Title: High-resolution suborganellar localization of Ca^{2+}-binding protein CAS, a novel regulator of CO_2-concentrating mechanism

Authors: Takashi Yamano, Chihana Toyokawa, and Hideya Fukuzawa

Affiliation: Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502, Japan

Corresponding author:
Hideya Fukuzawa
Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502, Japan
Phone: +81-75-753-4298
FAX: +81-75-753-9228
E-mail: fukuzawa@lif.kyoto-u.ac.jp

Abstract

Many aquatic algae induce a CO_2-concentrating mechanism (CCM) associated with active inorganic carbon transport to maintain high photosynthetic affinity using dissolved inorganic carbon even in low-CO_2 (LC) conditions. In the green alga *Chlamydomonas reinhardtii*, a Ca^{2+}-binding protein CAS was identified as a novel factor regulating the expression of CCM-related proteins including bicarbonate transporters. Although previous studies revealed that CAS associates with the thylakoid membrane and changes its localization in response to CO_2 and light availability, its detailed localization in the chloroplast has not been examined *in vivo*. In this study, high-resolution fluorescence images of CAS fused with a *Chlamydomonas*-adapted fluorescence protein, Clover, were obtained by using a sensitive hybrid detector and an image deconvolution method. In high-CO_2 (5% v/v) conditions, the fluorescence signals of Clover displayed a mesh-like structure in the chloroplast and part of the signals discontinuously overlapped with chlorophyll autofluorescence. The fluorescence signals gathered inside the pyrenoid as a distinct wheel-like structure at 2 h after transfer to LC-light condition, and then localized to the center of the pyrenoid at 12 h. These results suggest that CAS could move in the chloroplast along the thylakoid membrane in response to lowering CO_2 and gather inside the pyrenoid during the operation of the CCM.

Key words: Bicarbonate transporter, Ca^{2+}-binding protein, *Chlamydomonas*, CO_2-concentrating mechanism, Pyrenoid

Introduction

Photosynthetic organisms can sense and respond to changes of several environmental factors, such as light, CO_2, temperature, and various nutrient availabilities, to optimize and/or maintain their photosynthetic activity. Among these stresses, the shortage of CO_2 supply impacts many physiological aspects of plants, especially photosynthetic efficiency due to the low affinity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) against CO_2. In aquatic environments, CO_2-limiting stress is caused not only by the low catalytic activity of Rubisco but also by the 10,000-fold slower diffusion rate of CO_2 in aquatic conditions than that in atmospheric conditions (Jones 1992). To acclimate to this stress, many aquatic organisms possess a CO_2-concentrating mechanism (CCM), which involves the active transport of inorganic carbon (Ci; CO_2 and HCO_3^-) and enzymatic conversion between CO_2 and HCO_3^- to maintain the Ci pool and concentrate CO_2 in the vicinity of Rubisco (Fukuzawa et al. 2012; Wang et al. 2015).

Molecular aspects of the eukaryotic CCM have been mainly studied using the eukaryotic green alga *Chlamydomonas reinhardtii* as a model. So far, it was reported that high-light activated 3 (HLA3) and low-CO_2 (LC)-inducible protein A (LCIA) are associated with the HCO_3^- transport system, which facilitate HCO_3^- uptake from outside of cells to the chloroplast stroma across physiological barriers such as the plasma membrane and chloroplast envelope (Gao et al. 2015; Yamano et al. 2015). HLA3 belongs to a multidrug-resistance-related protein subfamily of the ATP-binding cassette transporter superfamily (Im and Grossman 2001) and localizes to the plasma membrane (Yamano et al. 2015). LCIA belongs to a formate-nitrite transporter family (Mariscal et al. 2006), in which proteins form a pentameric aquaporin-like channel rather than an active transporter (Wang et al. 2009), and localizes to the chloroplast envelope (Wang and Spalding 2014; Yamano et al. 2015).

Because simultaneous knockdown or knockout of *HLA3* and *LCIA* causes a dramatic decrease in photosynthetic Ci uptake, and simultaneous overexpression of these genes raised photosynthetic Ci affinity and internal Ci accumulation, HLA3 and LCIA are cooperatively associated with HCO_3^- transport to increase the Ci pool in the chloroplast stroma (Duanmu et al. 2009; Wang and Spalding 2014; Gao et al. 2015; Yamano et al. 2015). These LC-inducible proteins are regulated by zinc-containing regulatory protein CCM1/CIA5 (Fukuzawa et al. 2001; Xiang et al. 2001; Miura et al. 2004). Recently, we isolated a novel high-CO_2 (HC)-requiring mutant H82 (Wang et al. 2014) and revealed that a Ca^{2+}-binding protein, CAS, is also essential for the operation of the CCM by regulating the expression of *HLA3* and *LCIA* (Wang et al. 2016). CAS was initially identified in *Arabidopsis thaliana*

(Han et al. 2003) and was detected in the thylakoid membrane fraction (Nomura et al. 2008). By proteomic analysis and indirect immunofluorescence assays, *Chlamydomonas* CAS was also localized to the thylakoid membrane and especially inside the pyrenoid, which is a prominent structure in the chloroplast of the cells cultured in LC conditions (Wang et al. 2016). In many algae, the pyrenoid develops as a spherical proteinaceous structure surrounded with starch sheathes in the chloroplast. Some of the thylakoid membrane penetrates into the pyrenoid, termed pyrenoid tubules (Ohad et al. 1967), and multiple parallel minitubules are bundled within the pyrenoid tubule (Engel et al. 2015). Recently, *Chlamydomonas* CAS was also detected in the protein fraction of purified eyespot (Trippens et al. 2017). Considering that *Arabidopsis* CAS regulates nuclear-encoded genes related to plant immune responses (Nomura et al. 2012) and that *Chlamydomonas* CAS also regulates nuclear-encoded genes related to the CCM such as *HLA3* and *LCIA* (Wang et al. 2016), CAS-mediated retrograde signaling systems from the chloroplast to the nucleus appear to be conserved during the evolution of the plant lineage. However, the actual function of CAS and its detailed subcellular localization *in vivo* remain to be determined.

In this study, by combination of sensitive hybrid detector system, optimization of imaging parameters, and image deconvolution technique, we revealed distinct localization patterns of CAS in HC and LC conditions at high resolution. This result could help in understanding the function of CAS associated with the retrograde signal regulating stress-responsive genes.

Materials and Methods

Cell culture and growth conditions

Chlamydomonas reinhardtii strain C-9 (photosynthetically WT strain originally provided by the IAM Culture Collection held at Tokyo University, and available from the *Chlamydomonas* Resource Center as strain CC-5098), and transgenic lines were cultured in Tris-acetate-phosphate (TAP) medium for maintenance. For physiological and biochemical experiments, a 5 mL volume of cells were grown in liquid TAP medium for pre-cultivation, and diluted with modified high-salt medium supplemented with 20 mM 3-(N-Morpholino)propanesulfonic acid (HSM) to an OD_{730} of ~0.05. Then, the cells were grown under HC (5% v/v) conditions at 120 μmol photons m^{-2} s^{-1} until midlog phase with OD_{730} of 0.3 to 0.5. For LC induction, HC-acclimated cells were centrifuged at 600 × g, and pellets were resuspended in 50 mL of fresh HSM medium, and cultured in LC (0.04% v/v) conditions at 120 μmol photons m^{-2} s^{-1} for indicated time periods.

Plasmid construction and transformation

The genomic sequence of *CAS* was amplified by PCR with PrimeSTAR GXL (Takara Bio) using genomic DNA extracted from strain C-9 as a template with forward primer TP-clover-F (5'-TTTGCAGGATGCATATGCAGCTTGCTAACGCTCCT-3') and reverse primer gCAS-clover-R (5'-CGATGACGTCAGATCTCGAGCGGGGGGCGGGCAG-3'). The PCR products were purified and cloned into pOptimized Clover vector (Lauersen et al. 2015) digesting with *Nde*I and *Bgl*II using a SLiCE cloning method (Motohashi 2015). For the introduction of a flexible amino acid linker between CAS and Clover, two synthetic oligo nucleotides, gCAS_clover_linker-F (5'-CCCCCGCTCGAGATCTGGCGGCGCGGCCGCGGGCAGATCTGACGTCATCG-3') and gCAS_clover_linker-R (5'-CGATGACGTCAGATCTGCCCGCGGCCGCGCCGCCAGATCTCGAGCGGGGG-3') was annealed and then cloned into the above plasmid digesting with *Bgl*II using a SLiCE cloning method (18-bp nucleotide sequences encoding flexible linker are shown by underlines). This expression plasmid of CAS-Clover was transformed into the H82 mutant (Wang et al. 2014) by electroporation using a NEPA-21 electroporator (NEPAGENE), as described previously (Yamano et al. 2013). The transformants were incubated at 25°C for 24 h with gentle shaking and illumination of less than 1.5 μmol photons m^{-2} s^{-1} and spread over TAP plates containing 30 μg mL^{-1} hygromycin.

Immunoblotting analyses

Extracted total proteins suspended in SDS loading buffer containing 50 mM Tris HCl (pH 8.0), 25% (vol/vol) glycerol, 2% (wt/vol) SDS, and 0.1 M DTT were incubated at 37°C for 30 min and subsequently centrifuged at 13,000 × g for 5 min. The supernatant was loaded onto an SDS-polyacrylamide gel electrophoresis (SDS/PAGE) gel for the separation of proteins. Next, proteins were transferred to polyvinylidene fluoride (Pall Life Science) membranes using a semidry blotting system. Membranes were blocked with 5% (wt/vol) skim milk powder (Wako) in phosphate-buffered saline (PBS). Blocked membranes were washed with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T) and treated with anti-CAS (1:5,000 dilution) or anti-Histone H3 (1:20,000 dilution) antibodies. To recognize the primary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Life Technologies) was used as a secondary antibody in a dilution of 1:10,000. After washing with PBS-T, immunoreactive signals were detected using Luminata Crescendo Western HRP

substrate (Merck Millipore) and images were obtained using ImageQuant LAS-4010 (GE Healthcare).

Photosynthetic oxygen evolution

For evaluating the affinity for Ci, the rate of dissolved Ci-dependent photosynthetic O_2 evolution was measured. Cells harvested after growth in HC and LC conditions were suspended in Ci-depleted Hepes-NaOH buffer (pH 7.8) at 10 μg mL^{-1} chlorophyll. Photosynthetic O_2 evolution was measured by applying a Clark-type O_2 electrode (Hansatech Instruments), as described preciously (Yamano et al. 2008).

Capture of high-resolution fluorescence images

To reduce *Chlamydomonas* cell movement, 2.5 μL cells were placed between a coverslip and a thin agarose pad (Skinner et al. 2013), and then 16-bit digital fluorescence images were acquired with oil immersion objective lens (HC PL APO 63×/1.40; Leica) using an inverted laser-scanning confocal fluorescence microscope TCS SP8 (Leica) equipped with a sensitive hybrid detector (HyD). CAS-Clover was excited at 488 nm and emission was detected at 500–520 nm. Image scanning was performed with pinhole size of 0.6 Airy units, with z-stack distance of the scan at 150 nm, at a pixel size of 25 nm, and with a line scan speed of 200 Hz. Huygens Essential software (Scientific Volume Imaging B.V.) was used for data processing. Deconvolution of confocal datasets was performed using the point-spread function (PSF) theoretically calculated from the microscopic parameters attached to the data and classic maximum likelihood estimation (CMLE) algorithm (settings: maximum iterations: 100; signal-to-noise: 20; quality criterion: 0.05).

Results

Isolation of transgenic lines expressing CAS-Clover

To examine the subcellular localization of CAS *in vivo*, we generated transgenic lines expressing CAS fused with Clover (CAS-Clover), a *Chlamydomonas*-adapted modified green fluorescence protein (Lauersen et al. 2015). We modified the expression plasmid of CAS-Clover used previously (Wang et al. 2016) by introducing a flexible amino acid linker (Gly-Gly-Ala-Ala-Ala-Gly) between CAS and Clover to minimize interference by the protein fusion (Fig. 1a). This plasmid was used to transform the H82 mutant, from which 960 transformants showing paromomycin resistance were obtained, and nine transformants designated as CL-1–CL-9 showing fluorescence signals derived from CAS-Clover inside the

pyrenoid were screened. By immunoblotting analysis using an anti-CAS antibody, a band of approximately 63 kDa corresponding to the predicted size of the CAS-Clover fusion protein was detected (Fig. 1b). Among these transformants, strain CL-2 showed the strongest fluorescence signal and was selected for further analyses. The values of maximum O_2-evolving activity (V_{max}) and $K_{0.5}$ (Ci), the Ci concentration required for half V_{max}, of CL-2 were similar to those of wild-type (WT) cells (Fig. 1c), indicating that decreased photosynthetic Ci-affinity of H82 was complemented by expressing the CAS-Clover.

High-resolution suborganellar localization of CAS-Clover *in vivo*

High-resolution fluorescence images of the CL-2 cells expressing CAS-Clover were obtained using the combination of a sensitive hybrid detector and an image deconvolution technique. In HC conditions, the fluorescence signals were distributed across the entire chloroplast and several punctuate spots were also observed (Fig. 2a). By defocusing of confocal images, fluorescence signals displayed a mesh-like structure, and part of the signals discontinuously overlapped with chlorophyll autofluorescence (Fig. 2b). Considering that CAS was detected in the fraction enriched with the thylakoid membrane (Wang et al. 2016), CAS could be not uniformly but discontinuously distributed on the thylakoid membrane in HC conditions.

Next, when the cells were shifted from HC to LC conditions, the fluorescence signals were detected inside the pyrenoid as a distinct wheel-like structure at 2 h (Fig. 3a–c). When we shifted the focus along the z-axis direction, a strong fluorescent spot was also observed in the lateral region of the chloroplast, which overlapped with the region of eyespot observed in a differential interference contrast image (Fig. 3a). Although the autofluorescence signals of the eyespot were detected in the WT cells, their signal intensities were significantly weaker than that of CL-2 cells with the same microscopic conditions (Fig. 3b), indicating that the fluorescence signals of the eyespot region in CL-2 cells were mostly derived from CAS-Clover. By defocusing of confocal images in the pyrenoid region, the wheel-like structure consisting of several fibers were clearly observed (Fig. 3c). Inside the developed pyrenoid, chlorophyll autofluorescence were hardly detected (Fig. 3a), which was consistent with a previous report (Uniacke and Zerges 2007). This is possibly because the mean diameter of the pyrenoid tubule is very thin at 107 ± 26 nm (Engel et al. 2015), or the amount of chlorophyll could be much decreased in the pyrenoid tubules. By enhancing the contrast of fluorescence, thin fibers were observed, which could be derived from the structure of the pyrenoid tubules (Fig. 3e). In LC conditions after 12 h, the wheel-like structure had almost disappeared, and CAS-Clover was localized to the center of the pyrenoid (Fig. 3f).

Considering that part of the thylakoid membrane, termed the pyrenoid tubules, penetrates into the pyrenoid and fuses at the center of the pyrenoid, forming a knotted core (Engel et al. 2015; Meyer et al. 2016) and that relocation of CAS was not associated with *de novo* protein synthesis (Wang et al. 2016), dispersed CAS-Clover in the chloroplast in HC conditions could move and gather into the pyrenoid along the thylakoid membranes during CCM induction.

Discussion

In this study, we determined suborganellar localization of CAS based on fluorescence images of functional CAS-Clover *in vivo* at high resolution. CAS showed distinct different localization patterns between HC and LC conditions. Dispersed localization of CAS-Clover in HC conditions changed to a wheel-like structure in LC conditions at 2 h and aggregated inside the pyrenoid at 12 h. In particular, this wheel-like localization of CAS-Clover was clearly observed for the first time in this study, strengthening the hypothesis that CAS gathers inside the pyrenoid along the pyrenoid tubules during the operation of the CCM (Wang et al. 2016). Although the relocation of CAS in the chloroplast and its importance for regulation of the CCM has been proposed, it remains unclear how CAS moves along thylakoid membranes.

One possible mechanism is posttranslational modification. Other CCM-related proteins, such as LCIB and CAH3, also change their localization in response to CO_2 availability and undergo phosphorylation when CO_2 availability is limiting (Blanco-Rivero et al. 2012; Yamano et al. 2010). LCIB is an indispensable factor in the CCM and is observed as dispersed speckles in the chloroplast in HC conditions, but changes its localization as a ring-like structure in the vicinity of the pyrenoid in the LC-adapted cells (Yamano et al. 2010), which is distinctly different from the CAS localization pattern. Because *de novo* protein synthesis inhibits the relocation of LCIB (Yamano et al. 2014), but does not affect that of CAS (Wang et al. 2016), the regulatory mechanism of relocation could be different between these proteins. An α-type carbonic anhydrase, CAH3, is shown to be associated with dehydration of HCO_3^- to CO_2 within the lumen of pyrenoid tubules (Karlsson et al. 1998). Although CAH3 is associated with the donor side of PSII in the stroma of thylakoid membranes in HC conditions, CAH3 is partly concentrated in the pyrenoid tubules, which does not contain PSII, to provide CO_2 to Rubisco in LC conditions (Blanco-Rivero et al. 2012). Moreover, LCI5/EPYC1 was the first reported protein phosphorylated in response to CO_2-limiting conditions (Turkina et al. 2006). LCI5/EPYC1 is colocalized with Rubisco in

the pyrenoid matrix and assists in the formation of the pyrenoid and the packing of Rubisco in the pyrenoid in LC conditions by linking with Rubisco (Mackinder et al. 2016). In *Arabidopsis*, it is reported that a light-dependent thylakoid protein kinase STN8 phosphorylates a stroma-exposed Thr380 residue of CAS (flanking sequence is SGTKFLP and phosphorylated Threonine is underlined; Vainonen et al. 2008), which is also conserved as Thr370 (flanking sequence is TSTRRLP and putative phosphorylated Threonine is underlined) in *Chlamydomonas* CAS. Based on these results, phosphorylation could be an important factor to regulate the relocation and/or function of CCM-related proteins. Identifying kinases, phosphorylation sites, and obtaining high-resolution images of these proteins could lead to a better understanding of the regulatory mechanism of suborganellar protein relocation.

Another possible mechanism is the structural dynamics of thylakoid membranes. CAS has a hydrophobic sequence that separates the protein sequence into an N-terminus with a Ca^{2+}-binding region and a C-terminus with a rhodanese-like domain, and it is thought that CAS anchors to the thylakoid membrane via the hydrophobic sequence (Wang et al. 2016). A recent study revealed that both the structural stability and flexibility of thylakoid membranes is essential for dynamic protein reorganization (Iwai et al. 2014). It is possible that CAS also moves along with the membrane dynamics, although directional movement of the thylakoid membrane from dispersed chloroplast region into the pyrenoid and *vice versa* is unknown.

Recently, CAS was detected in a purified fraction of the *Chlamydomonas* eyespot and also involved in regulating the positive phototactic response under continuous illumination (Trippens et al. 2017). Consistent with this result, we first observed that the fluorescence signal of CAS-Clover overlapped with the eyespot *in vivo*. Ca^{2+} influx through the channel rhodopsins in the eyespot region play an important role for the regulation of phototactic behavior, but the primary Ca^{2+} sensing mechanism is unknown. Using our knockout mutant H82, the regulatory roles of CAS associated with the positive phototactic response could be more clarified.

It has become clear that the pyrenoid is important not only for CO_2 fixation but also for the regulation of the CCM (Meyer et al. 2017; Mitchell et al. 2017). So far, hundreds of proteins with unknown function have been identified in the purified pyrenoid (Mackinder et al. 2016), and there could be other CCM-related proteins that could relocate in the chloroplast in response to the CO_2 availability as reported previously (Yamano et al. 2010). Further screening of mutants showing aberrant localization patterns of these proteins could lead to understanding the regulatory mechanism of suborganellar relocation in response to

environmental stresses (Yamano et al. 2014). Obtaining high-resolution images described in this study could be useful for observing the suborganellar localization of proteins, especially for ones localized in small compartments such as the pyrenoid in a single cell.

Acknowledgments

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grants 16H04805 (to H.F.) and 16K07399 (to T.Y.) and the Japan Science and Technology Agency Advanced Low Carbon Technology Research and Development Program (to H.F.).

References

Blanco-Rivero A, Shutova T, Roman MJ, Villarejo A, Martinez F (2012) Phosphorylation controls the localization and activation of the luminal carbonic anhydrase in *Chlamydomonas reinhardtii*. PLoS One 7:e49063 doi:10.1371/journal.pone.0049063

Duanmu D, Miller AR, Horken KM, Weeks DP, Spalding MH (2009) Knockdown of limiting-CO_2-induced gene HLA3 decreases HCO_3^- transport and photosynthetic Ci affinity in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 106:5990–5995

Engel BD, Schaffer M, Cuellar LK, Villa E, Plitzko JM, Baumeister W (2015) Native architecture of the *Chlamydomonas* chloroplast revealed by *in situ* cryo-electron tomography. eLife 4:e04889 doi:10.7554/eLife.04889

Fukuzawa H, Miura K, Ishizaki K, Kucho K, Saito T, Kohinata T, Ohyama K (2001) *Ccm1*, a regulatory gene controlling the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2 availability. Proc Natl Acad Sci USA 98:5347–5352

Fukuzawa, H, Ogawa T, Kaplan A (2012) The uptake of CO_2 by cyanobacteria and microalgae. In: Eaton-Rye JJ, Tripathy BC, Sharkey TD (eds) Photosynthesis "plastid biology, energy conversion and carbon assimilation." Springer, Advances in Photosynthesis and Respiration 34:625–650

Gao H, Wang Y, Fei X, Wright DA, Spalding MH (2015) Expression activation and functional analysis of HLA3, a putative inorganic carbon transporter in *Chlamydomonas reinhardtii*. Plant J 82:1–11

Han S, Tang R, Anderson LK, Woerner TE, Pei ZM. (2003) A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells. Nature 425:196–200

Im CS, Grossman AR (2002) Identification and regulation of high light-induced genes in *Chlamydomonas reinhardtii*. Plant J 30:301-313

Iwai M, Yokono M, Nakano A (2014) Visualizing structural dynamics of thylakoid membranes. Sci Rep 4:3768 doi:10.1038/srep03768

Jones HG (1992) Plants and microclimate: A quantitative approach to environmental plant physiology, 2nd ed. Cambridge University Press: Cambridge, UK.

Karlsson J, Clarke AK, Chen ZY et al (1998) A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. EMBO J 17:1208-1216

Lauersen KJ, Kruse O, Mussgnug JH (2015) Targeted expression of nuclear transgenes in *Chlamydomonas reinhardtii* with a versatile, modular vector toolkit. Appl Microbiol Biotechnol 99:3491–3503

Mackinder LCM, Meyer M, Mettler-Altmann T et al (2016) A repeat protein links Rubisco to form the eukaryotic carbon-concentrating organelle. Proc Natl Acad Sci USA 113:5958–5963

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

Meyer MT, McCormick AJ, Griffiths H (2016) Will an algal CO_2-concentrating mechanism work in higher plants? Curr Opin Plant Biol 31:181–188

Meyer MT, Whittaker C, Griffiths H (2017) The algal pyrenoid: key unanswered questions. J Exp Bot 68:3739-3749

Mitchell MC, Metodieva G, Metodiev MV, Griffiths H, Meyer MT (2017) Pyrenoid loss impairs carbon-concentrating mechanism induction and alters primary metabolism in *Chlamydomonas reinhardtii*. J Exp Bot 68:3891-3902

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

Motohashi K (2015) A simple and efficient seamless DNA cloning method using SLiCE from *Escherichia coli* laboratory strains and its application to SLiP site-directed mutagenesis. BMC Biotechnol 15:47 doi:10.1186/s12896-015-0162-8

Nomura H, Komori T, Kobori M, Nakahira Y, Shiina T (2008) Evidence for chloroplast control of external Ca^{2+}-induced cytosolic Ca^{2+} transients and stomata closure. Plant J 53:988–998

Nomura H, Komori T, Uemura S et al (2012) Chloroplast-mediated activation of plant immune signaling in *Arabidopsis*. Nat Commun 3:926 doi:10.1038/ncomms1926

Ohad I, Siekevitz P, Palade GE (1967) Biogenesis of chloroplast membranes. I. Plastid dedifferentiation in a dark-grown algal mutant (*Chlamydomonas reinhardtii*). J Cell Biol 35:521–552

Skinner SO, Sepúlveda LA, Xu H, Golding I (2013) Measuring mRNA copy number in individual *Escherichia coli* cells using single-molecule fluorescent *in situ* hybridization. Nat Protoc 8:1100-1113

Trippens J, Reißenweber T, Kreimer G (2017) The chloroplast calcium sensor protein CAS affects phototactic behaviour in *Chlamydomonas reinhardtii* (Chlorophyceae) at low light intensities. Phycologia 56:261–270

Turkina MV, Blanco-Rivero A, Vainonen JP, Vener AV, Villarejo A (2006) CO_2 limitation induces specific redox-dependent protein phosphorylation in *Chlamydomonas reinhardtii*. Proteomics 6:2693–2704

Uniacke J and Zerges W (2007) Photosystem II assembly and repair are differentially localized in *Chlamydomonas*. Plant Cell 19:3640–3654

Vainonen JP, Sakuragi Y, Stael S et al (2008) Light regulation of CaS, a novel phosphoprotein in the thylakoid membrane of *Arabidopsis thaliana*. FEBS J 275:1767–1777

Wang L, Yamano T, Kajikawa M, Hirono M, Fukuzawa H (2014) Isolation and characterization of novel high-CO_2-requiring mutants of *Chlamydomonas reinhardtii*. Photosynth Res 121:175–184

Wang L, Yamano T, Takane S et al (2016) Chloroplast-mediated regulation of CO_2-concentrating mechanism by Ca^{2+}-binding protein CAS in the green alga *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 113:12586–12591

Wang Y, Huang Y, Wang J et al (2009) Structure of the formate transporter FocA reveals a pentameric aquaporin-like channel. Nature 462:467–472

Wang Y, Spalding MH (2014) Acclimation to very low CO_2: Contribution of limiting CO_2 inducible proteins, LCIB and LCIA, to inorganic carbon uptake in *Chlamydomonas reinhardtii*. Plant Physiol 166:2040–2050

Wang Y, Stessman DJ, Spalding MH (2015) The CO_2 concentrating mechanism and photosynthetic carbon assimilation in limiting CO_2: how Chlamydomonas works against the gradient. Plant J 82:429–448

Xiang Y, Zhang J, Weeks DP (2001) The *Cia5* gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA 98:5341–5346

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

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

Yamano T, Iguchi H, Fukuzawa H (2013) Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal. J Biosci Bioeng 115:691–694

Yamano T, Asada A, Sato E, Fukuzawa H (2014) Isolation and characterization of mutants defective in the localization of LCIB, an essential factor for the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Photosynth Res 121:193–200

Yamano T, Sato E, Iguchi H, Fukuda Y, Fukuzawa H (2015) Characterization of the cooperative bicarbonate uptake into chloroplast stroma in the green alga *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA 112:7315–7320

Figure legends

Fig. 1 Isolation of transgenic lines expressing CAS-Clover. (a) A schematic illustration of the expression plasmid of CAS-Clover. The plasmid was constructed based on the pOptimized Clover vector (Lauersen et al. 2015). Translation start (ATG) and stop (TAA) sites are shown. The genomic sequence of *CAS* is placed at the downstream of $P_{A/R}$, *HSP70A*/*RBCS2* tandem promoter, which is followed by first intron of *RBCS2*. The nucleotide acid sequence GGCGGCGCGGCCGCGGGC encoding the amino acid sequence Gly-Gly-Ala-Ala-Ala-Gly represents a synthetic flexible linker between CAS and Clover. The expression of CAS-Clover is terminated by the T_{RBCS2}, 3'-untranslated region of *RBCS2*. Restriction enzyme sites for cloning of *CAS* (*Nde*I and *Bgl*II) and for insertion check of the flexible linker (*Not*I) are shown. (b) Accumulation of CAS and CAS-Clover fusion protein in wild-type (WT), H82, and transformants (CL strains). Cells were grown in low-CO_2 (LC) conditions for 12 h. Histone H3 was used as a loading control. (c) Maximum photosynthetic O_2-evolving activity (V_{max}; left) and inorganic carbon (Ci) affinity (right) of WT, H82, and CL-2 cells grown in LC conditions for 12 h. Photosynthetic O_2-evolving activity was measured in externally dissolved Ci concentrations at pH 7.8, and the $K_{0.5}$ (Ci), the Ci concentrations required for half V_{max}, were calculated. Data in all experiments are mean values ± standard deviation from three biological replicates. **P*<0.001 by Student's t test.

Fig. 2 Fluorescence signals derived from CAS-Clover in high-CO_2 (HC) conditions. (a) CL-2 cells were adapted to HC conditions. Defocused images +1.0 μm from the focal plane are shown in the bottom row. Each image is placed with the flagella facing upward on the panel. DIC, differential interference contrast image. Scale bar, 2 μm. (b) Enlarged fluorescence images of the white boxed area in (a) obtained by defocusing the sample from –0.6 to +0.8 μm from the focal plane. Scale bar, 400 nm.

Fig. 3 Fluorescence signals derived from CAS-Clover in low-CO_2 (LC) conditions. (a) CL-2 cells grown in high-CO_2 conditions were transferred to LC conditions for 2 h. Defocused images +1.0 μm from the focal plane are shown in the bottom row. Each image is placed with the flagella facing upward on the panel. White arrowheads indicate the eyespot region. DIC, differential interference contrast image. Scale bar, 2 μm. (b) Autofluorescence image of wild-type (WT) cells grown in LC conditions for 2 h. White arrowheads indicate the eyespot region. Scale bar, 2 μm. (c) Enlarged fluorescence images of the pyrenoid region by defocusing the sample from –0.6 to +0.8 μm from the focal plane. Scale bar, 400 nm. (d)

Different images of CAS-Clover merged with chlorophyll grown in LC conditions for 2 h. (e) High-contrast image of chlorophyll autofluorescence in the pyrenoid region. Scale bar, 400 nm. (f) Fluorescence images derived from CAS-Clover in LC conditions for 12 h. Scale bar, 2 μm.

Title: High-resolution suborganellar localization of Ca^{2+}-binding protein CAS, a novel regulator of CO_2-concentrating mechanism

Authors: Takashi Yamano, Chihana Toyokawa, and Hideya Fukuzawa

Affiliation: Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502, Japan

Corresponding author:
Hideya Fukuzawa
Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502, Japan
Phone: +81-75-753-4298
FAX: +81-75-753-9228
E-mail: fukuzawa@lif.kyoto-u.ac.jp

Abstract

Many aquatic algae induce a CO_2-concentrating mechanism (CCM) associated with active inorganic carbon transport to maintain high photosynthetic affinity using dissolved inorganic carbon even in low-CO_2 (LC) conditions. In the green alga *Chlamydomonas reinhardtii*, a Ca^{2+}-binding protein CAS was identified as a novel factor regulating the expression of CCM-related proteins including bicarbonate transporters. Although previous studies revealed that CAS associates with the thylakoid membrane and changes its localization in response to CO_2 and light availability, its detailed localization in the chloroplast has not been examined *in vivo*. In this study, high-resolution fluorescence images of CAS fused with a *Chlamydomonas*-adapted fluorescence protein, Clover, were obtained by using a sensitive hybrid detector and an image deconvolution method. In high-CO_2 (5% v/v) conditions, the fluorescence signals of Clover displayed a mesh-like structure in the chloroplast and part of the signals discontinuously overlapped with chlorophyll autofluorescence. The fluorescence signals gathered inside the pyrenoid as a distinct wheel-like structure at 2 h after transfer to LC-light condition, and then localized to the center of the pyrenoid at 12 h. These results suggest that CAS could move in the chloroplast along the thylakoid membrane in response to lowering CO_2 and gather inside the pyrenoid ~~along the pyrenoid tubules, penetrated thylakoid membrane into pyrenoid,~~ during the operation of the CCM.

Key words: Bicarbonate transporter, Ca^{2+}-binding protein, *Chlamydomonas*, CO_2-concentrating mechanism, Pyrenoid

Introduction

Photosynthetic organisms can sense and respond to changes of several environmental factors, such as light, CO_2, temperature, and various nutrient availabilities, to optimize and/or maintain their photosynthetic activity. Among these stresses, the shortage of CO_2 supply impacts many physiological aspects of plants, especially photosynthetic efficiency due to the low affinity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) against CO_2. In aquatic environments, CO_2-limiting stress is caused not only by the low catalytic activity of Rubisco but also by the 10,000-fold slower diffusion rate of CO_2 in aquatic conditions than that in atmospheric conditions (Jones 1992). To acclimate to this stress, many aquatic organisms possess a CO_2-concentrating mechanism (CCM), which involves the active transport of inorganic carbon (Ci; CO_2 and HCO_3^-) and enzymatic conversion between CO_2 and HCO_3^- to maintain the Ci pool and concentrate CO_2 in the vicinity of Rubisco (Fukuzawa et al. 2012; Wang et al. 2015).

Molecular aspects of the eukaryotic CCM have been mainly studied using the eukaryotic green alga *Chlamydomonas reinhardtii* as a model. So far, it was reported that high-light activated 3 (HLA3) and low-CO_2 (LC)-inducible protein A (LCIA) are associated with the HCO_3^- transport system, which facilitate HCO_3^- uptake from outside of cells to the chloroplast stroma across physiological barriers such as the plasma membrane and chloroplast envelope (Gao et al. 2015; Yamano et al. 2015). HLA3 belongs to a multidrug-resistance-related protein subfamily of the ATP-binding cassette transporter superfamily (Im and Grossman 2001) and localizes to the plasma membrane (Yamano et al. 2015). LCIA belongs to a formate-nitrite transporter family (Mariscal et al. 2006), in which proteins form a pentameric aquaporin-like channel rather than an active transporter (Wang et al. 2009), and localizes to the chloroplast envelope (Wang and Spalding 2014; Yamano et al. 2015).

Because simultaneous knockdown or knockout of *HLA3* and *LCIA* causes a dramatic decrease in photosynthetic Ci uptake, and simultaneous overexpression of these genes raised photosynthetic Ci affinity and internal Ci accumulation, HLA3 and LCIA are cooperatively associated with HCO_3^- transport to increase the Ci pool in the chloroplast stroma (Duanmu et al. 2009; Wang and Spalding 2014; Gao et al. 2015; Yamano et al. 2015). These LC-inducible proteins are regulated by zinc-containing regulatory protein CCM1/CIA5 (Fukuzawa et al. 2001; Xiang et al. 2001; Miura et al. 2004). Recently, we isolated a novel high-CO_2 (HC)-requiring mutant H82 (Wang et al. 2014) and revealed that a Ca^{2+}-binding protein, CAS, is also essential for the operation of the CCM by regulating the expression of *HLA3* and *LCIA* (Wang et al. 2016). CAS was initially identified in *Arabidopsis thaliana*

(Han et al. 2003) and was detected in the thylakoid membrane fraction (Nomura et al. 2008). By proteomic analysis and indirect immunofluorescence assays, *Chlamydomonas* CAS was also localized to the thylakoid membrane and especially inside the pyrenoid, which is a prominent structure in the chloroplast of the cells cultured in LC conditions (Wang et al. 2016). In many algae, the pyrenoid develops as a spherical proteinaceous structure surrounded with starch sheathes in the chloroplast. Some of the thylakoid membrane penetrates into the pyrenoid, termed pyrenoid tubules (Ohad et al. 1967), and multiple parallel minitubules are bundled within the pyrenoid tubule (Engel et al. 2015). Recently, *Chlamydomonas* CAS was also detected in the protein fraction of purified eyespot (Trippens et al. 2017). Considering that *Arabidopsis* CAS regulates nuclear-encoded genes related to plant immune responses (Nomura et al. 2012) and that *Chlamydomonas* CAS also regulates nuclear-encoded genes related to the CCM such as *HLA3* and *LCIA* (Wang et al. 2016), CAS-mediated retrograde signaling systems from the chloroplast to the nucleus appear to be conserved during the evolution of the plant lineage. However, the actual function of CAS and its detailed subcellular localization *in vivo* remain to be determined.

In this study, by combination of sensitive hybrid detector system, optimization of imaging parameters, and image deconvolution technique, we revealed distinct localization patterns of CAS in HC and LC conditions at high resolution. This result could help in understanding the function of CAS associated with the retrograde signal regulating stress-responsive genes.

Materials and Methods

Cell culture and growth conditions

Chlamydomonas reinhardtii strain C-9 (photosynthetically WT strain originally provided by the IAM Culture Collection held at Tokyo University, and available from the *Chlamydomonas* Resource Center as strain CC-5098), and transgenic lines were cultured in Tris-acetate-phosphate (TAP) medium for maintenance. For physiological and biochemical experiments, a 5 mL volume of cells were grown in liquid TAP medium for pre-cultivation, and diluted with modified high-salt medium supplemented with 20 mM 3-(N-Morpholino)propanesulfonic acid (pH 7.0) to an OD_{730} of ~0.05 ~~for photoautotrophic growth~~. Then, the cells were grown under HC (5% v/v) conditions at 120 µmol photons m^{-2} s^{-1} until midlog phase with OD_{730} of 0.3 to 0.5. For LC induction, HC-acclimated cells were centrifuged at 600 × g, and pellets were resuspended in 50 mL of fresh HSM medium, and cultured in LC (0.04% v/v) conditions at 120 µmol photons m^{-2} s^{-1} for indicated time

periods.~~For all culture conditions, cells were cultured at 25°C with illumination at 120 μmol photons m^{-2} s^{-1}.~~

Plasmid construction and transformation

The genomic sequence of *CAS* was amplified by PCR with PrimeSTAR GXL (Takara Bio) using genomic DNA extracted from strain C-9 as a template with forward primer TP-clover-F (5'-TTTGCAGGATGCATATGCAGCTTGCTAACGCTCCT-3') and reverse primer gCAS-clover-R (5'-CGATGACGTCAGATCTCGAGCGGGGGGCGGGCAG-3'). The PCR products were purified and cloned into pOptimized Clover vector (Lauersen et al. 2015) digesting with *Nde*I and *Bgl*II using a SLiCE cloning method (Motohashi 2015). For the introduction of a flexible amino acid linker between CAS and Clover, two synthetic oligo nucleotides, gCAS_clover_linker-F (5'-CCCCCGCTCGAGATCTGGCGGCGCGGCCGCGGGCAGATCTGACGTCATCG-3') and gCAS_clover_linker-R (5'-CGATGACGTCAGATCTGCCCGCGGCCGCGCCGCCAGATCTCGAGCGGGGG-3') was annealed and then cloned into the above plasmid digesting with *Bgl*II using a SLiCE cloning method (18-bp nucleotide sequences encoding flexible linker are shown by underlines). This expression plasmid of CAS-Clover was transformed into the H82 mutant (Wang et al. 2014) by electroporation using a NEPA-21 electroporator (NEPAGENE), as described previously (Yamano et al. 2013). The transformants were incubated at 25°C for 24 h with gentle shaking and illumination of less than 1.5 μmol photons m^{-2} s^{-1} and spread over TAP plates containing 30 μg mL^{-1} hygromycin.

Immunoblotting analyses

Extracted total proteins suspended in SDS loading buffer containing 50 mM Tris HCl (pH 8.0), 25% (vol/vol) glycerol, 2% (wt/vol) SDS, and 0.1 M DTT were incubated at 37°C for 30 min and subsequently centrifuged at 13,000 × g for 5 min. The supernatant was loaded onto an SDS-polyacrylamide gel electrophoresis (SDS/PAGE) gel for the separation of proteins. Next, proteins were transferred to polyvinylidene fluoride (Pall Life Science) membranes using a semidry blotting system. Membranes were blocked with 5% (wt/vol) skim milk powder (Wako) in phosphate-buffered saline (PBS). Blocked membranes were washed with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T) and treated with anti-CAS (1:5,000 dilution) or anti-Histone H3 (1:20,000 dilution) antibodies. To recognize the primary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Life

Technologies) was used as a secondary antibody in a dilution of 1:10,000. After washing with PBS-T, immunoreactive signals were detected using Luminata Crescendo Western HRP substrate (Merck Millipore) and images were obtained using ImageQuant LAS-4010 (GE Healthcare).

Photosynthetic oxygen evolution

For evaluating the affinity for Ci, the rate of dissolved Ci-dependent photosynthetic O_2 evolution was measured. Cells harvested after growth in HC and LC conditions were suspended in Ci-depleted Hepes-NaOH buffer (pH 7.8) at 10 μg mL^{-1} chlorophyll. Photosynthetic O_2 evolution was measured by applying a Clark-type O_2 electrode (Hansatech Instruments), as described preciously (Yamano et al. 2008).

Capture of high-resolution fluorescence images

To reduce *Chlamydomonas* cell movement, 2.5 μL cells were placed between a coverslip and a thin agarose pad (Skinner et al. 2013), and then 16-bit digital fluorescence images were acquired with oil immersion objective lens (HC PL APO 63×/1.40; Leica) using an inverted laser-scanning confocal fluorescence microscope TCS SP8 (Leica) equipped with a sensitive hybrid detector (HyD). CAS-Clover was excited at 488 nm and emission was detected at 500–520 nm. Image scanning was performed with pinhole size of 0.6 Airy units, with z-stack distance of the scan at 150 nm, at a pixel size of 25 nm, and with a line scan speed of 200 Hz. Huygens Essential software (Scientific Volume Imaging B.V.) was used for data processing. Deconvolution of confocal datasets was performed using the point-spread function (PSF) theoretically calculated from the microscopic parameters attached to the data and classic maximum likelihood estimation (CMLE) algorithm (settings: maximum iterations: 100; signal-to-noise: 20; quality criterion: 0.05).

Results

Isolation of transgenic lines expressing CAS-Clover

To examine the subcellular localization of CAS *in vivo*, we generated transgenic lines expressing CAS fused with Clover (CAS-Clover), a *Chlamydomonas*-adapted modified green fluorescence protein (Lauersen et al. 2015). We modified the expression plasmid of CAS-Clover used previously (Wang et al. 2016) by introducing a flexible amino acid linker (Gly-Gly-Ala-Ala-Ala-Gly) between CAS and Clover to minimize interference by the protein fusion (Fig. 1a). This plasmid was used to transform the H82 mutant, from which 960

transformants showing paromomycin resistance were obtained, and nine transformants designated as CL-1–CL-9 showing fluorescence signals derived from CAS-Clover inside the pyrenoid were screened. By immunoblotting analysis using an anti-CAS antibody, a band of approximately 63 kDa corresponding to the predicted size of the CAS-Clover fusion protein was detected (Fig. 1b). Among these transformants, strain CL-2 showed the strongest fluorescence signal and was selected for further analyses. The values of maximum O_2-evolving activity (V_{max}) and $K_{0.5}$ (Ci), the Ci concentration required for half V_{max}, of CL-2 were similar to those of wild-type (WT) cells (Fig. 1c), indicating that decreased photosynthetic Ci-affinity of H82 was complemented by expressing the CAS-Clover.

High-resolution suborganellar localization of CAS-Clover *in vivo*

High-resolution fluorescence images of the CL-2 cells expressing CAS-Clover were obtained using the combination of a sensitive hybrid detector and an image deconvolution technique. In HC conditions, the fluorescence signals were distributed across the entire chloroplast and several punctuate spots were also observed (Fig. 2a). By defocusing of confocal images, fluorescence signals displayed a mesh-like structure, and part of the signals discontinuously overlapped with chlorophyll autofluorescence (Fig. 2b). Considering that CAS was detected in the fraction enriched with the thylakoid membrane (Wang et al. 2016), CAS could be not uniformly but discontinuously distributed on the thylakoid membrane in HC conditions.

Next, when the cells were shifted from HC to LC conditions, the fluorescence signals were detected inside the pyrenoid as a distinct wheel-like structure at 2 h (Fig. 3a–c). When we shifted the focus along the z-axis direction, a strong fluorescent spot was also observed in the lateral region of the chloroplast, which overlapped with the region of eyespot observed in a differential interference contrast image (Fig. 3a). Although the autofluorescence signals of the eyespot were detected in the WT cells, their signal intensities were significantly weaker than that of CL-2 cells with the same microscopic conditions (Fig. 3b), indicating that the fluorescence signals of the eyespot region in CL-2 cells were mostly derived from CAS-Clover. By defocusing of confocal images in the pyrenoid region, the wheel-like structure consisting of several fibers were clearly observed (Fig. 3c~~b~~). Inside the developed pyrenoid, chlorophyll autofluorescence were hardly detected (Fig. 3a), which was consistent with a previous report (Uniacke and Zerges 2007). This is possibly because the mean diameter of the pyrenoid tubule is very thin at 107 ± 26 nm (Engel et al. 2015), or the amount of chlorophyll could be much decreased in the pyrenoid tubules. By enhancing the contrast of fluorescence, thin fibers were observed, which could be derived from the structure of the

pyrenoid tubules (Fig. 3ed). In LC conditions after 12 h, the wheel-like structure had almost disappeared, and CAS-Clover was localized to the center of the pyrenoid (Fig. 3fe). Considering that part of the thylakoid membrane, termed the pyrenoid tubules, penetrates into the pyrenoid and fuses at the center of the pyrenoid, forming a knotted core (Engel et al. 2015; Meyer et al. 2016) and that relocation of CAS was not associated with *de novo* protein synthesis (Wang et al. 2016), dispersed CAS-Clover in the chloroplast in HC conditions could move and gather into the pyrenoid along the thylakoid membranes during CCM induction.

Discussion

In this study, we determined suborganellar localization of CAS based on fluorescence images of functional CAS-Clover *in vivo* at high resolution. CAS showed distinct different localization patterns between HC and LC conditions. Dispersed localization of CAS-Clover in HC conditions changed to a wheel-like structure in LC conditions at 2 h and aggregated inside the pyrenoid at 12 h. In particular, this wheel-like localization of CAS-Clover was clearly observed for the first time in this study, strengthening the hypothesis that CAS gathers inside the pyrenoid along the pyrenoid tubules during the operation of the CCM (Wang et al. 2016). Although the relocation of CAS in the chloroplast and its importance for regulation of the CCM has been proposed, it remains unclear how CAS moves along thylakoid membranes.

One possible mechanism is posttranslational modification. Other CCM-related proteins, such as LCIB and CAH3, also change their localization in response to CO_2 availability and undergo phosphorylation when CO_2 availability is limiting (Blanco-Rivero et al. 2012; Yamano et al. 2010). LCIB is an indispensable factor in the CCM and is observed as dispersed speckles in the chloroplast in HC conditions, but changes its localization as a ring-like structure in the vicinity of the pyrenoid in the LC-adapted cells (Yamano et al. 2010), which is distinctly different from the CAS localization pattern. Because *de novo* protein synthesis inhibits the relocation of LCIB (Yamano et al. 2014), but does not affect that of CAS (Wang et al. 2016), the regulatory mechanism of relocation could be different between these proteins. An α-type carbonic anhydrase, CAH3, is shown to be associated with dehydration of HCO_3^- to CO_2 within the lumen of pyrenoid tubules (Karlsson et al. 1998). Although CAH3 is associated with the donor side of PSII in the stroma of thylakoid membranes in HC conditions, CAH3 is partly concentrated in the pyrenoid tubules, which does not contain PSII, to provide CO_2 to Rubisco in LC conditions (Blanco-Rivero et al. 2012). Moreover, LCI5/EPYC1 was the first reported protein phosphorylated in response to

CO_2-limiting conditions (Turkina et al. 2006). LCI5/EPYC1 is colocalized with Rubisco in the pyrenoid matrix and assists in the formation of the pyrenoid and the packing of Rubisco in the pyrenoid in LC conditions by linking with Rubisco (Mackinder et al. 2016). In *Arabidopsis*, it is reported that a light-dependent thylakoid protein kinase STN8 phosphorylates a stroma-exposed Thr380 residue of CAS (flanking sequence is SGTKFLP and phosphorylated Threonine is underlined; Vainonen et al. 2008), which is also conserved as Thr370 (flanking sequence is TSTRRLP and putative phosphorylated Threonine is underlined) in *Chlamydomonas* CAS. Based on these results, phosphorylation could be an important factor to regulate the relocation and/or function of CCM-related proteins. Identifying kinases, phosphorylation sites, and obtaining high-resolution images of these proteins could lead to a better understanding of the regulatory mechanism of suborganellar protein relocation.

Another possible mechanism is the structural dynamics of thylakoid membranes. CAS has a hydrophobic sequence that separates the protein sequence into an N-terminus with a Ca^{2+}-binding region and a C-terminus with a rhodanese-like domain, and it is thought that CAS anchors to the thylakoid membrane via the hydrophobic sequence (Wang et al. 2016). A recent study revealed that both the structural stability and flexibility of thylakoid membranes is essential for dynamic protein reorganization (Iwai et al. 2014). It is possible that CAS also moves along with the membrane dynamics, although directional movement of the thylakoid membrane from dispersed chloroplast region into the pyrenoid and *vice versa* is unknown.

Recently, CAS was detected in a purified fraction of the *Chlamydomonas* eyespot and also involved in regulating the positive phototactic response under continuous illumination (Trippens et al. 2017). Consistent with this result, we first observed that the fluorescence signal of CAS-Clover overlapped with the eyespot *in vivo*. Ca^{2+} influx through the channel rhodopsins in the eyespot region play an important role for the regulation of phototactic behavior, but the primary Ca^{2+} sensing mechanism is unknown. Using our knockout mutant H82, the regulatory roles of CAS associated with the positive phototactic response could be more clarified.

It has become clear that the pyrenoid is important not only for CO_2 fixation but also for the regulation of the CCM (Meyer et al. 2017; Mitchell et al. 2017). So far, hundreds of proteins with unknown function have been identified in the purified pyrenoid (Mackinder et al. 2016), and there could be other CCM-related proteins that could relocate in the chloroplast in response to the CO_2 availability as reported previously (Yamano et al. 2010). Further screening of mutants showing aberrant localization patterns of these proteins could lead to

improved understand the regulatory mechanism of suborganellar relocation in response to environmental stresses (Yamano et al. 2014). Obtaining high-resolution images described in this study could be useful for observing the suborganellar localization of proteins, especially for ones localized in small compartments such as the pyrenoid in a single cell.

Acknowledgments

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grants 16H04805 (to H.F.) and 16K07399~~25840109~~ (to T.Y.) and the Japan Science and Technology Agency Advanced Low Carbon Technology Research and Development Program (to H.F.).

References

Blanco-Rivero A, Shutova T, Roman MJ, Villarejo A, Martinez F (2012) Phosphorylation controls the localization and activation of the luminal carbonic anhydrase in *Chlamydomonas reinhardtii*. PLoS One 7:e49063 doi:10.1371/journal.pone.0049063

Duanmu D, Miller AR, Horken KM, Weeks DP, Spalding MH (2009) Knockdown of limiting-CO_2-induced gene HLA3 decreases HCO_3^- transport and photosynthetic Ci affinity in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 106:5990–5995

Engel BD, Schaffer M, Cuellar LK, Villa E, Plitzko JM, Baumeister W (2015) Native architecture of the *Chlamydomonas* chloroplast revealed by *in situ* cryo-electron tomography. eLife 4:e04889 doi:10.7554/eLife.04889

Fukuzawa H, Miura K, Ishizaki K, Kucho K, Saito T, Kohinata T, Ohyama K (2001) *Ccm1*, a regulatory gene controlling the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2 availability. Proc Natl Acad Sci USA 98:5347–5352

Fukuzawa, H, Ogawa T, Kaplan A (2012) The uptake of CO_2 by cyanobacteria and microalgae. In: Eaton-Rye JJ, Tripathy BC, Sharkey TD (eds) Photosynthesis "plastid biology, energy conversion and carbon assimilation." Springer, Advances in Photosynthesis and Respiration 34:625–650

Gao H, Wang Y, Fei X, Wright DA, Spalding MH (2015) Expression activation and functional analysis of HLA3, a putative inorganic carbon transporter in *Chlamydomonas reinhardtii*. Plant J 82:1–11

Han S, Tang R, Anderson LK, Woerner TE, Pei ZM. (2003) A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells. Nature 425:196–200

Im CS, Grossman AR (2002) Identification and regulation of high light-induced genes in *Chlamydomonas reinhardtii*. Plant J 30:301-313

Iwai M, Yokono M, Nakano A (2014) Visualizing structural dynamics of thylakoid membranes. Sci Rep 4:3768 doi:10.1038/srep03768

Jones HG (1992) Plants and microclimate: A quantitative approach to environmental plant physiology, 2nd ed. Cambridge University Press: Cambridge, UK.

Karlsson J, Clarke AK, Chen ZY et al (1998) A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. EMBO J 17:1208-1216

Lauersen KJ, Kruse O, Mussgnug JH (2015) Targeted expression of nuclear transgenes in *Chlamydomonas reinhardtii* with a versatile, modular vector toolkit. Appl Microbiol Biotechnol 99:3491–3503

Mackinder LCM, Meyer M, Mettler-Altmann T et al (2016) A repeat protein links Rubisco to form the eukaryotic carbon-concentrating organelle. Proc Natl Acad Sci USA 113:5958–5963

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

Meyer MT, McCormick AJ, Griffiths H (2016) Will an algal CO_2-concentrating mechanism work in higher plants? Curr Opin Plant Biol 31:181–188

Meyer MT, Whittaker C, Griffiths H (2017) The algal pyrenoid: key unanswered questions. J Exp Bot 68:3739-3749

Mitchell MC, Metodieva G, Metodiev MV, Griffiths H, Meyer MT (2017) Pyrenoid loss impairs carbon-concentrating mechanism induction and alters primary metabolism in *Chlamydomonas reinhardtii*. J Exp Bot 68:3891-3902

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

Motohashi K (2015) A simple and efficient seamless DNA cloning method using SLiCE from *Escherichia coli* laboratory strains and its application to SLiP site-directed mutagenesis. BMC Biotechnol 15:47 doi:10.1186/s12896-015-0162-8

Nomura H, Komori T, Kobori M, Nakahira Y, Shiina T (2008) Evidence for chloroplast control of external Ca^{2+}-induced cytosolic Ca^{2+} transients and stomata closure. Plant J 53:988–998

Nomura H, Komori T, Uemura S et al (2012) Chloroplast-mediated activation of plant immune signaling in *Arabidopsis*. Nat Commun 3:926 doi:10.1038/ncomms1926

Ohad I, Siekevitz P, Palade GE (1967) Biogenesis of chloroplast membranes. I. Plastid dedifferentiation in a dark-grown algal mutant (*Chlamydomonas reinhardtii*). J Cell Biol 35:521–552

Skinner SO, Sepúlveda LA, Xu H, Golding I (2013) Measuring mRNA copy number in individual *Escherichia coli* cells using single-molecule fluorescent *in situ* hybridization. Nat Protoc 8:1100-1113

Trippens J, Reißenweber T, Kreimer G (2017) The chloroplast calcium sensor protein CAS affects phototactic behaviour in *Chlamydomonas reinhardtii* (Chlorophyceae) at low light intensities. Phycologia 56:261–270

Turkina MV, Blanco-Rivero A, Vainonen JP, Vener AV, Villarejo A (2006) CO_2 limitation induces specific redox-dependent protein phosphorylation in *Chlamydomonas reinhardtii*. Proteomics 6:2693–2704

Uniacke J and Zerges W (2007) Photosystem II assembly and repair are differentially localized in *Chlamydomonas*. Plant Cell 19:3640–3654

Vainonen JP, Sakuragi Y, Stael S et al (2008) Light regulation of CaS, a novel phosphoprotein in the thylakoid membrane of *Arabidopsis thaliana*. FEBS J 275:1767–1777

Wang L, Yamano T, Kajikawa M, Hirono M, Fukuzawa H (2014) Isolation and characterization of novel high-CO_2-requiring mutants of *Chlamydomonas reinhardtii*. Photosynth Res 121:175–184

Wang L, Yamano T, Takane S et al (2016) Chloroplast-mediated regulation of CO_2-concentrating mechanism by Ca^{2+}-binding protein CAS in the green alga *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 113:12586–12591

Wang Y, Huang Y, Wang J et al (2009) Structure of the formate transporter FocA reveals a pentameric aquaporin-like channel. Nature 462:467–472

Wang Y, Spalding MH (2014) Acclimation to very low CO_2: Contribution of limiting CO_2 inducible proteins, LCIB and LCIA, to inorganic carbon uptake in *Chlamydomonas reinhardtii*. Plant Physiol 166:2040–2050

Wang Y, Stessman DJ, Spalding MH (2015) The CO_2 concentrating mechanism and photosynthetic carbon assimilation in limiting CO_2: how Chlamydomonas works against the gradient. Plant J 82:429–448

Xiang Y, Zhang J, Weeks DP (2001) The *Cia5* gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA 98:5341–5346

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

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

Yamano T, Iguchi H, Fukuzawa H (2013) Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal. J Biosci Bioeng 115:691–694

Yamano T, Asada A, Sato E, Fukuzawa H (2014) Isolation and characterization of mutants defective in the localization of LCIB, an essential factor for the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Photosynth Res 121:193–200

Yamano T, Sato E, Iguchi H, Fukuda Y, Fukuzawa H (2015) Characterization of the cooperative bicarbonate uptake into chloroplast stroma in the green alga *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA 112:7315–7320

Figure legends

Fig. 1 Isolation of transgenic lines expressing CAS-Clover. (a) A schematic illustration of the expression plasmid of CAS-Clover. The plasmid was constructed based on the pOptimized Clover vector (Lauersen et al. 2015). Translation start (ATG) and stop (TAA) sites are shown. The genomic sequence of *CAS* is placed at the downstream of $P_{A/R}$, *HSP70A*/*RBCS2* tandem promoter, which is followed by first intron of *RBCS2*. The nucleotide acid sequence GGCGGCGCGGCCGCGGGC encoding the amino acid sequence Gly-Gly-Ala-Ala-Ala-Gly represents a synthetic flexible linker between CAS and Clover. The expression of CAS-Clover is terminated by the T_{RBCS2}, 3'-untranslated region of *RBCS2*. Restriction enzyme sites for cloning of *CAS* (*Nde*I and *Bgl*II) and for insertion check of the flexible linker (*Not*I) are shown. (b) Accumulation of CAS and CAS-Clover fusion protein in wild-type (WT), H82, and transformants (CL strains). Cells were grown in low-CO_2 (LC) conditions for 12 h. Histone H3 was used as a loading control. (c) Maximum photosynthetic O_2-evolving activity (V_{max}; left) and inorganic carbon (Ci) affinity (right) of WT, H82, and CL-2 cells grown in LC conditions for 12 h. Photosynthetic O_2-evolving activity was measured in externally dissolved Ci concentrations at pH 7.8, and the $K_{0.5}$ (Ci), the Ci concentrations required for half V_{max}, were calculated. Data in all experiments are mean values ± standard deviation from three biological replicates. **P*<0.001 by Student's t test.

Fig. 2 Fluorescence signals derived from CAS-Clover in high-CO_2 (HC) conditions. (a) CL-2 cells were adapted to HC conditions. Defocused images +1.0 µm from the focal plane are shown in the bottom row. Each image is placed with the flagella facing upward on the panel. DIC, differential interference contrast image. Scale bar, 2 µm. (b) Enlarged fluorescence images of the white boxed area in (a) obtained by defocusing the sample from –0.6 to +0.8 µm from the focal plane. Scale bar, 400 nm.

Fig. 3 Fluorescence signals derived from CAS-Clover in low-CO_2 (LC) conditions. (a) CL-2 cells grown in high-CO_2 conditions were transferred to LC conditions for 2 h. Defocused images +1.0 µm from the focal plane are shown in the bottom row. Each image is placed with the flagella facing upward on the panel. White arrowheads indicate the eyespot. DIC, differential interference contrast image. Scale bar, 2 µm. (b) Autofluorescence image of wild-type (WT) cells grown in LC conditions for 2 h. White arrowheads indicate the eyespot region. Scale bar, 2 µm. (~~b~~c) Enlarged fluorescence images of the pyrenoid region by defocusing the sample from –0.6 to +0.8 µm from the focal plane. Scale bar, 400 nm. (~~c~~d)

Different images of CAS-Clover merged with chlorophyll grown in LC conditions for 2 h. (~~d~~c) High-contrast image of chlorophyll autofluorescence in the pyrenoid region. Scale bar, 400 nm. (~~e~~f) Fluorescence images derived from CAS-Clover in LC conditions for 12 h. Scale bar, 2 μm.

Conflict of Interest:

The authors declare that they have no conflict of interest.

Figure1

Click here to download Figure Fig.1.tif

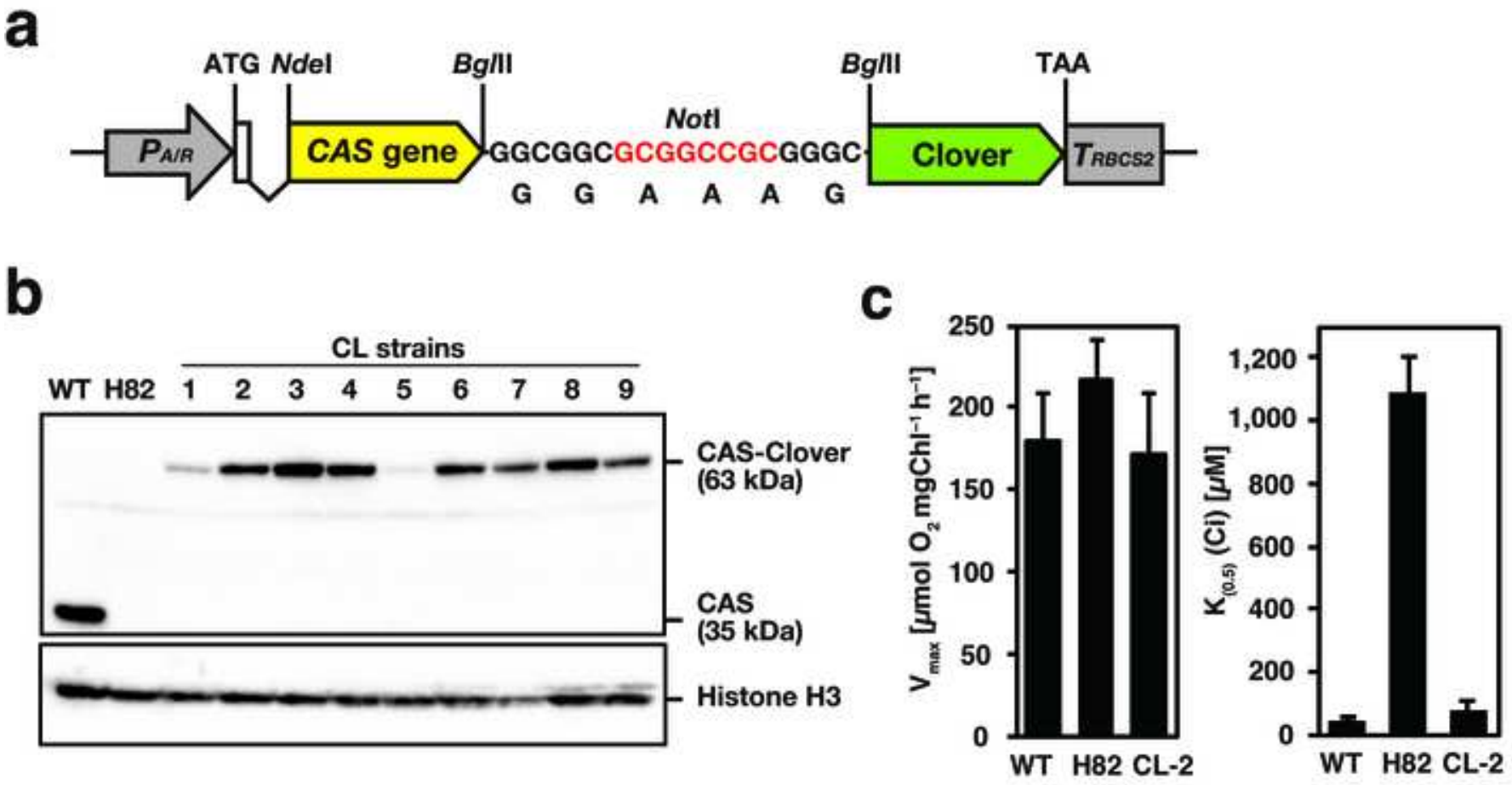

Figure2

Click here to download Figure Fig.2.tif

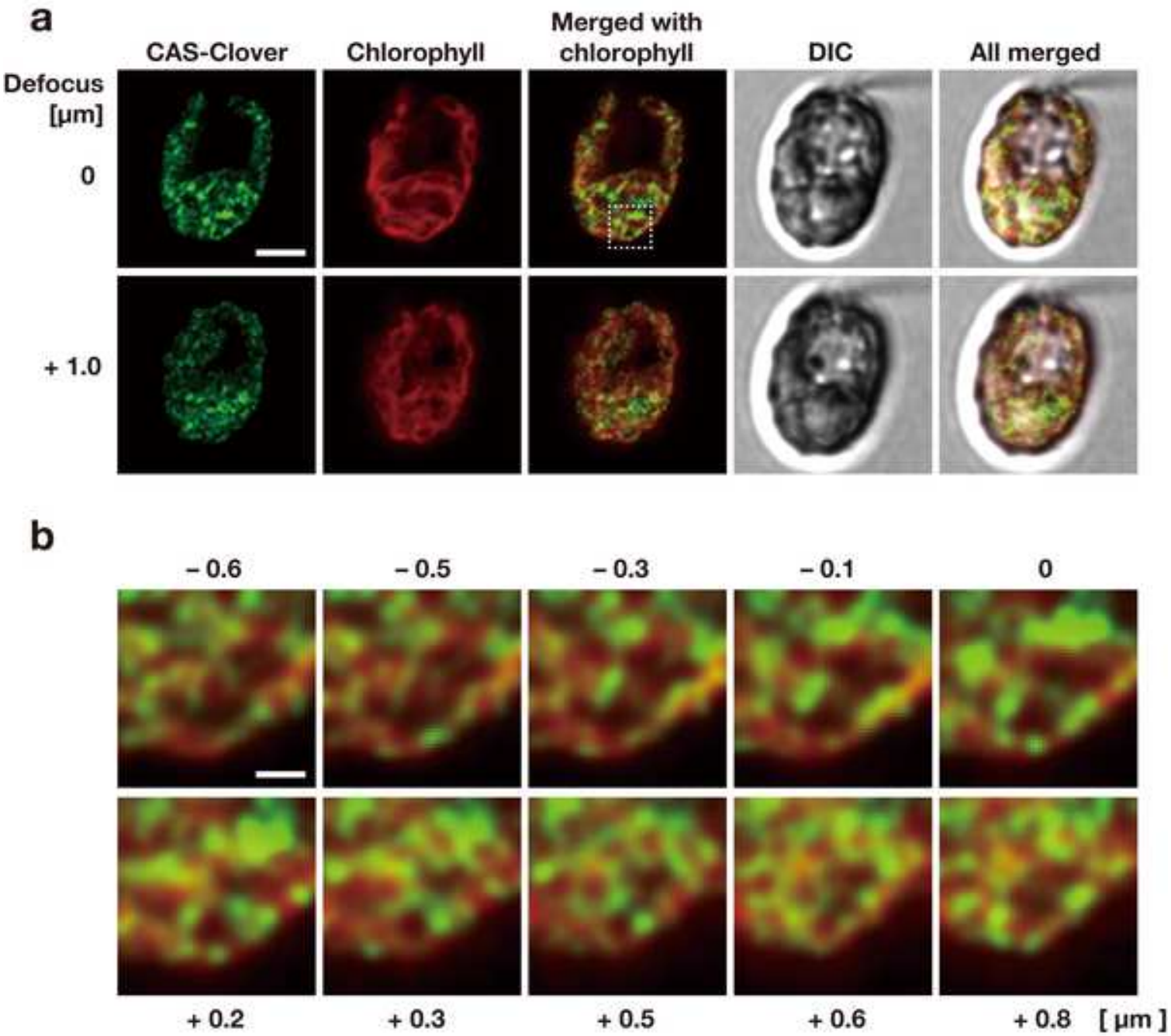

Figure3

Click here to download Figure Fig.3.tif