_2$ (HC) until the mid-log phase. When changing the concentrations of CO$_2$, HC-acclimated cells were centrifuged at 600 g for 5 min, resuspended in 50 mL of fresh MOPS-P medium, and cultured with aeration with ordinary air (0.04% CO$_2$) or 0.001% CO$_2$ for the indicated periods. To create 0.001% CO$_2$ gas, ordinary air was passed through 100 mL of 2 N NaOH solution two times while monitoring the CO$_2$ concentrations using an infrared CO$_2$ analyzer (model LI-7000; LI-COR). Unless indicated otherwise, cells were cultured at 25°C with continuous illumination using white fluorescent light at ~120 μmol photons m$^{-2}$ s$^{-1}$.

**Genetic crosses**

*Chlamydomonas* parental strains with opposite mating types were cultured for 4 d on an agar plate with 1/5 N TAP medium in which the nitrogen (N) source was diluted to one-fifth. Cultured cells on the agar plates were resuspended in 10 mL of N-free gamete-induction medium (Dutcher, 1995) two times and grown for 3 h with illumination at 80 μmol photons m$^{-2}$ s$^{-1}$. A mixture containing 5 mL of each parental strain was left undisturbed at 80 μmol photons m$^{-2}$ s$^{-1}$ for 1 h. The mixture of 500 μL was plated on a 3% TAP agar plate and left overnight with illumination at 80 μmol photons m$^{-2}$ s$^{-1}$, followed by 7 d incubation in the dark.
7 d, the vegetative cells were removed using a sterile razor blade and the plate was incubated in the light until zygote colonies appeared. Individual zygote colonies were isolated with the use of an inverted microscope (BX41; Olympus, Tokyo, Japan) equipped with a micromanipulator (Narisighe, Tokyo, Japan) and a glass needle (Singer Instruments, Somerset, UK).

Immunoblotting analysis

Cell suspensions were centrifuged at 800 g at 4°C for 5 min, resuspended in chilled phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂O₄, pH 7.4) supplemented with a Complete protease inhibitor cocktail (Roche, Basel, Switzerland), and then disrupted for 1 min on ice using a handy sonicator (UR-20P; TOMY). Unbroken cells and cell debris were removed by centrifugation at 17,000 g at 4°C for 20 min, and the resulting supernatant was used as a protein crude extract. After measuring and normalizing the concentrations of the extract by Bradford assay, the extract was resuspended in an equal volume of 2 × SDS gel loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue (w/v), 20% glycerol (v/v)) and incubated at 65°C for 10 min. After heating, 10 μg of the extract was separated by 10% (w/v) acrylamide SDS–PAGE. After electrophoresis, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, California, USA). Membranes were blocked with 5% (w/v) non-fat skim milk (Wako) in PBS for 1 h at room temperature. Blocked membranes were washed with PBS containing 0.1% (v/v) Tween-20 (PBS-T) and incubated with the following antibodies in PBS-T for 1 h at 20°C: rabbit anti-LCIB (1:5,000 dilution), rabbit anti-LCIC (1:10,000), rabbit anti-GFP (1:10,000), rabbit anti-histone H3 (1:20,000; Abcam, Cambridge, UK). A horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Life Technologies, Carlsbad, California, USA) was used as a secondary antibody at a dilution of 1:10,000. Immunologically positive signals were visualized using an enhanced chemiluminescence system in accordance with the manufacturer’s instructions (GE Healthcare).

Spot test analysis

Cells cultured in MOPS-P medium aerated with 5% CO₂ at the mid-log phase were diluted with fresh MOPS-P medium to OD₇₃₀ of 0.30, 0.15, and 0.07. Then, 3 μL of each cell suspension was spotted onto an agar plate with MOPS-P medium. The plates were kept in a growth chamber supplied with HC (5% CO₂), LC (0.04% CO₂), or VLC (0.01% CO₂) at ~120 μmol photons m⁻² s⁻¹ for 3–8 d. To create 0.01% CO₂ gas, ordinary air was passed through 100 mL of 2N NaOH solution once and by monitoring the CO₂ concentrations in the total CO₂ concentrations were calculated using the Henderson–Hasselbalch equation:

\[
pH = \text{pKa} + \log_{10}\left[\frac{\text{HCO}_3^-}{\text{CO}_2}\right]
\]

where pKa is the acid dissociation constant of 6.35 and using an HCO₃⁻/CO₂ ratio of 4.46 at pH 7.0, 11.22 at pH 7.4, 35.48 at pH 7.9, and 112.2 at pH 8.4.

Observation and quantification of subcellular localization of LCIB-Clover

For observation of LCIB-Clover, 2.5 μL of cultured cells were placed between a coverslip and a thin agarose pad. Then, 16-bit digital fluorescence images were acquired with an oil immersion objective lens (HC PL APO 63 × /1.40; Leica) using an inverted laser-scanning confocal fluorescence microscope TCS SP8 (Leica) equipped with a sensitive hybrid detector (for detecting LCIB-Clover) and photomultiplier tube detector (for detecting chlorophyll autofluorescence). LCIB-Clover and chlorophyll were excited at 488 nm, and the emissions derived from Clover and chlorophyll were detected at wavelength ranges of 500–520 nm and 648–700 nm, respectively. Image scanning was performed with a pinhole size of 0.5 or 0.6 Airy units, with the z-stack distance of the scan at 150 nm, at a pixel size of 35–45 nm, and with a line scan speed of 200 or 400 Hz. Huygens Essential software (Scientific Volume Imaging BV) was used for the deconvolution of images. The deconvolution of confocal datasets was performed using the point-spread function theoretically calculated from the microscopic parameters attached to the data and a classic maximum likelihood estimation algorithm (settings: maximum iterations: 100; signal-to-noise: 20; quality criterion: 0.05).
To quantify the localization pattern of LCIB-Clover, the CV values of LCIB-Clover fluorescence signals in the chloroplast were calculated. The CV value was defined as the standard deviation (σ) ratio to the mean (μ) of LCIB-Clover fluorescence signals. Using ImageJ (Fiji), a cup-shaped chloroplast area except for the pyrenoid region was defined as the region of interest (ROI) for an individual cell using the chlorophyll channel of confocal fluorescence microscopy images. The σ and μ of LCIB-Clover fluorescence intensity were quantified using the above-defined ROI, and μ/σ was calculated.

Accession numbers

The accession numbers in the Phytozome database for Chlamydomonas genes LCIB and LCIIC are Cre10.g452800 and Cre06.g307500, respectively.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Z-stack images of confocal sections of LCIB-Clover, merged with the chlorophyll, and DIC in MBC-3 cells grown in VLC conditions.

Supplemental Figure S2. Different images of LCIB-Clover in C16-BC cells and quantification of the speckled structures with no fluorescence at their cores.

Supplemental Table S1. Photosynthetic parameters of WT and transformants cells.

Supplemental Movie S1. Z-stack of confocal sections of LCIB-Clover merged with the chlorophyll in MBC-3 cells grown in VLC conditions.

Supplemental Movie S2. Z-stack of confocal sections of LCIB-Clover merged with the chlorophyll in C16-BC cells grown in VLC conditions with light for 6 h.

Supplemental Movie S3. Z-stack of confocal sections of LCIB-Clover merged with the chlorophyll in C16-BC cells grown in VLC conditions in the dark for 6 h.

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