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Kyoto University
Chloroplast-mediated regulation of CO₂-concentrating mechanism by Ca²⁺-binding protein CAS in the green alga *Chlamydomonas reinhardtii*

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Aquatic photosynthetic organisms, including the green alga *Chlamydomonas reinhardtii*, induce a CO₂-concentrating mechanism (CCM) to maintain photosynthetic activity in CO₂-limiting conditions by sensing environmental CO₂ and light availability. Previously, a novel high-CO₂-requiring mutant, H82, defective in the induction of the CCM, was isolated. A homolog of calcium (Ca²⁺)-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 Ca²⁺ 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 the expression levels of nuclear-encoded CO₂-limiting-inducible genes, including the HCO₃⁻ transporters high-light activated 3 (HLA3) and low-CO₂–inducible gene A (LCIA). CAS changed its localization from dispersed across the thylakoid membrane in high-CO₂ conditions or in the dark to being associated with tubule-like structures in the pyrenoid in CO₂-limiting conditions, along with a significant increase of the fluorescent signals of the Ca²⁺ indicator in the pyrenoid. *Chlamydomonas* CAS had Ca²⁺–binding activity, and the perturbation of intracellular Ca²⁺ homeostasis by a Ca²⁺-chelator or calmodulin antagonist impaired the accumulation of HLA3 and LCIA. These results suggest that *Chlamydomonas* CAS is a Ca²⁺-mediated regulator of CCM-related genes via a retrograde signal from the pyrenoid in the chloroplast to the nucleus.

Significance

Ca²⁺ and CO₂ are fundamental biological signaling molecules in microbes, animals, and plants. Although Ca²⁺ was proposed to act as a second messenger in CO₂ signaling in guard cells of terrestrial plants, the role of Ca²⁺ in CO₂ signal transduction pathways in aquatic photosynthetic organisms remains largely unknown. We show here that a chloroplast Ca²⁺-binding protein, CAS, changes its localization in response to environmental CO₂ conditions and regulates the expression of nuclear-encoded limiting-CO₂-induced genes, including two key bicarbonate transporters. These findings led us to propose a model for the participation of Ca²⁺ signals in chloroplast-regulated CO₂ signal transduction pathways in aquatic photosynthetic organisms and help us to further understand the role of Ca²⁺ in CO₂ signal transduction in eukaryotes.


The authors declare no conflict of interest.

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of Arabidopsis thaliana and to regulate plant immune responses and stomatal closure (20-22). In Chlamydomonas, an ortholog of CAS (Cre12.g497300) was also detected in the thylakoid membrane fraction (23, 24). It was proposed that CAS is required for photoacclimation by regulating induction of light-harvesting complex stress-related protein 3 (LHCISR3) (25) and for forming a super complex for photosynthetic cyclic electron flow (CEF) in anaerobic conditions (26). From Chlamydomonas insertion mutant library screening experiments, we previously isolated a mutant strain, H82, in which a hygromycin resistance gene cassette was inserted into CAS (27). H82 cells showed decreased Ci affinity and did not accumulate HLA3 or LCIA in LC conditions.

In this study, we show the suborganellar localization of CAS in the chloroplast in vivo and its Ca2+-binding activity in vitro. Furthermore, using complemented strains of H82, the link between CAS and regulation of the CCM is elucidated by examining the expression patterns of HLA3 and LCIA in response to CO2 and Ca2+ changes. From these results, we propose the existence of chloroplast-mediated regulation of the CCM by Ca2+-binding protein CAS in parallel with regulation by CCM1/CIA5.

Results

Complementation of H82 Mutant Phenotype by CAS Gene. To examine whether disruption of CAS by insertion of a hygromycin resistance gene cassette is responsible for the phenotype of H82, an 8.2-kb PCR-amplified genomic DNA fragment containing the WT CAS gene with its own predicted promoter was cotransformed into H82 cells with a paromomycin resistance gene cassette, aphVIII (Fig. S1A). From 774 paromomycin-resistant transformants, two strains showing similar growth rates to that of WT cells in LC conditions were isolated and designated as C-1 and C-2 (Fig. S1B). In parallel, 12 strains from 92 paromomycin-resistant transformants generated by transforming a plasmid pTY2b-CAS to H82 exhibited recovered growth rate (Fig. S1C) and accumulated CAS (Fig. S1D) in LC conditions. Then, C-1 was used for further analyses. The HC-requiring phenotype of H82 cells was dependent on light intensity (Fig. S1E and SI Results and Discussion), and the addition of excess Ca2+ did not recover the retarded growth rate of the H82 cells (Fig. S1B and SI Results and Discussion).

Because it was reported that CEF activity increases in LC conditions possibly for increased ATP demand for ABC-type bicarbonate transporter HLAS in CCM (12, 28) and that CAS is associated with the CEF complex formation in anaerobic conditions (26), it was possible that CAS-dependent CEF might contribute to CCM. However, CEF activity was not affected by the lack of CAS protein in the LC conditions at 120 μmol photons·m−2·s−1 that we examined (Fig. S1F and SI Results and Discussion). This result coincides with the previous finding that down-regulation of CAS did not impair CEF activity in aerobic conditions (26).

To evaluate induction of the CCM in LC conditions, photosynthetic affinities for Ci were measured by monitoring 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. L4 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 6.5-fold and 19.7-fold lower than that of H82 cells (Fig. S1G 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. 1B 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-LCIA 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. S1H). 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 NH2 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. 1H 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 CA3 mutation were screened by RNA-seq analysis (Table S2). WT, HS2, and C-1 cells were grown in HC or LC conditions for 0.3 and 2 h, and the respective transcription profiles were compared. After exposure to HC and LC conditions for 0.3 h, no gene other than CA3 was affected by the mutation. In contrast, in LC conditions for 2 h, the expression levels of 44 genes in addition to CA3 were significantly different [FDR (false discovery rate) < 0.05] in HS2 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 CA3 mutation, including the following: HL3A; LC1A; DNL31 encoding putative DnaJ-like chaperonin; CAH4 and CAH5 encoding mitochondrial carbonyl 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 Cre26.g756747). Eventually, the transcriptional abundances of these 12 genes, except for gene Cre12.g541550, were decreased by the CA3 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. S1D). 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 HS2 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 LC1A, were transiently induced at the mRNA levels at 0.3 h in HS2 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 LCIB (Fig. S1A), suggesting that CAS could be required for maintaining the mRNA levels of HL3A and LC1A after the initial induction of these genes by CCM1/CA3.

Requirement of Ca2+ for LC-Induced Accumulation of HL3A and LC1A. As in the case of AtCAS, the N terminus of CrCAS also has 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 LC1A in WT cells grown in the presence of 1,2-bis(o-aminophenoxo)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 LC1A was dramatically decreased in the presence of 0.5 mM BAPTA (Fig. 2A) as in the case of LHCSR3 (25, 30), and concomitantly $K_{0.5}$ (Ci) increased twofold (72 ± 9 mM to 144 ± 14 mM) (Fig. 2B and Table S1). Considering that the addition of 0.75 mM CaCl2 rescued the accumulation of HL3A and LC1A 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 LC1A and photosynthetic Ci affinity was examined. In the presence of W-7, concentration-dependent effects of decreased accumulation of HL3A and LC1A were observed, as in the case of LHCSR3 (Fig. S2D) (25, 30), and concomitantly $K_{0.5}$ (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 LC1A.

Fig. 2. The effects of extracellular calcium (Ca2+) chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N,N’-tetraacetic acid (BAPTA) on the accumulation of HL3A, LC1A, LHCSR3, LCIB, and CAS (A) and on inorganic carbon (Ci) affinity in low-CO2 (LC) conditions at pH 7.8 (B). Histone H3 was used as a loading control. High-CO2 (HC)-grown WT cells were centrifuged, resuspended by fresh high-salt medium supplemented with 20 mM 3-(N-morpholino)propanesulfonic acid (HSM) medium in the presence of the indicated chemicals and cultured in LC conditions for 2 h. For the culturing medium without any indications, HSM liquid medium contained 0.068 mM CaCl2. Data in all experiments indicate mean values ± SD from three biological replicates.

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 LCIA (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–2·s–1 (HC-light), fluorescent signals were observed as dispersed speckles 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 also distinctly aggregated and observed as a tubule-like structure in the pyrenoid in LC-light conditions at 12 h (Fig. S3f). The aggregated tubule-like signals were dispersed throughout the chloroplast when transferred to HC-light conditions (Fig. S3 I and J). 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. S3A). 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-dimethyleurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) (Fig. 3B and Fig. S3 F, 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 (CG-1) at room temperature for 30 min. Fluorescence images derived from CG-1 treatments and chlorophyll (CHL) are shown. Each image is placed with the flagella facing upward on the panel. Merged images show the fluorescence image superimposed with its differential interference contrast image. (Scale bars: 5 μm.)

![Figure 3](Image 306x124 to 542x241)

Fig. 3. Subcellular localization of CAS. (A) Localization of CAS in WT and H82 cells was assessed using an indirect immunofluorescence assay with an anti-CAS antibody. Cells grown in high-CO₂ (HC) conditions were shifted to low-CO₂ (LC) conditions for 2 h or 12 h in light, and then the LC-acclimated cells (12 h) were transferred from light to dark for 2 h in the LC condition. (B) The effect of dichlorophenyl-dimethyleurea (DCMU) (10 μM), 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) (10 μM), or cycloheximide (CHX) (10 μg mL⁻¹) on the localization of CAS in WT cells. The cells cultured in each condition used in A were also used for these drug treatments. The HC-grown cells were incubated under LC-light conditions in the presence of DCMU or DBMIB for 2 h. For CHX treatments, HC-grown cells were transferred to LC-light conditions in the presence of CHX for 12 h. The LC-light-acclimated cells were subjected to LC-dark conditions in the presence of CHX for 2 h. The LC-dark-acclimated cells were transferred to LC-light conditions with or without CHX for 2 h. (C) Localization of CAS in a CCM1 insertion mutant, C16, and its parental 5D strain using an indirect immunofluorescence assay. Cells grown in HC conditions were shifted to LC-light conditions for 2 h or 12 h. For all panels, each image is placed with the flagella facing upward on the panel. Merged images show the fluorescence image superimposed with its differential interference contrast image. (Scale bars: 5 μm.)

![Figure 4](Image 12589)

Fig. 4. Calcium Green-1, AM fluorescence in WT and H82 cells. Cells were grown in high-CO₂ (HC) or low-CO₂ (LC) conditions for 12 h in light, or cells in LC-light were transferred to LC-dark conditions for 2 h, and then incubated with Calcium Green-1, AM (CG-1) at room temperature for 30 min. Fluorescence images derived from CG-1 treatments and chlorophyll (CHL) are shown. Each image is placed with the flagella facing upward on the panel. DIC, differential interference contrast image. (Scale bars: 5 μm.)
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 Ci 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. 3A and Fig. S3), 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 along the 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, on the basis of 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\(^{2+}\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{tub}}\) (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\(^{2+}\)]\(_{\text{cyt}}\) and stomatal closing were attenuated by chelating or without adding external Ca\(^{2+}\) (19) leads to a hypothesis for the participation of [Ca\(^{2+}\)]\(_{\text{tub}}\) in CO\(_2\) signal transduction in guard cells. Considering that CAS also mediates transient elevation of [Ca\(^{2+}\)]\(_{\text{tub}}\) and [Ca\(^{2+}\)]\(_{\text{tub}}\) 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\(^{2+}\)]\(_{\text{tub}}\) 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. S4A). 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 chloroplastic 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\(^{2+}\)-indicated in the pyrenoid in LC-light conditions was also observed in H82 cells (Fig. 4 and Fig. S4A), 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 (Fig. 2A and SI Tables 1I and J), supporting the previous finding that CAS knock-down *Chlamydomonas* strains showed decreased levels of LHCSR3 accumulation (31). Considering that simultaneous overexpression of CAS and LCIA in 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). CCM1 insertion mutant C16, as well as its parental 5D strain (6), and a PGRL1 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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