Expression of a Low CO₂-Inducible Protein, LCI1, Increases Inorganic Carbon Uptake in the Green Alga

*Chlamydomonas reinhardtii*

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Aquatic photosynthetic organisms can modulate their photosynthesis to acclimate to CO₂-limiting stress by inducing a carbon-concentrating mechanism (CCM) that includes carbonic anhydrases and inorganic carbon (Ci) transporters. However, to date, Ci-specific transporters have not been well characterized in eukaryotic algae. Previously, a *Chlamydomonas reinhardtii* mutant (*lcr1*) was identified that was missing a Myb transcription factor. This mutant had reduced light-dependent CO₂ gas exchange (LCE) activity when grown under CO₂-limiting conditions and did not induce the CAH1 gene encoding a periplasmic carbonic anhydrase, as well as two as yet uncharacterized genes, LCI1 and LCI6. In this study, LCI1 was placed under the control of the nitrate reductase promoter, allowing for the induction of LCI1 expression by nitrate in the absence of other CCM components. When the expression of LCI1 was induced in the *lcr1* mutant under CO₂-enriched conditions, the cells showed an increase in LCE activity, internal Ci accumulation, and photosynthetic affinity for Ci. From experiments using indirect immunofluorescence, LCI1-green fluorescent protein fusions, and cell fractionation procedures, it appears that LCI1 is mainly localized to the plasma membrane. These results provide strong evidence that LCI1 may contribute to the CCM as a component of the Ci transport machinery in the plasma membrane.

INTRODUCTION

Aquatic photosynthetic organisms acclimate to different environmental changes, such as light intensity, light quality, temperature, and nutrient availability. The supply of inorganic carbon (Ci; CO₂ and HCO₃⁻) is one of the environmental factors that can limit photosynthetic performance because the environmental Ci concentration is usually lower than that required for the optimal carboxylase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. To overcome the difficulty in acquiring Ci at low CO₂ concentrations, cyanobacteria and green algae, including *Chlamydomonas reinhardtii*, induce a transport system called the carbon-concentrating mechanism (CCM) to increase Ci uptake (Badger et al., 1980; Giordano et al., 2005; Spalding, 2008; Yamano and Fukuzawa, 2009). In cyanobacteria, five types of cytoplasmic Ci transporters have been identified (Price et al., 2008), including three bicarbonate transporters and two NAD(P)H dehydrogenase-dependent CO₂ uptake systems. In the model eukaryotic algae C. *reinhardtii*, only one possible HCO₃⁻ transporter, HLA3, has been characterized in some detail (Duann et al., 2009b), although several candidate genes responsible for Ci uptake have been identified in transcriptomic analyses (Miura et al., 2004; Moroney and Ynalvez, 2007; Spalding, 2008; Yamano et al., 2008; Yamano and Fukuzawa, 2009).

Several key mutants for understanding the CCM in *C. reinhardtii* are known, including the high CO₂–requiring mutants cia5 (Moroney et al., 1989), C16 (Fukuzawa et al., 1998), *pmp1-1* (Spalding et al., 1983), *ppg1* (Suzuki et al., 1990), HCR89 (Nakamura et al., 2005), and *lcr1* (Yoshioka et al., 2004). Notably, the regulatory gene CCM1/CIA5 encoding a zinc binding protein has been shown to be essential for the expression of >50 low CO₂ (LC)–inducible genes (Fukuzawa et al., 2001; Xiang et al., 2001; Miura et al., 2004; Kohinata et al., 2008). Included in this group of genes regulated by CCM1/CIA5 are many of the genes encoding Ci transporter candidates identified by transcriptomic analyses. These genes include *LCI1*, *LCA1*, *LCIB*, and *HLA3* (Yamano and Fukuzawa, 2009).

The LC-inducible protein LCIA, also called NAR1.2, shows significant sequence similarity to a chloroplast nitrite transporter, NAR1.1, and is also induced under LC conditions and may function as a bicarbonate transporter whose subcellular location is not identified (Miura et al., 2004). Although it was reported that expression of LCIA in oocytes from *Xenopus laevis* facilitated the uptake of bicarbonate as well as nitrite (Mariscal et al., 2006), the function of LCIA in vivo remains to be elucidated. Recently, it was shown that the *mp1-1* mutation was complemented by the LC-inducible gene *LCIB* (Wang and Spalding, 2006). Although the protein deficiency in *mp1-1* was expected to be in a Ci transporter, LCIB does not have any transmembrane domains and
colocalized with a homologous protein, LCIC, in the vicinity of the pyrenoid (Yamano et al., 2010). Moreover, LCIB is genetically shown to function downstream of CAH3 in the CCM. Thus, this protein is assumed to act after the accumulated stromal HCO$_3$-$^-$ is dehydrated to CO$_2$ rather than as a Ci transporter (Duanmu et al., 2009a). In addition, HLA3, which is an LC-inducible ABC-type transporter, was shown to play a role in Ci uptake in C. reinhardtii (Duanmu et al., 2009b). Although other CO$_2$-responsive membrane proteins, including LIP36 (Razamov et al., 1993), CemA (previously designated as Ycf10; Rolland et al., 1997), and Rh1 (Soupene et al., 2004), might also be involved in Ci transport, further biochemical evidence is needed to elucidate their functions in the CCM.

In a previous study, we isolated the regulatory mutant lcr1, which lacks a Myb-DNA binding transcription factor, LCR1. The loss of LCR1 greatly reduces the expression of three LC-inducible genes, namely, CAH1, LCI1, and LCI6 (Yoshioka et al., 2004). Whereas CAH1 is known to encode a periplasmic carbonic anhydrase, LCI1 and LCI6 have not been characterized biochemically. In addition, the lcr1 mutant showed a 30% decrease in growth rate and a higher apparent K$_1$/2(Ci) value compared to the plasma membrane. Together, these observations support the idea that LCI1 is, in part, responsible for Ci uptake in the plasma membrane. Furthermore, the detected 17-kD band was assumed to correspond to the mature LCI1 protein without the first hydrophobic domain, although it is not clear which amino acid residue corresponds to the mature LCI1 protein in vivo. In the HC-lacking the LC-inducible periplasmic carbonic anhydrase (Van and Spalding, 1999) showed a similar LCE activity under LC conditions (Figure 1, Table 1). On the other hand, the lcr1 mutant showed a decreased level of LCE activity, but the activity of the LCR1-complemented strain (lcr1+G) (Yoshioka et al., 2004) was equivalent to that of Q304P3 (Figure 1, Table 1). Although the lcr1 mutant cannot induce 13 genes in LC conditions, the induction of only three genes, namely, LCI1, LCI6, and CAH1, were recovered in the LCR1-complemented strain (Yoshioka et al., 2004). Together, these results suggest that LCR1-regulated genes other than CAH1 are necessary for high LCE activity under CO$_2$-limiting conditions.

**RESULTS**

**LCE Activity of Low and High CO$_2$-Grown Cells**

To evaluate the effects of mutations in LCR1 and CAH1 on photosynthesis, we compared the LCE activities of cells of the photosynthetically wild-type transgenic strain Q304P3 (Yoshioka et al., 2004) and mutants lcr1 (Yoshioka et al., 2004) and cah1 using an open gas exchange system with an air supply containing 50 ppm CO$_2$ (Yamano et al., 2008) (Figure 1, Table 1). Previously, it was shown that LCE activity mirrors total Ci consumption, which includes Ci uptake and CO$_2$ fixation (Spalding and Ogren, 1985) and provides a reasonably good indirect estimate of the CCM in cells (Yamano et al., 2008). The photosynthetically wild-type strain Q304P3 showed a LCE activity of 72.6 μmol CO$_2$ mg Chl$^{-1}$ h$^{-1}$ under LC conditions and 13.1 μmol CO$_2$ mg Chl$^{-1}$ h$^{-1}$ in high-CO$_2$ (HC) conditions. The cah1 mutant lacking the LC-inducible periplasmic carbonic anhydrase (Van and Spalding, 1999) showed a similar LC activity under LC conditions (Figure 1, Table 1). On the other hand, the lcr1 mutant showed a decreased level of LCE activity, but the activity of the LCR1-complemented strain (lcr1+G) (Yoshioka et al., 2004) was equivalent to that of Q304P3 (Figure 1, Table 1). Although the lcr1 mutant cannot induce 13 genes in LC conditions, the induction of only three genes, namely, LCI1, LCI6, and CAH1, were recovered in the LCR1-complemented strain (Yoshioka et al., 2004). Together, these results suggest that LCR1-regulated genes other than CAH1 are necessary for high LCE activity under CO$_2$-limiting conditions.

**Induction of LCI1 by Changing Nitrogen Source**

To determine whether the lcr1 mutant can change the synthesis of the LCI1 protein, we performed protein immunoblot analysis using an affinity-purified antibody against synthetic polypeptides corresponding to parts of LCI1 (Figure 2). A 17-kD protein band was detected by the antibody against LCI1 more abundantly in LC-grown Q304P3 cells compared with HC-grown Q304P3 cells. Although LCI1 encodes a putative 21-kD protein with four hydrophobic domains, the first domain in the N-terminal region was predicted to be a signal peptide by the prediction program SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004). Thus, the detected 17-kD band was assumed to correspond to the mature LCI1 protein without the first hydrophobic domain, although it is not clear which amino acid residue is the N terminus of the mature LCI1 protein in vivo. In the HC- and LC-grown lcr1 mutant, a faint signal was observed near 17 kD, but its level was equivalent to that in HC-grown Q304P3 and was not affected by changes in the level of CO$_2$ supplied (Figure 2). Because the faint bands at 17 kD were detected by both oligopeptide affinity-purified antibody and antiserum (Figures 2 and 3C, respectively), it is possible that a trace amount of LCI1...
accumulates in HC-grown Q304P3 and even in both HC- and LC-grown lcr1 mutant cells, although these results do not rule out a possibility that the antibody used in this study may recognize a 17-kD protein other than LCI1.

To analyze whether LCI1 is involved in LCE activity, we generated a chimeric gene in which tandemly duplicated NIA1 regulatory elements (Loppes and Radoux, 2002) and the minimal promoter of TUB2 encoding β-tubulin (Davies and Grossman, 1994) were fused to the LCI1 coding region followed by the 3′-untranslated region (UTR) of RBCS2 (Figure 3A). This construct allowed us to induce the expression of LCI1 by switching the nitrogen source from NH₄⁺ to NO₃⁻ irrespective of the CO₂ conditions. Although Q304P3 is photosynthetically a wild-type strain, this strain cannot use NO₃⁻ as a nitrogen source because of a deficiency in the NIA1 gene encoding nitrate reductase. By contrast, the lcr1 mutant can use NO₃⁻ as a nitrogen source because this mutant was generated by introducing the NIA1 gene into Q304P3 as a tag (Yoshioka et al., 2004), and it does not induce endogenous LCI1 protein in the presence of NO₃⁻ (Figure 2). Thus, this lcr1 mutant is appropriate to use as the host strain for this gain-of-function analysis of LCI1. After transformation of the chimeric plasmid into the lcr1 mutant, Zeocin-resistant colonies were selected and used for RNA gel blot analyses with a probe specific to the LCI1 coding region (Figure 3B). The level of endogenous LCI1 mRNAs of 1.3 kb (Burow et al., 1996; Yoshioka et al., 2004) increased in LC-grown Q304P3. In the lcr1 mutant, there was no significant accumulation of the LCI1 mRNA either under LC conditions or in the presence of NO₃⁻, although this mutant previously was shown to accumulate a trace amount of LCI1 mRNA under LC conditions (Yoshioka et al., 2004). The LCI1 mRNA in the LC-grown lcr1 mutant could not be detected at this exposure level because the exposure period of the autoradiograph was too short to detect such a low amount of mRNA. On the other hand, in the LCI1 transformants designated as C2 and E4, the level of the 0.8-kb mRNA, which corresponds to the predicted LCI1-encoding mRNA from the transcription initiation site of TUB2 to the predicted poly(A) signal (Davies and Grossman, 1994) in the 3′-UTR of RBCS2, increased in the presence of NO₃⁻ irrespective of changes in the level of CO₂ supplied to the cells (Figure 3B).

We also analyzed the accumulation of LCI1 protein in the LCI1 transformants by protein immunoblot analysis (Figure 3C). Both LCI1 transformants grown in high salt minimal medium containing NO₃⁻, referred to as HSM(NO3), accumulated LCI1 protein under both HC and LC conditions. Although the levels of LCI1 protein in HC- and LC-grown LCI1 transformant cells cultured in HSM(NO3) media were ~25 and 50% of that in LC-grown Q304P3, respectively, the level of LCI1 protein was 10- to 20-fold higher than in those cells grown with NH₄⁺ as the nitrogen source. Because the combination of LC conditions and NO₃⁻ did not affect the level of LCI1 protein in the lcr1 mutant (Figure 2), the accumulation of LCI1 protein seen in the transformants should be ascribed to the exogenously introduced LCI1 gene fused to NIA1 regulatory elements.

### LCE Activity of the lcr1 Transformants

To evaluate the apparent photosynthetic capacity of the lcr1 mutant and the LCI1 transformants, we measured the LCE activity of the cells grown in high salt minimal medium containing either NH₄⁺ or NO₃⁻, referred to as HSM(NH4) or HSM(NO3), respectively (Figure 4, Table 2). Under HC conditions, there were no meaningful differences among the LCE activities of the lcr1 mutant, C2, and E4 grown in HSM(NH4) (Table 2). When the cells were grown in HSM(NO3), it was notable that the LCE activity of the LCI1 transformants C2 and E4 increased 2.2- and 3.6-fold, respectively, in the presence of NO₃⁻, whereas the lcr1 mutant showed only a 1.3-fold increase in activity.

LCE activity was also measured for cells grown under LC conditions in the presence of NH₄⁺ or NO₃⁻. Comparisons of LC-grown lcr1 mutant cultured in HSM(NH4) and HSM(NO3) indicated that a change of nitrogen source had no effect on LCE activity in the lcr1 mutant (Table 2). On the other hand, transformants C2 and E4 cultured in HSM(NH4) had almost the same LCE activities as the lcr1 mutant but showed a slight increase in LCE activity (Figure 4, Table 2) when they were grown in HSM (NO3). Whereas artificial induction of LCI1 alone does not completely restore LCE activity to wild-type levels, these results reveal that the expression of LCI1 increases the LCE activity in both HC and LC conditions.

To analyze whether the expression of LCI1 preferentially increased CO₂ or HCO₃⁻ uptake, we measured the LCE activity

### LCE Activity of Q304P3, lcr1, cah1, and lcr1+G Cells at pH 7.0

| Strain | Q304P3 | lcr1 | cah1 | lcr1+G |
|--------|--------|------|------|--------|
| High CO₂ | 13.1 ± 1.7 | 11.6 ± 0.9 | 15.2 ± 1.2 | n.d. |
| Low CO₂ | 72.6 ± 2.2 | 36.9 ± 6.7 | 70.5 ± 1.7 | 69.7 ± 5.1 |

The data are shown as μmol CO₂ mg Chl⁻¹ h⁻¹, ±SD, obtained from three independent experiments. lcr1+G, the LCR1-complemented strain; n.d., not determined.

![Figure 2](image-url)
of the lcr1 mutant and C2 cells grown in HC conditions at two other external pH values: pH 6.2 and 7.8 (Table 3). At pH 6.2, HSM(NH4)-grown lcr1 mutant cells showed comparable LCE activity to that of HSM(NO3)-grown cells, indicating that changing the nitrogen species in the medium did not affect LCE activity in the parent strain. By contrast, HSM(NO3)-grown C2 cells showed a 2.1-fold increase in the LCE activity compared with that in HSM(NH4)-grown cells. At pH 7.8, a similar increase in the LCE activity of the C2 cells was observed when the culture medium was changed from HSM(NH4) to HSM(NO3). These results mean that the increase in the LCE activity in HSM(NO3)-grown C2 cells was observed in both acidic and alkaline pH conditions. Interestingly, although the LCE activities at pH 7.8 in HSM(NH4)-grown cells decreased by ~50% compared with those at pH 6.2 in both strains, the increase in the LCE activities of HSM(NO3)-grown C2 cells compared with that of HSM(NO3)-grown lcr1 mutant cells was almost constant (shown as [C2 (NO3)]-[lcr1(NO3)] in Table 3). Unfortunately, because the cells were easily broken by bubbling during the measurements under more alkaline conditions (pH 8.6 and 9.0), we could not evaluate the effect of the expression of LCI1 on LCE activity under more alkaline conditions.

**Dissolved Ci-Dependent Photosynthesis of the LCI1 Transformants**

We also analyzed the photosynthetic affinity for Ci in the LCI1 transformants by measuring the rate of Ci-dependent O2-evolving activity at two different pH values, namely, pH 7.0 and 7.8. At pH 7.0, the K1/2 value of the HC-grown lcr1 mutant was comparable to that in the HC-grown Q304P3 cells, the photosynthetically wild-type strain, whereas that of LC-grown lcr1 mutant was higher than that of LC-grown Q304P3 cells (Figure 5A; see Figure 4). The LCE activities are summarized in Table 2.

**Figure 4. LCE Profiles of the lcr1 Mutant and LCI1 Transformants C2 and E4.**

The LCE activities of cells grown under HC or LC conditions in HSM(NH4) or HSM(NO3) were measured at pH 7.0. Arrowheads indicate when actinic light was turned on (open arrowheads) and off (closed arrowheads), respectively. The dotted lines represent the light-dependent decrease in the CO2 levels of the lcr1 mutant, which was used as the host strain for the LCI1 transformants. The LCE activities are summarized in Table 2.
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Table 2. LCE Activity of the lcr1 Mutant and LCI1 Transformants at pH 7.0

| Strain | lcr1 | C2 | E4 |
|--------|------|----|----|
| N source | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ |
| High CO₂ | 11.6 ± 0.9 | 15.0 ± 2.7 | 11.3 ± 2.3 | 24.7 ± 2.9 | 7.4 ± 1.5 | 26.3 ± 4.2 |
| Low CO₂ | 36.9 ± 6.7 | 38.7 ± 7.1 | 35.7 ± 8.3 | 54.1 ± 7.1 | 33.1 ± 4.6 | 54.5 ± 8.5 |

The data are shown as μmol CO₂ mg Chl⁻¹ h⁻¹, ± SD, obtained from three independent experiments.

Supplemental Table 1online). The LC-grown lcr1 mutant showed a lower Ci affinity compared with that of Q304P3, as described previously (Yoshioka et al., 2004). In addition, the K₁/₂ values of both HC- and LC-grown lcr1 mutant cells were not affected by the nitrogen source in the culture media (Figure 5A; see Supplemental Table 1 online). By contrast, the affinities of C2 and E4 cells for Ci increased when the cells were grown in HSM(NO₃). The K₁/₂ values of C2 and E4 cells grown in HSM(NO₃) under HC conditions were decreased compared with those of the cells grown in HSM(NH₄), indicating that the Ci affinity of the LCI1 transformants increased when exogenous LCI1 was expressed. However, the K₁/₂ values of all the strains grown in LC conditions, except for Q304P3, were ~40 μM. This value was not affected by the nitrogen source, although the increase in LCE activity was observed in both LCI1 transformants (Figure 4).

At pH 7.8, the photosynthetic affinities for Ci decreased in all the examined strains compared with those at pH 7.0. The K₁/₂ value of the HC-grown lcr1 mutant cells was not affected by the nitrogen source (Figure 5B; see Supplemental Table 2 online). By contrast, the K₁/₂ values of C2 and E4 cells grown in HSM(NO₃) under HC conditions were apparently decreased when compared with those of the cells grown in HSM(NH₄), indicating that the Ci affinity of the LCI1 transformants increased in the presence of NO₃⁻. These results were consistent with the increases observed in LCE activities (Figure 4). However, in LC conditions, the K₁/₂ values of all the strains except for Q304P3 were ~100 μM and were not affected by the nitrogen source (Figure 5B; see Supplemental Table 2 online).

Inorganic Carbon Uptake of the LCI1 Transformants

To evaluate the contribution of LCI1 to Ci transport activity, we compared the accumulation and fixation of [¹⁴C]-labeled Ci by the transformants with those of the lcr1 mutant using a silicone oil layer centrifugation method (Figure 6). We performed the analysis at pH 7.8 using HC-grown cells because the effects of the artificial expression of LCI1 on Ci affinity were more marked under those conditions (Figure 5; see Supplemental Tables 1 and 2 online). The lcr1 mutant showed almost the same levels of Ci accumulation (~0.1 mM) and CO₂ fixation (~1.0 nmol L⁻¹ SIS⁻¹) after 80 s with either nitrogen source in the media. By contrast, the levels of Ci accumulation of HSM(NO₃)-grown LCI1 transformants C2 and E4 were more than twice the levels observed in the transformants cultured in HSM(NH₄). In addition, both HSM(NO₃)-grown LCI1 transformants showed an ~1.3-fold increase in CO₂ fixation, although the lcr1 mutant cells did not show any apparent effects of nitrogen source on Ci accumulation and fixation. These results reveal that nitrate-induced expression of the LCI1 protein enhanced the Ci uptake and/or CO₂ fixation of the transformants grown under HC conditions.

Growth of the LCI1 Transformants

The effects of the expression of LCI1 on the growth of cells were also analyzed at both pH 7.0 and 7.8. At pH 7.0, Q304P3 cells showed a similar growth rate in both HC and LC conditions, where the doubling time was 6.5 h (Figure 7). By contrast, at pH 7.0, the growth of the lcr1 mutant was slower in LC conditions than in HC conditions, which is consistent with the deficiency in the CCM of this mutant, as described previously (Yoshioka et al., 2004). The growth of the lcr1 mutant at pH 7.0 was not affected by the nitrogen source in the media in either HC or LC conditions. At pH 7.0, the growth of the LCI1 transformant C2 was similar to that of the lcr1 mutant and was not affected by the nitrogen source, although increases in LCE activity (Figure 4) and Ci affinity (Figure 5) were observed at pH 7.0.

Table 3. The pH-Dependent HCO₃⁻/CO₂ Ratio and the LCE Activity of the HC-Grown lcr1 Mutant and LCI1 Transformant C2 at Three pH Values

| pH | HCO₃⁻/CO₂ Ratioa | Strain | N Source | LCE activity |
|----|------------------|--------|---------|--------------|
| 6.2 | 0.7 | lcr1 | NH₄⁺ | 14.1 ± 3.0 | 11.6 ± 0.9 | 6.7 ± 0.1 |
| 7.0 | 4.5 | | NO₃⁻ | 15.3 ± 1.3 | 15.0 ± 2.7 | 8.9 ± 1.3 |
| 7.8 | 28.2 | C2 | NH₄⁺ | 12.5 ± 0.4 | 11.3 ± 2.3 | 6.8 ± 0.4 |
| | | | NO₃⁻ | 26.3 ± 0.7 | 24.7 ± 2.9 | 19.8 ± 1.9 |
| | | [C2 (NO₃)⁻]-lcr1 (NO₃)⁻] | 11.0 | 9.7 | 11.1 |

The data are shown as μmol CO₂ mg Chl⁻¹ h⁻¹, ± SD, obtained from three independent experiments.

aHCO₃⁻/CO₂ ratio was calculated using the equation [pH = pK₆+log₁₀[HCO₃⁻]/[CO₂]], where the pK₆ is an acid dissociation constant of 6.35.
At pH 7.8, Q304P3 cells grew more slowly, even in HC conditions, with a doubling time of 9 h, which was longer than that of the growth at pH 7.0 (Figure 7). A similar increase in doubling time was observed in both the lcr1 mutant and the LCI1 transformant C2. The lcr1 mutant and C2 cells were grown in both HSM(NH4) and HSM(NO3) in HC conditions. Although the apparent increases in the Ci affinity and the Ci uptake in HSM(NO3)-grown C2 cells were observed at pH 7.8 (Figures 5 and 6), the growth rate for those cells was almost the same as that of cells cultured in HSM(NH4) (Figure 7).

Subcellular Localization of LCI1 Protein

To analyze the localization of the LCI1 protein, three methods were applied: visualization using indirect immunofluorescence, a C. reinhardtii–adapted green fluorescent protein (CrGFP) fused to LCI1 (Fuhrmann et al., 1999), and a cell fractionation method, in which LCI1 was detected using a specific antibody. Indirect immunofluorescence analyses with anti-LCI1 were applied to photosynthetically grown Q304P3 cells. As expected, signals from fluorescently labeled anti-LCI1 antibodies were detected in LC-grown cells but not in HC-grown cells (see Supplemental Figure 1 online). In LC-grown cells, fluorescence was detected around the nucleus as well as at the cell periphery. Similar fluorescence signals were observed in the HSM(NO3)-grown LCI1 transformants. Because C. reinhardtii has a single, large, cup-shaped chloroplast that closely appresses to the plasma membrane (Ohad et al., 1967), it was difficult to determine whether the fluorescence arose from the chloroplast envelope or the plasma membrane. To resolve this question, CrGFP was fused to the C terminus of LCI1 and further studies were conducted.

To express LCI1 fused with CrGFP, the genomic region of LCI1 was fused in frame with the CrGFP coding region, which was also placed under the control of regulatory elements of the NIA1 gene (Figures 3A and 8A). The generated plasmid was transformed into the wild-type strain C9 and then Zeocin-resistant colonies were screened for fluorescent signals using a microscope. One typical transformant was selected and analyzed using an anti-LCI1 antibody to examine whether the cell expressed the LCI1-CrGFP fusion protein in the presence of NO32 (Figure 8B). A positive band of ~45 kD, which corresponded to the predicted size of the LCI1-CrGFP fusion protein, was detected when the transgenic cells were cultured in HSM(NO3) but not when they were cultured in HSM(NH4). Although the level of the LCI1-CrGFP fusion protein in cells grown in HSM(NO3) was only a small fraction of that of the endogenous LCI1 protein in LC grown cells (Figure 8B), green fluorescence signals were observed (Figure 8C). In these experiments, GFP fluorescence was detected in two regions. In many cells, a prominently labeled region surrounding the nucleus was observed. The second region of the cell showing prominent fluorescence was the cell periphery.

Figure 5. Typical Oxygen-Evolving Activity of the lcr1 Mutant and LCI1 Transformants in Response to External Dissolved Ci Concentrations.

Cells were grown in HSM(NH4) (closed circles) or HSM(NO3) (open circles) under HC or LC conditions. Photosynthetic oxygen evolution was measured using a Clark-type oxygen electrode in the presence of various concentrations of NaHCO3 at pH 7.0 (A) or pH 7.8 (B). The dotted lines indicate 50% of the maximum photosynthetic oxygen-evolving activity.
Because the chloroplast envelope is appressed to the plasma membrane, it was not possible to determine whether the LCI1-CrGFP fluorescence is associated with the chloroplast envelope or the plasma membrane. However, we found that the label was observed surrounding the entire cell, including the region of the cell with the open end of the cup-shaped chloroplast (indicated by arrows in Figure 8C). In addition, no fluorescence was detected in the region of the chloroplast envelope furthest from the plasma membrane.

To clarify further the localization of LCI1, we isolated both chloroplast envelope and plasma membrane fractions. We probed the proteins in the total cell, chloroplast envelope, and plasma membrane fractions with antibodies against LCI1, CCP1, and D1 (Figure 9). Using equal protein loading, we detected all the proteins in the total protein fraction from LC-grown cells. CCP1 was enriched in the chloroplast envelope, which was consistent with the chloroplast envelope localization of this protein (Ramazanov et al., 1993). In contrast with CCP1, a notable enrichment of LCI1 was observed in the plasma membrane fraction (Figure 9). D1 protein was detected only in the total cell protein fraction, indicating very little thylakoid membrane contamination was present in the chloroplast envelope and plasma membrane fractions. Considering the results from fractionation studies, as well as the GFP fluorescence, it is reasonable to conclude that LCI1 is mainly localized to the plasma membrane.

**DISCUSSION**

**LCI1 Protein Is Involved in Light-Dependent Ci Uptake**

In this study, we demonstrated that the level of LCE activity as well as the rate of Ci accumulation increased in transgenic *C. reinhardtii* cells expressing *LCI1* grown in HSM(NO3) compared with cells grown in HSM(NH4) even under HC conditions (Figures 4 and 6). Because NO3\(^{-}\) did not affect LCE activity or Ci accumulation in control cells, it is reasonable to conclude that the increases in LCE activity and the rate of Ci accumulation observed in the LCI1 transformants are caused by the LCI1 protein accumulating in response to the change of the nitrogen source from NH4\(^{+}\) to NO3\(^{-}\) in the culture medium. Although the increase in the Ci accumulation in the cells grown in HSM(NO3) was ~2-fold compared with cells grown in HSM(NH4) at the 80 s time point, CO2 fixation increased ~1.3-fold in transformants grown in HSM(NO3) (Figure 6). These results suggest that LCI1 is involved in the active uptake of Ci rather than directly in CO2 fixation, although our results do not rule out the possibility that some components required for CO2 fixation are activated after 80 s when LCI1 is expressed in the absence of other CCM components. The localization data showing that LCI1 is present on the plasma membrane are also consistent with a hypothesis that LCI1 plays a role in the uptake of Ci but not directly in CO2 fixation. The positive effects of artificial expression of LCI1 can be seen in the LCE activities shown in Figure 4 where the expression of LCI1 under HC conditions in the C2 and E4 transformants increased LCE activity 2-fold. Similarly, Ci affinity showed an apparent increase in HC-grown cells cultured in HSM(NO3) (Figure 5). However, when these transformants were grown under LC conditions, almost no effect of the artificial expression of LCI1 was observed on Ci-dependent O2 evolution. This discrepancy between the LCE and Ci-dependent O2...
The measurement of O2 evolution is performed in a closed system, where the Ci concentration is constantly decreasing as it is assimilated by the cells. Therefore, at low Ci concentrations, this results in highly variable and underestimated rates of O2 evolution in the closed system. By contrast, more reliable and sensitive rates of LCE activity are obtained compared with that of O2 evolution measurement for detecting changes in the characteristics of the CCM.

At pH 7.0, LCE activity and Ci affinity increased in all the strains examined compared with the same strains at pH 7.8. Although an increase in the LCE activity of LC-grown LC1 transformant cells was observed (Figure 4), the K1/2 value was not affected by the nitrogen source in the media. Because *C. reinhardtii* cells prefer to use CO2 rather than HCO3− (Spalding, 1998), the high affinity of the cells for CO2 possibly increased the background, obscuring the effects of the expression of LCI1. On the other hand, the difference in K1/2 values was only 10 μM between Q304P3 and the lcr1 mutant at pH 7.0 (see Supplemental Table 1 online), while the LCE activity of the lcr1 mutant was ~60% of that of wild-type Q304P3 cells in LC conditions at the same pH (Table 1). Considering the results for Q304P3 and the lcr1 mutant, the measurement of LCE activity with an air supply of 50 ppm CO2 could be a more sensitive method than Ci-dependent O2 evolution for detecting changes in the characteristics of the CCM as described above.

In a previous study, it was shown that the lcr1 mutant had reduced Ci affinity and failed to induce the expression of three genes, namely, *CAH1*, *LC1*, and *LCI6*, under LC conditions (Yoshioka et al., 2004). The *cah1* mutant is missing only the periplasmic carbonic anhydrase. We found that while the loss of *CAH1* in the *cah1* mutant resulted in some kinetic changes to the LCE activity (Figure 1), the overall extent of LCE activity remained...
unchanged. Because the reduction in LCE activity in the cah1 mutant was minimal, this implied that LCI1 and/or LCI6 significantly contributed to LCE activity under normal conditions. Therefore, LC11 was placed under the control of the NIA1 promoter and put back into the lcr1 mutant. This allowed us to express LCI1 selectively: under HC conditions when most of the CCM components are absent and also under LC conditions, when most of the components of the CCM are present. The effect of the selective expression of LCI1 could be observed more clearly under HC conditions, even though the level of LCI1 protein was not high in the transformants. Although the upregulation of LCE activity, Ci affinity, and Ci uptake/accumulation in the LCI1 transformants grown in HSM(NO3) were apparent (Figures 4 to 6), the levels of LCE activity and Ci affinity were still lower than those of LC-grown wild-type cells (Tables 1 and 2). This may be because the level of LCI1 protein accumulation in these transformants (Figure 3C) was ~50% of the wild-type level, which may not have been sufficient to support all levels of LCE activity and Ci affinity. Another possibility is that other LC-inducible proteins, including CAH1 and LCI6, were needed to achieve similar levels of CCM activity compared with that of wild-type cells. The upregulation of LCE activity, Ci affinity, and Ci uptake/accumulation in the LCI1 transformants grown in HSM (NO3) was apparent as described above, but the growth of these transformants was not affected by the nitrogen source in this study (Figure 7). Measurements of LCE activity, O2 evolution, and Ci uptake were performed with a severely limited supply of Ci (e.g., air containing 50 ppm CO2 for LCE activity measurements) and relatively strong illumination of actinic light at intensities of 1000, 750, and 300 μmol photons m⁻² s⁻¹, respectively. On the other hand, during growth assays, cells were illuminated at 80 μmol photons m⁻² s⁻¹ with bubbling of ordinary air containing 370 ppm CO2. These differences in light intensity and level of CO2 supplied for the measurement may be important for observing the effects of the artificial expression of LCI1 resulting from the change in nitrogen source.

The absolute increase in the LCE activity of LC-grown LCI1 transformants was ~15 μmol CO2 mg Chl⁻¹ h⁻¹ compared with the HSM(NO3)-grown lcr1 mutant (Figure 4, Table 2), which was slightly higher than that in HC-grown cells (10 μmol CO2 mg Chl⁻¹ h⁻¹), although the level of NO3⁻-induced LCI1 protein in LC-grown cells was 2-fold compared with that in HC-grown cells (Figure 3C). Because only the LCI1 gene was introduced into the transformants, LCI1 could act alone, but its function is possibly limited in the absence of other LC-inducible proteins, including CAH1 and LCI6. Considering the fact that the cah1 mutant showed very little decrease in LCE activity (Figure 1), LCI6 could also play some role in the CCM. LCI6 contains a Pro-rich region that is involved in protein–protein interactions (Harauz and Libich, 2009). This protein might interact with LCI1 through this region and support its function. The decrease in growth rates of Q304P3 at pH 7.8 compared with that at pH 7.0 might be due not only to Ci utilization but also other factors controlling cell growth. Thus, the expression of LCI1 alone was unable to enhance the growth of cells, indicating that the contribution of LCI1 by itself to the CCM may not be especially large. Alternatively, the growth of the lcr1 mutant might be already partially compensated for by other Ci transporters, obscuring the effects of the expression of LCI1.

Interestingly, the mRNA expression level of the chimeric LCI1 gene was quite high in the presence of NO3⁻ (Figure 3B), but the level of LCI1 protein accumulation was lower than that in LC-grown Q304P3 (Figure 3C). Therefore, the accumulation of LCI1 protein might possibly be regulated at the level of either translation or stability, in addition to normal transcriptional control. This regulatory pattern seen in the artificially expressing LCI1 transformants may be caused by the exchange of the 5' and 3'–UTRs of the LCI1 or lack of other proteins, including LCI6, regulated by LCR1. To understand the detailed molecular mechanism of the regulation of LCI1, it will be important to reveal whether it is mediated by LCR1 or not. It is also possible that LCI1 participates in a protein complex of which the stable accumulation is dependent on other components. In the LCI1 transformants grown in HSM(NO3) under LC conditions, most LC-inducible proteins, other than LCR1-regulated proteins, such as CAH1 and LCI6, should accumulate. CAH1 is a periplasmic carbonic anhydrase that does not appear critical to the function of the CCM (Van and Spalding, 1999). However, the potential role of LCI6 in the CCM and its possible interaction with LCI1 merits further detailed analyses.

**LCI1 Is Localized Mainly to the Plasma Membrane**

In this study, we showed that fluorescent signals from CrGFP-fused LCI1 were detected on the periphery of the cell, in the vicinity of the chloroplast, and on the outer perimeter of the nucleus (Figure 8). The GFP fluorescence that originated from the cell periphery could be from the plasma membrane. The internal signals consisted of a ring-like arrangement around the outer perimeter of the nuclei and patches in the vicinity of the chloroplast. When the chloroplast envelope and the plasma membrane were separated, LCI1 was strongly enriched in the plasma membrane fraction but not in the chloroplast envelope fraction (Figure 9). These fluorescent images of CrGFP-fused LCI1 and the immunoblotting data using separated membrane fractions strongly suggest that LCI1 is localized mainly to the plasma membrane. LCI1 localized at the plasma membrane could be involved in LCE activity to support the CCM. On the other hand, it is unlikely that LCI1 molecules localized around the nuclear envelope and cytosolic vesicles contribute to the CCM. Therefore, it is possible that the internal GFP signals originated from a precursor of LCI1, which underwent transportation to the plasma membrane. The fluorescence signals from either GFP-fused proteins or immunolabeled proteins are not specifically quantitative; thus, the precursor may be undetectable or undistinguishable from the mature CrGFP-fused LCI1 in immunoblot analyses (Figure 8A).

**Possible Role of LCI1**

It remains to be elucidated how LCI1 is involved in Ci transport. When the LCE activity was compared between HSM(NO3)-grown lcr1 and C2 in HC conditions, the increase in the LCE activity in C2 cells grown in HC conditions in HSM(NO3) was constant, at ~10 μmol CO2 mg Chl⁻¹ h⁻¹, between the examined pH conditions despite obvious changes in the basic LCE activity in the HSM(NH4)-grown cells (see Table 3. [C2 (NO3)]; [lcr1 (NO3)]). From this observation of the pH independence of LCE activity, it appeared that LCI1 could be involved in the
transport of both Ci species. On the other hand, the decreases in the $K_{\text{ci}}$ value in HSM(NO$_3$)-grown LCI1 transformants were ~30 and 250 $\mu$M at pH 7.0 and 7.8, respectively (see Supplemental Tables 1 and 2 online), indicating a more obvious increase in the Ci affinity in the HSM(NO$_3$)-grown LCI1 transformants at pH 7.8 than at pH 7.0. Therefore, it also appeared that LCI1 functions well at alkaline pH. However, at pH 7.0, the high affinity of $C.\ reinhardtii$ cells for CO$_2$ (Spalding, 1998) possibly obscured the effects of the expression of LCI1 on the Ci affinity as described above. Considering these results from the LCE activity and the Ci affinity, it is difficult to conclude whether or not LCI1 increased the flux of a specific species of Ci.

According to prediction programs, such as SMART (http://smart.embl-heidelberg.de/), LCI1 does not contain any domains typical of known transporters. However, the Chlamydomonas, Chlorella, Cocomysxa, and Volvox genomes, as described in the Joint Genome Initiative database (http://genome.jgi-psf.org/), contain several genes and hypothetical genes that are predicted to encode membrane proteins with three or four transmembrane helices, of which the domain structure and hydrophobicity are similar to LCI1 (see Supplemental Figure 2 online). Four regions of LCI1 (Leu-18 to Gln-34, Gln-77 to Phe-88, Arg-108 to Ile-126, and Arg-146 to Gly-169) seemed to be conserved between these proteins, although it is also possible that the similarity is due to convergent evolution. The functions of most of these proteins are unknown, but one of them encodes CST1, which is annotated as a putative transporter protein (Merchant et al., 2007) containing four hydrophobic domains, as does LCI1 (see Supplemental Figure 3 online). Based on the similarity of the domain structure, LCI1 might function as a small transporter or permease for Ci. In addition, LCI1 also shows a low level of similarity to the transmembrane domains of Sho1 in yeast, which plays a crucial role in the osmotic stress response (Saito and Tatebayashi, 2004) by interacting with Hkr1/Msb2 through its transmembrane domains and activating the MAPK cascade (Tatebayashi et al., 2007). Alternatively, LCI1 might enhance Ci uptake by interacting with unknown Ci transporter(s) through the transmembrane helices. In any case, the silicone oil centrifugation experiments clearly showed an increase in the Ci pool sizes of the LCI1 transformants grown in HSM(NO$_3$) (Figure 6).

It is generally expected that multiple Ci uptake and/or accumulation systems function in $C.\ reinhardtii$, as can be seen in cyanobacteria. Recently, it was reported that the LC-inducible proteins HLA3 (Duanmu et al., 2009b) and LCI1 (Wang and Spalding, 2006) play crucial roles in Ci transport and/or accumulation and that HLA3-dependent and LCI1-dependent processes act partially in overlapping, complementary systems. Whereas the Ci transport and/or accumulation processes involving either LCI1 or HLA3 work mainly at alkaline pH, the LCI1-dependent mechanism appeared to function at both acidic and alkaline pH, at least between pH 6.2 and 7.8. Therefore, the LCI1-dependent Ci transport and/or accumulation process may interact with, or act in parallel with, either LCI1 or HLA3.

As proposed previously (Giordano et al., 2005; Spalding, 2008), Ci needs to be transported from the outside of the cells across at least three kinds of membranes, namely, the plasma membrane, chloroplast envelope, and thylakoid membranes, because ribulose-1,5-bisphosphate carboxylase/oxygenase and CAH3 are localized mainly in the pyrenoid and on the luminal side of pyrenoid tubules, respectively (Borkhensive et al., 1998; Moroney and Ynalvez, 2007; Duanmu et al., 2009a). Therefore, distinct Ci transporters and accumulation machinery should be localized to each membrane and subcellular compartment. Ci transport and/or accumulation related to LCI1 should be a part of the mechanism. Unfortunately, we could not determine whether LCI1 contributes mostly to CO$_2$ or HCO$_3^-$ transport/accumulation. This is likely due to the fact that the amount of the LCI1 protein was lower than in LC-grown wild-type cells. It also remains unclear whether LCI1 is involved in Ci transport or accumulation. However, our results strongly suggest that LCI1 contributes to Ci transport or accumulation at the plasma membrane. Further biochemical analyses on the structure and functional relationships of LCI1 could provide new perspectives on Ci transport and/or accumulation mechanisms in eukaryotic photosynthetic organisms.

**METHODS**

**Strains and Culture Conditions**

*Chlamydomonas reinhardtii* strains C9 (wild-type strain), CC503 (photosynthetically wild type, wall-less strain), Q304P3 (photosynthetically wild type, cell wall-less strain; Yoshioka et al., 2004), lcr1 (generated from Q304P3; Yoshioka et al., 2004), lcr1+G (lcr1 mutant complemented with LCR1; Yoshioka et al., 2004), and ca1 (Van and Spalding, 1999) were grown in Tris-acetate-phosphate medium and diluted with modified HSM (NH$_4$) supplemented with 20 mM MOPS, pH 7.0, to an OD$_{730}$ of ~0.01 (~7 × 10$^4$ cells mL$^{-1}$) for photoautotrophic growth. Subsequently, the cells were cultured at 28°C with illumination at 80 mol photons m$^{-2}$ s$^{-1}$ under HC conditions for 24 h until the cell density reached an OD$_{730}$ of ~0.1. The cells were further cultured for 12 h under HC or LC conditions until they reached logarithmic growth phase. To induce the gene expression of the transgene, cells grown in HSM(NH$_4$) containing 9.35 mM NH$_4$Cl for 24 h were collected by centrifugation and then resuspended in fresh HSM(NO$_3$) containing 9.35 mM KNO$_3$. After the change in media, cells for RNA analysis were grown for an additional 6 h in HC or LC conditions, while cells used for physiological measurements or protein immunoblot analyses were grown for 12 h under HC or LC conditions. The cell density (OD$_{730}$) per 5 g Chl mL$^{-1}$ was ~0.6 for all the examined strains, justifying normalization on the basis of chlorophyll content in the physiological measurements.

**LCE Activity with an Open Gas Exchange System**

The cells grown as described above were collected by centrifugation at 700g for 10 min and resuspended in fresh HSM(NH$_4$) or HSM(NO$_3$) media. To analyze the effects of pH, the collected cells were also resuspended in HSM media of pH 6.2 and 7.8, which were prepared using 20 mM MES, pH 6.2, or 20 mM HEPES, pH 7.8. The light-dependent CO$_2$ gas exchange activity of cells (5 $\mu$g mL$^{-1}$ of chlorophyll) was measured with bubbling air containing 50 ppm CO$_2$ under actinic light illumination of 1000 mol photons m$^{-2}$ s$^{-1}$ at 28°C using an open infrared gas analysis system that records the rate of CO$_2$ exchange as a function of time as described previously (Yamano et al., 2008).

**Intercellular Concentration of Dissolved Ci**

The intercellular concentration of dissolved Ci was measured by the silicone oil centrifugation method (Fukuzawa et al., 1998). Cells grown as
described above were collected by centrifugation (700g at 28°C) and subsequently suspended at a cell density of 25 μg mL⁻¹ chlorophyll in 50 mM HEPES-NaOH buffer, pH 7.8, that had been bubbled with N₂ gas. One milliliter of cell suspension was bubbled with N₂ gas for 10 min in an O₂ electrode until they no longer produced O₂. First, a 60-μL silicone oil layer (SH550/SH556 = 4/7 [v/v]) was overlaid on a 20-μL layer of the termination solution containing 1 M glycine-NaOH, pH 10.0, and 0.75% SDS (w/v). Then, the cells (300 μL) were overlaid further on the silicone oil layer. SH550 and SH556 were purchased from Toray Dow Corning Silicone and solution containing 1 M glycine-NaOH, pH 10.0, and 0.75% SDS (w/v). The other aliquot was added to 0.5N HCl, desiccated until they no longer produced O₂. First, a 60-s illumination at 10 mol photons m⁻² s⁻¹ and the reaction was terminated by centrifugation. Control cells that had been supplied with nonradioactive NaHCO₃ were directly subjected to a liquid scintillation counting, which was described above were collected by centrifugation (700g at 28°C) and subsequently centrifuged at 13,000g for 3 min. The supernatant containing solubilized proteins was subjected to SDS-PAGE with a 15% polyacrylamide gel that contained 6 M urea (Ohnishi and Takahashi, 2001). In the case of protein immunoblot analysis for the cell fractionation experiments, samples containing equal amounts of protein solubilized in a buffer containing 5% SDS, 0.1 M DTT, 0.1 M Na₂CO₃, 7 M urea, 0.02% mercaptoethanol, and 25 mM Tris were run on 12% SDS-PAGE gels. Separated polypeptides were blotted electrotheroically onto polyvinylidene difluoride membranes and probed with polyclonal antibodies against LC1 (1 × 10⁻³ dilution), CCP1 (1 × 10⁻³ dilution; Ramazanov et al., 1993), and D1 protein of photosystem II (1 × 10⁻⁴ dilution; product number AS05 084 from Agrisera). The antibodies against LC1 (AS-F and AS-M) were raised in rabbits using synthetic oligopeptides as the antigens, which were FDTQEGIDKYPFYV (from Phe-43 to Tyr-56) and DAEESHAMPNVHVTSDGATKV (from Asp-172 to Val-192), respectively. The former oligopeptide was also used for affinity purification of the antibody against LC1. The rabbit antibody against CCP1 was raised against the peptide CFKQVMSKHGIKGLYRGFTST. Signals were visualized using an enhanced chemiluminescence reagent (ECL; GE Healthcare) and detected using a LAS Image analyzer (FUJI FILM). Three typical blots were detected using a densitometric quantification. ECL-detectable recombinant protein mixture (The MagicMark XP Western Protein Standard; Invitrogen) was used as a size marker.

Protein Immunoblot Analysis
Whole-cell proteins corresponding to 2 μg of chlorophyll were suspended in 50 mM Tris-HCl, pH 8.0, buffer containing 25% glycerol (v/v) to a volume of 10 μL. The cells were solubilized by incubation at 100°C for 1 min in the presence of 2% SDS and 0.1 M DTT and subsequently centrifuged at 13,000g for 3 min. The supernatant containing solubilized proteins was subjected to SDS-PAGE with a 15% polyacrylamide gel that contained 6 M urea (Ohnishi and Takahashi, 2001). In the case of protein immunoblot analysis for the cell fractionation experiments, samples containing equal amounts of protein solubilized in a buffer containing 5% SDS, 0.1 M DTT, 0.1 M Na₂CO₃, 7 M urea, 0.02% mercaptoethanol, and 25 mM Tris were run on 12% SDS-PAGE gels. Separated polypeptides were blotted electrotheroically onto polyvinylidene difluoride membranes and probed with polyclonal antibodies against LC1 (1 × 10⁻³ dilution), CCP1 (1 × 10⁻³ dilution; Ramazanov et al., 1993), and D1 protein of photosystem II (1 × 10⁻⁴ dilution; product number AS05 084 from Agrisera). The antibodies against LC1 (AS-F and AS-M) were raised in rabbits using synthetic oligopeptides as the antigens, which were FDTQEGIDKYPFYV (from Phe-43 to Tyr-56) and DAEESHAMPNVHVTSDGATKV (from Asp-172 to Val-192), respectively. The former oligopeptide was also used for affinity purification of the antibody against LC1. The rabbit antibody against CCP1 was raised against the peptide CFKQVMSKHGIKGLYRGFTST. Signals were visualized using an enhanced chemiluminescence reagent (ECL; GE Healthcare) and detected using a LAS Image analyzer (FUJI FILM). Three typical blots were detected using a densitometric quantification. ECL-detectable recombinant protein mixture (The MagicMark XP Western Protein Standard; Invitrogen) was used as a size marker.

GFP Fluorescence Imaging
For GFP fluorescence imaging, live cells were immobilized in 1% low-melting-point agarose and observed using a laser scanning microscope LSM710 system at an excitation of 405 nm and emission at 490 to 550 nm for GFP image capture and at an excitation of 488 nm and emission at 600 to 700 nm for the chlorophyll image capture.

Isolation of Chloroplast Envelope and Plasma Membrane
Intact chloroplasts were isolated from cells of C503 according to Mason et al. (2006). Isolated chloroplasts were then used for chloroplast membrane fractionation by the method of Clemenson and Boschetti (1988). The chloroplast envelope proteins were solubilized in buffer containing detergent. The procedure followed that for the isolation of plasma membrane adopted from Norling et al. (1998). Protein concentration was determined using the bichinconic acid assay (Pierce) before loading on SDS-PAGE gels.

Accession Numbers
Sequence data from this article can be found in the DDBJ/NCBI data libraries under accession numbers AB447355 (pTY2b), U31976 (LC11), and XP_001693802 (CST1).

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure 1. Indirect Immunofluorescence Analyses of LC1 Protein in C. reinhardtii Cells.
Supplemental Figure 2. Comparison of Amino Acid Sequences between LC1 and Proteins That Contain Similar Domain Structure to LC1.
Supplemental Figure 3. Hydropathy Profiles of LC11 and CST1.

Supplemental Table 1. Ci Affinity of the Photosynthetically Wild-Type Q304P3, the icr1 Mutant, and LC11 Transformants at pH 7.0.

Supplemental Table 2. Ci Affinity of the Photosynthetically Wild-Type Q304P3, the icr1 Mutant, and LC11 Transformants at pH 7.8.

Supplemental Methods. RNA Gel Blot Analysis, Indirect Immunofluorescence Analysis, and Amino Acid Sequence Alignment.

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Expression of a Low CO₂–Inducible Protein, LCI1, Increases Inorganic Carbon Uptake in the Green Alga Chlamydomonas reinhardtii
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