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Chloroplast-mediated regulation of CO$_2$-concentrating mechanism by Ca$^{2+}$-binding protein CAS in the green alga *Chlamydomonas reinhardtii*

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**Abstract**

Aquatic photosynthetic organisms, including the green alga *Chlamydomonas reinhardtii*, induce a CO$_2$-concentrating mechanism (CCM) to maintain photosynthetic activity in CO$_2$-limiting conditions by sensing environmental CO$_2$ and light availability. Previously, a novel high-CO$_2$-requiring mutant, H82, defective in the induction of the CCM, was isolated. A homolog of calcium (Ca$^{2+}$)-binding protein CAS, originally found in *Arabidopsis thaliana*, was disrupted in H82 cells. Although *Arabidopsis* CAS is reported to be associated with stomatal closure or immune responses via a chloroplast-mediated retrograde signal, the relationship between a Ca$^{2+}$ signal and the CCM associated with the function of CAS in an aquatic environment is still unclear. In this study, the introduction of an intact CAS gene into H82 cells restored photosynthetic affinity for inorganic carbon, and RNA-seq analyses revealed that CAS could function in maintaining homeostasis by a Ca$^{2+}$-binding protein, CAS, has been shown to mediate the transient elevation of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{cyt}$), as well as stromal Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{strom}$), in guard cells.

**Significance**

Ca$^{2+}$ and CO$_2$ are fundamental biological signaling molecules in microbes, animals, and plants. Although Ca$^{2+}$ was proposed to act as a second messenger in CO$_2$ signaling in guard cells of terrestrial plants, the role of Ca$^{2+}$ in CO$_2$ signal transduction pathways in aquatic photosynthetic organisms remains largely unknown. We show here that a chloroplast Ca$^{2+}$-binding protein, CAS, changes its localization in response to environmental CO$_2$ conditions and regulates the expression of nuclear-encoded limiting-CO$_2$-induced genes, including two key bicarbonate transporters. These findings led us to propose a model for the participation of Ca$^{2+}$ signals in chloroplast-regulated CO$_2$ signal transduction of aquatic photosynthetic organisms and help us to further understand the role of Ca$^{2+}$ in CO$_2$ signal transduction in eukaryotes.

**Author contributions**: L.W., T.Y., and H.F. designed research; L.W., T.Y., S.T., Y.N., C.T., S.-i.O., R.T., Y.K., and H.F. performed research; T.Y., T.M., H.Y., and H.F. contributed new reagents/analytic tools; L.W., T.Y., Y.N., and H.F. analyzed data; and L.W., T.Y., and H.F. wrote the paper.

The authors declare no conflict of interest. This article is PNAS Direct Submission. Freely available online through the PNAS open access option.

Data deposition: The RNA-seq raw data (Tables S2 and S3) in this paper have been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) (accession no. DRA004677).

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Characterization of CAS insertion mutant H82 and its complemented strain C-1. (A) Inorganic carbon (Ci) affinity of WT, H82, C-1, and transgenic strain AH-1 with or without the CAS gene. (B) Accumulation of CAS, LCIA, HLA3, and LCIB in WT, H82, and C-1 cells. Cells were grown in LC conditions for 12 h, and the intracellular Ci concentration and carbon fixation were measured using a silicone-oil layer method. SIS, sorbitol impermeable space. (D) Accumulation of CAS, LCIA, HLA3, and LCIB in WT, H82, and C-1 cells. Cells were grown in high-CO2 (HC) or LC conditions for 12 h. Histone H3 was used as a loading control.

To evaluate induction of the CCM in LC conditions, photosynthetic affinities for Ci were evaluated by measuring the rates of photosynthetic oxygen (O2) evolution of WT, H82, and C-1 cells. At pH 7.8 (ratio of HCO3−:CO2 = 28:1), the Ci concentration required for half maximal velocity [K0.5 (Ci)] in C-1 cells was 58 ± 8 µM, which was similar to that of 50 ± 8 µM in WT cells and ~19 times lower than that of 1,087 ± 113 µM in H82 cells (Fig. 4A and Table S1). Even at pH 6.2 (HCO3−:CO2 = 0.7:1) and pH 7.0 (HCO3−:CO2 = 4:3:1), the respective K0.5 (Ci) values of C-1 were 65-fold and 19.7-fold lower than that of H82 cells (Fig. S1C and Table S1). Because maximum rates of photosynthesis (Vmax) of the three strains WT, H82, and C-1 were similar with each other at each pH condition (Table S1), the decreased Ci affinity in H82 cells could be partly explained by a defect in Ci uptake activity. The accumulation and fixation of [14C]-labeled Ci in H82 cells were 0.13 mM and 0.34 nmol per microliter of sorbitol impermeable space (SIS) after 80 s of illumination, respectively, which was lower than 0.22 mM and 1.34 nmol µL SIS−1 of C-1 cells (Fig. 1A and C).

In addition to recovery of the accumulation of CAS and photosynthetic affinities for Ci in C-1, LC-induced accumulation of HLA3 and LCIA was also restored (Fig. 1D). To further evaluate the contribution of the defect in the accumulation of HLA3 and LCIA to the decreased Ci affinity in H82 cells, strain AH-1 was generated, in which two recombinant plasmids, pTY2b-CAS and pTY2b-HLA3 (12), were introduced into H82 cells and in which HLA3 and LCIA could be induced by switching the nitrogen source from NH4+ to NO3− (Fig. S1F). Although the K0.5 (Ci) value of 1.008 ± 70 µM in AH-1 cells was similar to that of 1,087 ± 113 µM in H82 cells in NH4+ conditions, that of AH-1 cells decreased to 430 µM from 793 ± 91 µM in H82 cells by expression of both HLA3 and LCIA in NO3− conditions (Fig. 4C and Table S1). However, the K0.5 (Ci) value in AH-1 cells was still ~10-fold higher than those in WT and C-1 cells. These results suggested that decreased Ci affinity in H82 cells was partially caused by a defect in the accumulation of HLA3 and LCIA, but other additional factors could be responsible for the HC-requiring phenotype of H82 cells.

**CAS-Dependent Regulation of Nuclear-Encoded LC-Inducible Genes.** To evaluate the cause of decreased Ci affinity of H82 cells other than HLA3 and LCIA, genes whose expression was affected by
the CAS mutation were screened by RNA-seq analysis (Table S2). WT, H82, and C-1 cells were grown in HC or LC conditions for 0.3 and 2 h, and the respective transcriptome profiles were compared. After exposure to HC and LC conditions for 0.3 h, no gene other than CAS3 was affected by the mutation. In contrast, in LC conditions for 2 h, the expression levels of 44 genes in addition to CAS3 were significantly different (FDR false discovery rate) < 0.05 in H82 cells compared with those in WT and C-1 cells (Table S3). Among them, the expression levels of 13 genes were decreased more than fourfold by the CAS3 mutation, including the following: HL3A; LCI4; DN31 encoding putative DNAJ-like chaperonin; CAH4 and CAH5 encoding mitochondrial carbonic anhydrases; PPP30 encoding type 2C protein phosphatase; chloroplast carrier protein 1 (CCP1) and CCP2 encoding putative chloroplast envelope membrane proteins; low-CO2-inducible gene D (LCID); LHCSR3.1 and LHCSR3.2 encoding LHCSR3; and two unknown genes (Cre12.g541550 and Cre26g756747). Eventually, the transcriptional abundances of these 12 genes, except for gene Cre12.g541550, were decreased by the CIA5 mutation (9).

In Chlamydomonas, accumulation of LHCSR3, which is essential for energy-dependent quenching (eQ), is shown to be dependent on CAS in high-light condition (25). Because its function during LC acclimation related to CAS is unclear, we examined LHCSR3 accumulation in LC conditions in a time-dependent manner (Fig. S1J). After shifting to LC conditions, significant accumulation of LHCSR3 was detected within 2 h in WT cells, supporting the previous report that LHCSR3 is induced by CO2-limiting stress (29, 30). Although the accumulation of LHCSR3 in H82 cells was twofold lower than that in WT and C-1 cells in LC at 2 h or 4 h, similar accumulation levels of LHCSR3 were detected in each strain at 12 h (Fig. S1J). These results suggested that CAS regulates LHCSR3 accumulation at an early stage of CCM induction. In contrast, other CCM-related genes, including HL3A and LCI4, were transiently induced at the mRNA levels at 0.3 h in H82 cells, but their mRNA levels could not be maintained at the same levels as in WT and C-1 cells at 2 h in contrast to LCI4 (Fig. S1K), suggesting that CAS could be required for maintaining the mRNA levels of HL3A and LCI4 after the initial induction of these genes by CCM1/CIA5.

Requirement of Ca2+ for LC-Induced Accumulation of HL3A and LCI4. As in the case of AtCAS, the N terminus of CrCAS has also Ca2+-binding activity (Fig. S2A–C and SI Results and Discussion). To know the link between Ca2+ signal via CAS and regulation of HCO3− transporters, the accumulation of HL3A and LCI4 in WT cells grown in the presence of 1,2-bis(o-aminophenoxy)ethane-N,N,N,N'-tetraacetic acid (BAPTA), a membrane-impermeable Ca2+-specific chelator (31), was measured in LC conditions. The accumulation of HL3A and LCI4 was dramatically decreased in the presence of 0.5 mM BAPTA (Fig. 2A) as in the case of LHCSR3 (25, 30), and concomitantly K0.5 (CI) increased twofold (72 ± 9 μM to 144 ± 14 μM) (Fig. 2B and Table S1). Considering that the addition of 0.75 mM CaCl2 rescued the accumulation of HL3A and LCI4 and decreased CI affinity, extracellular Ca2+ is necessary for the accumulation of these HCO3− transporters and for the photosynthetic CI affinity. To further examine the regulation of these HCO3− transporters by an intracellular Ca2+ signal, the impact of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), a membrane-permeable calmodulin antagonist, on the accumulation of HL3A and LCI4 and photosynthetic CI affinity was examined. In the presence of W-7, concentration-dependent effects of decreased accumulation of HL3A and LCI4 were observed, as in the case of LHCSR3 (Fig. S2D) (25, 30), and concomitantly K0.5 (CI) increased from 59 ± 18 μM (mock) to 235 ± 48 μM in the presence of 75 μM W-7 (Fig. S2E and Table S1).

In contrast, addition of N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5), a biologically inactive calmodulin antagonist, did not show any significant effects. As a control, LCIB was normally induced in the presence of both chemicals. These results suggested that a calmodulin-mediated Ca2+ signal could play roles in the LC-induced accumulation of HL3A and LCI4.

Relocation of Thylakoid Membrane-Localized CAS by LC in Light. To examine the localization of CAS, total protein, soluble/insoluble, chloroplast envelope, and thylakoid membrane fractions were isolated and probed with antibodies against CAS, thylakoid membrane-localized D1, soluble protein LCIB, plasma membrane-localized H+-ATPase (12, 32), and chloroplast envelope-localized LCI4 (12) (Fig. S3 A–C). CAS was mainly detected in the insoluble and thylakoid membrane fractions where D1 was enriched, as shown in the previous proteome analyses (23, 24). Similar to the case of AtCAS, both the N and C terminus of CrCAS were exposed to the stromal side of thylakoid membrane (Fig. S3D and SI Results and Discussion). Next, to elucidate the detailed subcellular localization of CAS in vivo, an indirect immunofluorescence assay using an anti-CAS antibody was performed (Fig. 3A). In HC conditions with light illumination at 120 μmol photons·m·s−1·s−1 (HC-light), fluorescent signals were observed as dispersed specks in the chloroplast. In contrast, after shifting to LC conditions in light (LC-light), the fluorescent signals were aggregated in the pyrenoid after 2 h, and the aggregation was observed as tubule-like structures inside the pyrenoid at 12 h. These localization patterns were consistent with the results using a complemented strain FN-1 expressing FLAG-tag fused CAS and an anti-FLAG antibody (Fig. S3 E–G). Furthermore, to observe CAS localization in living cells, we also generated a complemented strain, CL-1, expressing exogenous CAS tagged with clover, a Chlamydomonas-adapted modified GFP (33) (Fig. S3 E and H). Fluorescent signals of CAS-Clover were distinctly aggregated and observed as a tubule-like structure in the pyrenoid in LC-light conditions at 12 h (Fig. S3I). The aggregated tubule-like signals were dispersed throughout the chloroplast when transferred to HC-light conditions (Fig. S3 J and D). Changing localization in the chloroplast in response to CO2 was also shown in the case of LCIB, and light as well as CO2 could affect its localization (17). Similarly, the aggregated fluorescent signals of CAS in LC-acclimated cells became diffuse...
After transferring from LC-light to LC conditions in the dark (LC-dark) within 2 h (Fig. 3A and Fig. S3). Considering the fact that the localization of CAS to the pyrenoid (as well as the accumulation of HLA3, LCIA, and LCIB) was inhibited in the presence of dichlorophenyl-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) (Fig. 3B and Fig. S3 I, K, and L) and that these inhibitors also suppress the CCM (34), the localization of CAS to the pyrenoid could be important for regulation of the CCM in LC-light conditions where the CCM was active.

Next, to determine whether the aggregation of CAS in LC-light is associated with de novo protein synthesis, the effect of cycloheximide (CHX) was examined. By switching from HC-light to LC-light conditions or from LC-light to LC-dark conditions, a change in the localization of fluorescent signals derived from CAS was observed in the presence of CHX (Fig. 3B). These results suggested that the LC-induced CAS relocation was not associated with de novo protein synthesis, in contrast to the case of LCIB (35). Moreover, the addition of CHX inhibited the accumulation of LCIB but not CAS in LC conditions (Fig. S3M), and the addition of BAPTA and W-7 did not affect CAS localization (Fig. S3N). Because the accumulation of CAS and its relocation in response to CO2 were not impaired in strain C16, a CCM1 insertion mutant (6) (Fig. 3C and Fig. S3O), relocation of CAS was regulated by external CO2 concentration irrespective of CCM1/CIA5 function. Additionally, the impaired photosynthetic Ci affinity in H82 cells caused poor Ci consumption in the culture medium, affecting the localization of LCIB in LC conditions (Fig. S3 P-S and SI Results and Discussion).

Increased Ca2+ Concentrations in the Pyrenoid in LC-light Conditions. Because CAS had low Ca2+-binding affinity (20), a high Ca2+ concentration should be required to bind Ca2+. This aspect of CAS raised the possibility that the subcellular regions where free Ca2+ is enriched could be related to CAS localization. To test this hypothesis, we monitored the fluorescence of Calcium Green-1, AM, a Ca2+-sensitive fluorescent dye, in WT and H82 cells (Fig. 4 and Fig. S4). In both HC and LC conditions, apparent fluorescent signals were detected in regions that overlapped with chlorophyll. Notably, distinct high levels of fluorescent signals were observed in the region of the pyrenoid in both WT and H82 cells, especially in LC-light conditions, thereby implying that free Ca2+ might be concentrated in the pyrenoid. These increased fluorescent signals in LC-light conditions were not impaired by the CAS mutation (Fig. 4 and Fig. S4A) as well as BAPTA and W-7 (Fig. S4B). In contrast, the fluorescent signals in the pyrenoid were decreased after shifting from LC-light to LC-dark conditions for 2 h. Additionally, the signal intensities of Ca2+ indicator in chloroplast were slightly stronger in H82 than in WT cells after switching to LC conditions for 12 h (Fig. 4 and Fig. S4I), suggesting a higher free Ca2+ concentration in the chloroplast of H82 cells. Considering that depletion of CAS protein does not affect the total cellular
Ca\(^{2+}\) content (25), increased free Ca\(^{2+}\) could be caused by the redistribution of internal Ca\(^{2+}\).

**Discussion**

In this study, we identified a Ca\(^{2+}\)-binding protein as a regulator of the CCM by characterization of a CAS insertion mutant, H82, and its complemented strain, C-1. RNA-seq analyses of these strains revealed that CAS is required for maintaining the expression of 13 nuclear-encoded LC-induced genes after induction (Table S3). Of those genes regulated by CAS, HLA3 and LCIA, whose accumulation was decreased in H82 cells (Fig. 1D), are involved in HCO\(_3\)-uptake for operation of the CCM (11–14). However, simultaneous expression of HLA3 and LCIA could only partially rescue the decreased affinity of H82 cells (Fig. 1A), suggesting that some of the 13 CO\(_2\)-limiting-inducible genes other than HLA3 and LCIA could contribute to the operation of the CCM in *Chlamydomonas* cells.

Thylakoid-membrane–localized CAS changed its localization from dispersed in HC-light or LC-dark conditions, where the CCM was inactive, to an aggregated tubule-like structure in the pyrenoid in LC-light conditions, where the CCM is active (Fig. 34 and Fig. S3F), which means that the aggregation of CAS to the pyrenoid in response to the availability of environmental light and LC is important for regulation of the CCM. Considering that some of the thylakoid membrane penetrates the pyrenoid, which are termed pyrenoid tubules (36), it is possible that CAS could be localized in or among such pyrenoid tubules in LC-light conditions and could change its localization reversibly in response to CO\(_2\) conditions. The change in CAS localization was independent of de novo protein synthesis (Fig. 3B), which was unlike the case of LCIB (35), suggesting that aggregated CAS is not newly synthesized and that CAS itself could move in response to light and CO\(_2\) conditions. This distinct localization of CAS could be explained by the thylakoid membrane remodeling observed previously in varying light conditions (37) although the actual mechanism for the change in localization requires further analysis.

Previously, based on the fact that the accumulation of chloroplast envelope-localized LCIA is required for the expression of HLA3, unknown retrograde signals from the chloroplast to nuclear gene have been suggested to support the CCM (12). This study further revealed a regulatory pathway related to chloroplast-retrograde signaling from the thylakoid- and/or pyrenoid-tubule–localized CAS to nuclear genes because CAS was essential to maintain the expression of 13 genes possibly important for operation of the CCM, including both HLA3 and LCIA. Additionally, the fact that accumulation of CCM1 was not inhibited by the loss of CAS protein (27), and vice versa (Fig. S3O), and that CAS could relocate to pyrenoid tubules in response to LC-light irrespective of CCM1 suggest that CCM1 and CAS could function in parallel to each other in the regulation of CCM, including HCO\(_3\)-transporters (Fig. S5 and SI Results and Discussion).

When chelating external Ca\(^{2+}\) by application of BAPTA, the accumulation of HLA3 and LCIA was decreased, and the addition of Ca\(^{2+}\) restored accumulation (Fig. 2A). Because BAPTA cannot permeate the plasma membrane, it remains in the extracellular space (31) and prevents the elevation of both [Ca\(^{2+}\)]_ext and [Ca\(^{2+}\)]_tub (22), meaning that the elevation of intracellular Ca\(^{2+}\) is required for the accumulation of these two HCO\(_3\)-transporters. In terrestrial plants, the observation that CO\(_2\)-induced changes in [Ca\(^{2+}\)]_ext and stomatal closing are attenuated by chelating or without adding external Ca\(^{2+}\) (19) leads to a hypothesis for the participation of [Ca\(^{2+}\)]_tub in CO\(_2\) signal transduction in guard cells. Considering that CAS also mediates transient elevation of [Ca\(^{2+}\)]_tub and [Ca\(^{2+}\)]_cyt in *Arabidopsis* (21, 22), it is possible that the regulation of these HCO\(_3\)-transporters by CAS could be through Ca\(^{2+}\) signals resulting from the influx of [Ca\(^{2+}\)]_ext into *Chlamydomonas* cells.

Moreover, with the use of a Ca\(^{2+}\) indicator, free Ca\(^{2+}\) could be concentrated in the pyrenoid, especially in LC-light conditions (Fig. 4 and Fig. S44). Because the Ca\(^{2+}\)-binding characteristics of CAS involve low affinity and high capacity (20), the coexistence of CAS and higher concentrations of Ca\(^{2+}\) in the pyrenoid could be important to activate CAS function. A rhodamine-like domain conserved in the C terminus of CAS is thought to exhibit a regulatory function rather than an enzymatic one (18), which might account for the consequent signal transduction followed by binding of Ca\(^{2+}\) at its N terminus. Furthermore, because the chloroplastic Ca\(^{2+}\) has been shown to be important for the chloroplast metabolism and the function of the thylakoid (18), where CAS protein is localized (Fig. S3B), it is possible that the absence of CAS could cause damage on thylakoid or pyrenoid structure in H82 cells in LC conditions. Of note, stronger fluorescent signals derived from a Ca\(^{2+}\) indicator in the pyrenoid in LC-light conditions was also observed in H82 cells (Fig. 4 and Fig. S44), which was not affected by perturbation of intracellular Ca\(^{2+}\) homeostasis (Fig. S4B), suggesting that the change in Ca\(^{2+}\) concentration is not directly regulated by CAS and could act upstream of the Ca\(^{2+}\) signal for regulation of the CCM.

In addition to HLA3 and LCIA, the mRNA abundance and accumulation of LHCSR3 were significantly decreased by the impairment of CAS in LC conditions at 2 h (Table S3 and Fig. S1 I and J), supporting the previous finding that CAS knock-down *Chlamydomonas* strains showed decreased levels of LHCSR3 accumulation (23). Considering the simultaneous upregulation of other nuclear-encoded genes in CAS mutants (24), LHCSR3 was simultaneously decreased by the application of BAPTA or W-7 in LC at 2 h, the expression of these proteins could be regulated by CAS protein or Ca\(^{2+}\) signal in a similar way.

In contrast to CCM1/CAS, CAS is highly conserved in vascular plants, as well as green eukaryotic algae, over the course of evolution. Our findings showed that chloroplast-mediated retrograde signaling pathways via CAS were already developed in the green algae lineage, which could throw light on understanding the cross-talk between Ca\(^{2+}\)- and CO\(_2\)-dependent signal transduction pathways in photosynthetic organisms. Furthermore, considering that the induction of the CCM is dependent on limiting-CO\(_2\) conditions, as well as on light intensity (29), the relationship between CO\(_2\)-limiting stress and high-light stress could be clarified by further analyses of this CAS mutant. The trigger of relocation of CAS into the pyrenoid, as well as Ca\(^{2+}\) in response to changes in the availability of CO\(_2\) and light, could be a key regulatory factor for the CO\(_2\)-sensing mechanism in photosynthetic eukaryotes.

**Materials and Methods**

*C. reinhardtii* strain C9 (mt-) was used as a WT line (6). *CMM1* insertion mutant C16, as well as its parental 5D strain (6), and a *PGLR1* insertion mutant (38) were characterized previously. For the complementation assay, the DNA genomic fragment was amplified using specific primers (Table S4). The genotypes of strains used in this study are listed in Table S5. In physiological assays, cells were cultured in Tris-acetate-phosphate liquid medium for preculture and subcultured in modified high-salt medium (NH\(_4\)) supplemented with 20 mM 3-(N-morpholino)propanesulfonic acid (Mops) to midlog phase (OD\(_{730}\) 0.3 to 0.5) for photoautotrophic growth. For all culture conditions without other conditions specified, cells were cultured at 25 °C with illumination at 120 μmol photons m\(^{-2}\) s\(^{-1}\).

Additional information is described in the SI Materials and Methods.

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