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1 **Title:** High-resolution suborganellar localization of Ca²⁺-binding protein CAS, a novel
2 regulator of CO₂-concentrating mechanism
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14 **Abstract**

15 Many aquatic algae induce a CO₂-concentrating mechanism (CCM) associated with active
16 inorganic carbon transport to maintain high photosynthetic affinity using dissolved inorganic
17 carbon even in low-CO₂ (LC) conditions. In the green alga *Chlamydomonas reinhardtii*, a
18 Ca²⁺-binding protein CAS was identified as a novel factor regulating the expression of CCM-
19 related proteins including bicarbonate transporters. Although previous studies revealed that
20 CAS associates with the thylakoid membrane and changes its localization in response to CO₂
21 and light availability, its detailed localization in the chloroplast has not been examined *in*
22 *vivo*. In this study, high-resolution fluorescence images of CAS fused with a
23 *Chlamydomonas*-adapted fluorescence protein, Clover, were obtained by using a sensitive
24 hybrid detector and an image deconvolution method. In high-CO₂ (5% v/v) conditions, the
25 fluorescence signals of Clover displayed a mesh-like structure in the chloroplast and part of
26 the signals discontinuously overlapped with chlorophyll autofluorescence. The fluorescence
27 signals gathered inside the pyrenoid as a distinct wheel-like structure at 2 h after transfer to
28 LC-light condition, and then localized to the center of the pyrenoid at 12 h. These results
29 suggest that CAS could move in the chloroplast along the thylakoid membrane in response to
30 lowering CO₂ and gather inside the pyrenoid during the operation of the CCM.

31

32 **Key words:** Bicarbonate transporter, Ca²⁺-binding protein, *Chlamydomonas*, CO₂-
33 concentrating mechanism, Pyrenoid

34

35 Introduction

36 Photosynthetic organisms can sense and respond to changes of several environmental factors,
37 such as light, CO₂, temperature, and various nutrient availabilities, to optimize and/or
38 maintain their photosynthetic activity. Among these stresses, the shortage of CO₂ supply
39 impacts many physiological aspects of plants, especially photosynthetic efficiency due to the
40 low affinity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) against CO₂. In
41 aquatic environments, CO₂-limiting stress is caused not only by the low catalytic activity of
42 Rubisco but also by the 10,000-fold slower diffusion rate of CO₂ in aquatic conditions than
43 that in atmospheric conditions (Jones 1992). To acclimate to this stress, many aquatic
44 organisms possess a CO₂-concentrating mechanism (CCM), which involves the active
45 transport of inorganic carbon (Ci; CO₂ and HCO₃⁻) and enzymatic conversion between CO₂
46 and HCO₃⁻ to maintain the Ci pool and concentrate CO₂ in the vicinity of Rubisco (Fukuzawa
47 et al. 2012; Wang et al. 2015).

48 Molecular aspects of the eukaryotic CCM have been mainly studied using the eukaryotic
49 green alga *Chlamydomonas reinhardtii* as a model. So far, it was reported that high-light
50 activated 3 (HLA3) and low-CO₂ (LC)-inducible protein A (LCIA) are associated with the
51 HCO₃⁻ transport system, which facilitate HCO₃⁻ uptake from outside of cells to the
52 chloroplast stroma across physiological barriers such as the plasma membrane and
53 chloroplast envelope (Gao et al. 2015; Yamano et al. 2015). HLA3 belongs to a multidrug-
54 resistance-related protein subfamily of the ATP-binding cassette transporter superfamily (Im
55 and Grossman 2001) and localizes to the plasma membrane (Yamano et al. 2015). LCIA
56 belongs to a formate-nitrite transporter family (Mariscal et al. 2006), in which proteins form a
57 pentameric aquaporin-like channel rather than an active transporter (Wang et al. 2009), and
58 localizes to the chloroplast envelope (Wang and Spalding 2014; Yamano et al. 2015).

59 Because simultaneous knockdown or knockout of *HLA3* and *LCIA* causes a dramatic
60 decrease in photosynthetic Ci uptake, and simultaneous overexpression of these genes raised
61 photosynthetic Ci affinity and internal Ci accumulation, HLA3 and LCIA are cooperatively
62 associated with HCO₃⁻ transport to increase the Ci pool in the chloroplast stroma (Duanmu et
63 al. 2009; Wang and Spalding 2014; Gao et al. 2015; Yamano et al. 2015). These LC-
64 inducible proteins are regulated by zinc-containing regulatory protein CCM1/CIA5
65 (Fukuzawa et al. 2001; Xiang et al. 2001; Miura et al. 2004). Recently, we isolated a novel
66 high-CO₂ (HC)-requiring mutant H82 (Wang et al. 2014) and revealed that a Ca²⁺-binding
67 protein, CAS, is also essential for the operation of the CCM by regulating the expression of
68 *HLA3* and *LCIA* (Wang et al. 2016). CAS was initially identified in *Arabidopsis thaliana*

69 (Han et al. 2003) and was detected in the thylakoid membrane fraction (Nomura et al. 2008).
70 By proteomic analysis and indirect immunofluorescence assays, *Chlamydomonas* CAS was
71 also localized to the thylakoid membrane and especially inside the pyrenoid, which is a
72 prominent structure in the chloroplast of the cells cultured in LC conditions (Wang et al.
73 2016). In many algae, the pyrenoid develops as a spherical proteinaceous structure
74 surrounded with starch sheathes in the chloroplast. Some of the thylakoid membrane
75 penetrates into the pyrenoid, termed pyrenoid tubules (Ohad et al. 1967), and multiple
76 parallel minitubules are bundled within the pyrenoid tubule (Engel et al. 2015). Recently,
77 *Chlamydomonas* CAS was also detected in the protein fraction of purified eyespot (Trippens
78 et al. 2017). Considering that *Arabidopsis* CAS regulates nuclear-encoded genes related to
79 plant immune responses (Nomura et al. 2012) and that *Chlamydomonas* CAS also regulates
80 nuclear-encoded genes related to the CCM such as *HLA3* and *LCIA* (Wang et al. 2016), CAS-
81 mediated retrograde signaling systems from the chloroplast to the nucleus appear to be
82 conserved during the evolution of the plant lineage. However, the actual function of CAS and
83 its detailed subcellular localization *in vivo* remain to be determined.

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

89 90 **Materials and Methods**

91 **Cell culture and growth conditions**

92 *Chlamydomonas reinhardtii* strain C-9 (photosynthetically WT strain originally provided by
93 the IAM Culture Collection held at Tokyo University, and available from the
94 *Chlamydomonas* Resource Center as strain CC-5098), and transgenic lines were cultured in
95 Tris-acetate-phosphate (TAP) medium for maintenance. For physiological and biochemical
96 experiments, a 5 mL volume of cells were grown in liquid TAP medium for pre-cultivation,
97 and diluted with modified high-salt medium supplemented with 20 mM 3-(N-
98 Morpholino)propanesulfonic acid (HSM) to an OD₇₃₀ of ~0.05. Then, the cells were grown
99 under HC (5% v/v) conditions at 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ until midlog phase with OD₇₃₀ of
100 0.3 to 0.5. For LC induction, HC-acclimated cells were centrifuged at 600 \times g, and pellets
101 were resuspended in 50 mL of fresh HSM medium, and cultured in LC (0.04% v/v)
102 conditions at 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for indicated time periods.

103 **Plasmid construction and transformation**

104 The genomic sequence of *CAS* was amplified by PCR with PrimeSTAR GXL (Takara Bio)
105 using genomic DNA extracted from strain C-9 as a template with forward primer TP-clover-F
106 (5'-TTTGCAGGATGCATATGCAGCTTGCTAACGCTCCT-3') and reverse primer gCAS-
107 clover-R (5'-CGATGACGTCAGATCTCGAGCGGGGGCGGGCAG-3'). The PCR
108 products were purified and cloned into pOptimized Clover vector (Lauersen et al. 2015)
109 digesting with *NdeI* and *BglIII* using a SLiCE cloning method (Motohashi 2015). For the
110 introduction of a flexible amino acid linker between CAS and Clover, two synthetic oligo
111 nucleotides, gCAS_clover_linker-F (5'-
112 CCCCCGCTCGAGATCTGGCGGCGCGGCCGCGGGCAGATCTGACGTCATCG-3') and
113 gCAS_clover_linker-R (5'-
114 CGATGACGTCAGATCTGCCCGCGGCCGCGCCGCCAGATCTCGAGCGGGGG-3')
115 was annealed and then cloned into the above plasmid digesting with *BglIII* using a SLiCE
116 cloning method (18-bp nucleotide sequences encoding flexible linker are shown by
117 underlines). This expression plasmid of CAS-Clover was transformed into the H82 mutant
118 (Wang et al. 2014) by electroporation using a NEPA-21 electroporator (NEPAGENE), as
119 described previously (Yamano et al. 2013). The transformants were incubated at 25°C for 24
120 h with gentle shaking and illumination of less than 1.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and spread over
121 TAP plates containing 30 $\mu\text{g mL}^{-1}$ hygromycin.

123 **Immunoblotting analyses**

124 Extracted total proteins suspended in SDS loading buffer containing 50 mM Tris HCl (pH
125 8.0), 25% (vol/vol) glycerol, 2% (wt/vol) SDS, and 0.1 M DTT were incubated at 37°C for
126 30 min and subsequently centrifuged at 13,000 $\times g$ for 5 min. The supernatant was loaded
127 onto an SDS-polyacrylamide gel electrophoresis (SDS/PAGE) gel for the separation of
128 proteins. Next, proteins were transferred to polyvinylidene fluoride (Pall Life Science)
129 membranes using a semidry blotting system. Membranes were blocked with 5% (wt/vol)
130 skim milk powder (Wako) in phosphate-buffered saline (PBS). Blocked membranes were
131 washed with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T) and treated with anti-CAS
132 (1:5,000 dilution) or anti-Histone H3 (1:20,000 dilution) antibodies. To recognize the
133 primary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Life
134 Technologies) was used as a secondary antibody in a dilution of 1:10,000. After washing
135 with PBS-T, immunoreactive signals were detected using Luminata Crescendo Western HRP

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

138

139 **Photosynthetic oxygen evolution**

140 For evaluating the affinity for C_i , the rate of dissolved C_i -dependent photosynthetic O_2

141 evolution was measured. Cells harvested after growth in HC and LC conditions were

142 suspended in C_i -depleted HEPES-NaOH buffer (pH 7.8) at $10 \mu\text{g mL}^{-1}$ chlorophyll.

143 Photosynthetic O_2 evolution was measured by applying a Clark-type O_2 electrode (Hansatech

144 Instruments), as described previously (Yamano et al. 2008).

145

146 **Capture of high-resolution fluorescence images**

147 To reduce *Chlamydomonas* cell movement, $2.5 \mu\text{L}$ cells were placed between a coverslip and

148 a thin agarose pad (Skinner et al. 2013), and then 16-bit digital fluorescence images were

149 acquired with oil immersion objective lens (HC PL APO 63 \times /1.40; Leica) using an inverted

150 laser-scanning confocal fluorescence microscope TCS SP8 (Leica) equipped with a sensitive

151 hybrid detector (HyD). CAS-Clover was excited at 488 nm and emission was detected at

152 500–520 nm. Image scanning was performed with pinhole size of 0.6 Airy units, with z-stack

153 distance of the scan at 150 nm, at a pixel size of 25 nm, and with a line scan speed of 200 Hz.

154 Huygens Essential software (Scientific Volume Imaging B.V.) was used for data processing.

155 Deconvolution of confocal datasets was performed using the point-spread function (PSF)

156 theoretically calculated from the microscopic parameters attached to the data and classic

157 maximum likelihood estimation (CMLE) algorithm (settings: maximum iterations: 100;

158 signal-to-noise: 20; quality criterion: 0.05).

159

160 **Results**

161 **Isolation of transgenic lines expressing CAS-Clover**

162 To examine the subcellular localization of CAS *in vivo*, we generated transgenic lines

163 expressing CAS fused with Clover (CAS-Clover), a *Chlamydomonas*-adapted modified green

164 fluorescence protein (Lauersen et al. 2015). We modified the expression plasmid of CAS-

165 Clover used previously (Wang et al. 2016) by introducing a flexible amino acid linker (Gly-

166 Gly-Ala-Ala-Ala-Gly) between CAS and Clover to minimize interference by the protein

167 fusion (Fig. 1a). This plasmid was used to transform the H82 mutant, from which 960

168 transformants showing paromomycin resistance were obtained, and nine transformants

169 designated as CL-1–CL-9 showing fluorescence signals derived from CAS-Clover inside the

170 pyrenoid were screened. By immunoblotting analysis using an anti-CAS antibody, a band of
171 approximately 63 kDa corresponding to the predicted size of the CAS-Clover fusion protein
172 was detected (Fig. 1b). Among these transformants, strain CL-2 showed the strongest
173 fluorescence signal and was selected for further analyses. The values of maximum O₂-
174 evolving activity (V_{\max}) and $K_{0.5}$ (Ci), the Ci concentration required for half V_{\max} , of CL-2
175 were similar to those of wild-type (WT) cells (Fig. 1c), indicating that decreased
176 photosynthetic Ci-affinity of H82 was complemented by expressing the CAS-Clover.

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

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

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

204 Considering that part of the thylakoid membrane, termed the pyrenoid tubules, penetrates into
205 the pyrenoid and fuses at the center of the pyrenoid, forming a knotted core (Engel et al.
206 2015; Meyer et al. 2016) and that relocation of CAS was not associated with *de novo* protein
207 synthesis (Wang et al. 2016), dispersed CAS-Clover in the chloroplast in HC conditions
208 could move and gather into the pyrenoid along the thylakoid membranes during CCM
209 induction.

210

211 Discussion

212 In this study, we determined suborganellar localization of CAS based on fluorescence images
213 of functional CAS-Clover *in vivo* at high resolution. CAS showed distinct different
214 localization patterns between HC and LC conditions. Dispersed localization of CAS-Clover
215 in HC conditions changed to a wheel-like structure in LC conditions at 2 h and aggregated
216 inside the pyrenoid at 12 h. In particular, this wheel-like localization of CAS-Clover was
217 clearly observed for the first time in this study, strengthening the hypothesis that CAS gathers
218 inside the pyrenoid along the pyrenoid tubules during the operation of the CCM (Wang et al.
219 2016). Although the relocation of CAS in the chloroplast and its importance for regulation of
220 the CCM has been proposed, it remains unclear how CAS moves along thylakoid
221 membranes.

222 One possible mechanism is posttranslational modification. Other CCM-related
223 proteins, such as LCIB and CAH3, also change their localization in response to CO₂
224 availability and undergo phosphorylation when CO₂ availability is limiting (Blanco-Rivero et
225 al. 2012; Yamano et al. 2010). LCIB is an indispensable factor in the CCM and is observed
226 as dispersed speckles in the chloroplast in HC conditions, but changes its localization as a
227 ring-like structure in the vicinity of the pyrenoid in the LC-adapted cells (Yamano et al.
228 2010), which is distinctly different from the CAS localization pattern. Because *de novo*
229 protein synthesis inhibits the relocation of LCIB (Yamano et al. 2014), but does not affect
230 that of CAS (Wang et al. 2016), the regulatory mechanism of relocation could be different
231 between these proteins. An α -type carbonic anhydrase, CAH3, is shown to be associated with
232 dehydration of HCO₃⁻ to CO₂ within the lumen of pyrenoid tubules (Karlsson et al. 1998).
233 Although CAH3 is associated with the donor side of PSII in the stroma of thylakoid
234 membranes in HC conditions, CAH3 is partly concentrated in the pyrenoid tubules, which
235 does not contain PSII, to provide CO₂ to Rubisco in LC conditions (Blanco-Rivero et al.
236 2012). Moreover, LCI5/EPYC1 was the first reported protein phosphorylated in response to
237 CO₂-limiting conditions (Turkina et al. 2006). LCI5/EPYC1 is colocalized with Rubisco in

238 the pyrenoid matrix and assists in the formation of the pyrenoid and the packing of Rubisco
1 239 in the pyrenoid in LC conditions by linking with Rubisco (Mackinder et al. 2016). In
2
3 240 *Arabidopsis*, it is reported that a light-dependent thylakoid protein kinase STN8
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5 241 phosphorylates a stroma-exposed Thr380 residue of CAS (flanking sequence is SGTKFLP
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7 242 and phosphorylated Threonine is underlined; Vainonen et al. 2008), which is also conserved
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9 243 as Thr370 (flanking sequence is TSTRRLP and putative phosphorylated Threonine is
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11 244 underlined) in *Chlamydomonas* CAS. Based on these results, phosphorylation could be an
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13 245 important factor to regulate the relocation and/or function of CCM-related proteins.
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15 246 Identifying kinases, phosphorylation sites, and obtaining high-resolution images of these
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17 247 proteins could lead to a better understanding of the regulatory mechanism of suborganellar
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19 248 protein relocation.

20 249 Another possible mechanism is the structural dynamics of thylakoid membranes. CAS
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22 250 has a hydrophobic sequence that separates the protein sequence into an N-terminus with a
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24 251 Ca²⁺-binding region and a C-terminus with a rhodanese-like domain, and it is thought that
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26 252 CAS anchors to the thylakoid membrane via the hydrophobic sequence (Wang et al. 2016). A
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28 253 recent study revealed that both the structural stability and flexibility of thylakoid membranes
29
30 254 is essential for dynamic protein reorganization (Iwai et al. 2014). It is possible that CAS also
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32 255 moves along with the membrane dynamics, although directional movement of the thylakoid
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34 256 membrane from dispersed chloroplast region into the pyrenoid and *vice versa* is unknown.

35 257 Recently, CAS was detected in a purified fraction of the *Chlamydomonas* eyespot and
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37 258 also involved in regulating the positive phototactic response under continuous illumination
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39 259 (Trippens et al. 2017). Consistent with this result, we first observed that the fluorescence
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41 260 signal of CAS-Clover overlapped with the eyespot *in vivo*. Ca²⁺ influx through the channel
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43 261 rhodopsins in the eyespot region play an important role for the regulation of phototactic
44
45 262 behavior, but the primary Ca²⁺ sensing mechanism is unknown. Using our knockout mutant
46
47 263 H82, the regulatory roles of CAS associated with the positive phototactic response could be
48
49 264 more clarified.

50 265 It has become clear that the pyrenoid is important not only for CO₂ fixation but also
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52 266 for the regulation of the CCM (Meyer et al. 2017; Mitchell et al. 2017). So far, hundreds of
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54 267 proteins with unknown function have been identified in the purified pyrenoid (Mackinder et
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56 268 al. 2016), and there could be other CCM-related proteins that could relocate in the chloroplast
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58 269 in response to the CO₂ availability as reported previously (Yamano et al. 2010). Further
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60 270 screening of mutants showing aberrant localization patterns of these proteins could lead to
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62 271 understanding the regulatory mechanism of suborganellar relocation in response to
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272 environmental stresses (Yamano et al. 2014). Obtaining high-resolution images described in
273 this study could be useful for observing the suborganelle localization of proteins, especially
274 for ones localized in small compartments such as the pyrenoid in a single cell.

275

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280

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391 **Figure legends**

392 **Fig. 1** Isolation of transgenic lines expressing CAS-Clover. (a) A schematic illustration of the
393 expression plasmid of CAS-Clover. The plasmid was constructed based on the pOptimized
394 Clover vector (Lauersen et al. 2015). Translation start (ATG) and stop (TAA) sites are
395 shown. The genomic sequence of *CAS* is placed at the downstream of *P_{A/R}*, *HSP70A/RBCS2*
396 tandem promoter, which is followed by first intron of *RBCS2*. The nucleotide acid sequence
397 GGCGGCGCGGCCGCGGGC encoding the amino acid sequence Gly-Gly-Ala-Ala-Ala-Gly
398 represents a synthetic flexible linker between CAS and Clover. The expression of CAS-
399 Clover is terminated by the *T_{RBCS2}*, 3'-untranslated region of *RBCS2*. Restriction enzyme sites
400 for cloning of *CAS* (*NdeI* and *BglII*) and for insertion check of the flexible linker (*NotI*) are
401 shown. (b) Accumulation of CAS and CAS-Clover fusion protein in wild-type (WT), H82,
402 and transformants (CL strains). Cells were grown in low-CO₂ (LC) conditions for 12 h.
403 Histone H3 was used as a loading control. (c) Maximum photosynthetic O₂-evolving activity
404 (V_{max} ; left) and inorganic carbon (Ci) affinity (right) of WT, H82, and CL-2 cells grown in
405 LC conditions for 12 h. Photosynthetic O₂-evolving activity was measured in externally
406 dissolved Ci concentrations at pH 7.8, and the $K_{0.5}$ (Ci), the Ci concentrations required for
407 half V_{max} , were calculated. Data in all experiments are mean values \pm standard deviation from
408 three biological replicates. * $P < 0.001$ by Student's t test.

410 **Fig. 2** Fluorescence signals derived from CAS-Clover in high-CO₂ (HC) conditions. (a) CL-2
411 cells were adapted to HC conditions. Defocused images +1.0 μ m from the focal plane are
412 shown in the bottom row. Each image is placed with the flagella facing upward on the panel.
413 DIC, differential interference contrast image. Scale bar, 2 μ m. (b) Enlarged fluorescence
414 images of the white boxed area in (a) obtained by defocusing the sample from -0.6 to +0.8
415 μ m from the focal plane. Scale bar, 400 nm.

417 **Fig. 3** Fluorescence signals derived from CAS-Clover in low-CO₂ (LC) conditions. (a) CL-2
418 cells grown in high-CO₂ conditions were transferred to LC conditions for 2 h. Defocused
419 images +1.0 μ m from the focal plane are shown in the bottom row. Each image is placed with
420 the flagella facing upward on the panel. White arrowheads indicate the eyespot region. DIC,
421 differential interference contrast image. Scale bar, 2 μ m. (b) Autofluorescence image of wild-
422 type (WT) cells grown in LC conditions for 2 h. White arrowheads indicate the eyespot
423 region. Scale bar, 2 μ m. (c) Enlarged fluorescence images of the pyrenoid region by
424 defocusing the sample from -0.6 to +0.8 μ m from the focal plane. Scale bar, 400 nm. (d)

425 Different images of CAS-Clover merged with chlorophyll grown in LC conditions for 2 h. (e)
1
2 426 High-contrast image of chlorophyll autofluorescence in the pyrenoid region. Scale bar, 400
3
4 427 nm. (f) Fluorescence images derived from CAS-Clover in LC conditions for 12 h. Scale bar,
5 428 2 μm .
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1 **Title:** High-resolution suborganellar localization of Ca²⁺-binding protein CAS, a novel
2 regulator of CO₂-concentrating mechanism
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14 **Abstract**

15 Many aquatic algae induce a CO₂-concentrating mechanism (CCM) associated with active
16 inorganic carbon transport to maintain high photosynthetic affinity using dissolved inorganic
17 carbon even in low-CO₂ (LC) conditions. In the green alga *Chlamydomonas reinhardtii*, a
18 Ca²⁺-binding protein CAS was identified as a novel factor regulating the expression of CCM-
19 related proteins including bicarbonate transporters. Although previous studies revealed that
20 CAS associates with the thylakoid membrane and changes its localization in response to CO₂
21 and light availability, its detailed localization in the chloroplast has not been examined *in vivo*.
22 In this study, high-resolution fluorescence images of CAS fused with a *Chlamydomonas*-
23 adapted fluorescence protein, Clover, were obtained by using a sensitive hybrid detector and
24 an image deconvolution method. In high-CO₂ (5% v/v) conditions, the fluorescence signals
25 of Clover displayed a mesh-like structure in the chloroplast and part of the signals
26 discontinuously overlapped with chlorophyll autofluorescence. The fluorescence signals
27 gathered inside the pyrenoid as a distinct wheel-like structure at 2 h after transfer to LC-light
28 condition, and then localized to the center of the pyrenoid at 12 h. These results suggest that
29 CAS could move in the chloroplast along the thylakoid membrane in response to lowering
30 CO₂ and gather inside the pyrenoid ~~along the pyrenoid tubules, penetrated thylakoid~~
31 ~~membrane into pyrenoid,~~ during the operation of the CCM.

32
33
34 **Key words:** Bicarbonate transporter, Ca²⁺-binding protein, *Chlamydomonas*, CO₂-
35 concentrating mechanism, Pyrenoid

36 Introduction

37 Photosynthetic organisms can sense and respond to changes of several environmental factors,
38 such as light, CO₂, temperature, and various nutrient availabilities, to optimize and/or
39 maintain their photosynthetic activity. Among these stresses, the shortage of CO₂ supply
40 impacts many physiological aspects of plants, especially photosynthetic efficiency due to the
41 low affinity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) against CO₂. In
42 aquatic environments, CO₂-limiting stress is caused not only by the low catalytic activity of
43 Rubisco but also by the 10,000-fold slower diffusion rate of CO₂ in aquatic conditions than
44 that in atmospheric conditions (Jones 1992). To acclimate to this stress, many aquatic
45 organisms possess a CO₂-concentrating mechanism (CCM), which involves the active
46 transport of inorganic carbon (Ci; CO₂ and HCO₃⁻) and enzymatic conversion between CO₂
47 and HCO₃⁻ to maintain the Ci pool and concentrate CO₂ in the vicinity of Rubisco (Fukuzawa
48 et al. 2012; Wang et al. 2015).

49 Molecular aspects of the eukaryotic CCM have been mainly studied using the eukaryotic
50 green alga *Chlamydomonas reinhardtii* as a model. So far, it was reported that high-light
51 activated 3 (HLA3) and low-CO₂ (LC)-inducible protein A (LCIA) are associated with the
52 HCO₃⁻ transport system, which facilitate HCO₃⁻ uptake from outside of cells to the
53 chloroplast stroma across physiological barriers such as the plasma membrane and
54 chloroplast envelope (Gao et al. 2015; Yamano et al. 2015). HLA3 belongs to a multidrug-
55 resistance-related protein subfamily of the ATP-binding cassette transporter superfamily (Im
56 and Grossman 2001) and localizes to the plasma membrane (Yamano et al. 2015). LCIA
57 belongs to a formate-nitrite transporter family (Mariscal et al. 2006), in which proteins form a
58 pentameric aquaporin-like channel rather than an active transporter (Wang et al. 2009), and
59 localizes to the chloroplast envelope (Wang and Spalding 2014; Yamano et al. 2015).

60 Because simultaneous knockdown or knockout of *HLA3* and *LCIA* causes a dramatic
61 decrease in photosynthetic Ci uptake, and simultaneous overexpression of these genes raised
62 photosynthetic Ci affinity and internal Ci accumulation, HLA3 and LCIA are cooperatively
63 associated with HCO₃⁻ transport to increase the Ci pool in the chloroplast stroma (Duanmu et
64 al. 2009; Wang and Spalding 2014; Gao et al. 2015; Yamano et al. 2015). These LC-
65 inducible proteins are regulated by zinc-containing regulatory protein CCM1/CIA5
66 (Fukuzawa et al. 2001; Xiang et al. 2001; Miura et al. 2004). Recently, we isolated a novel
67 high-CO₂ (HC)-requiring mutant H82 (Wang et al. 2014) and revealed that a Ca²⁺-binding
68 protein, CAS, is also essential for the operation of the CCM by regulating the expression of
69 *HLA3* and *LCIA* (Wang et al. 2016). CAS was initially identified in *Arabidopsis thaliana*

70 (Han et al. 2003) and was detected in the thylakoid membrane fraction (Nomura et al. 2008).
71 By proteomic analysis and indirect immunofluorescence assays, *Chlamydomonas* CAS was
72 also localized to the thylakoid membrane and especially inside the pyrenoid, which is a
73 prominent structure in the chloroplast of the cells cultured in LC conditions (Wang et al.
74 2016). In many algae, the pyrenoid develops as a spherical proteinaceous structure
75 surrounded with starch sheathes in the chloroplast. Some of the thylakoid membrane
76 penetrates into the pyrenoid, termed pyrenoid tubules (Ohad et al. 1967), and multiple
77 parallel minitubules are bundled within the pyrenoid tubule (Engel et al. 2015). Recently,
78 *Chlamydomonas* CAS was also detected in the protein fraction of purified eyespot (Trippens
79 et al. 2017). Considering that *Arabidopsis* CAS regulates nuclear-encoded genes related to
80 plant immune responses (Nomura et al. 2012) and that *Chlamydomonas* CAS also regulates
81 nuclear-encoded genes related to the CCM such as *HLA3* and *LCIA* (Wang et al. 2016), CAS-
82 mediated retrograde signaling systems from the chloroplast to the nucleus appear to be
83 conserved during the evolution of the plant lineage. However, the actual function of CAS and
84 its detailed subcellular localization *in vivo* remain to be determined.

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

91 **Materials and Methods**

92 **Cell culture and growth conditions**

93 *Chlamydomonas reinhardtii* strain C-9 (photosynthetically WT strain originally provided by
94 the IAM Culture Collection held at Tokyo University, and available from the
95 *Chlamydomonas* Resource Center as strain CC-5098), and transgenic lines were cultured in
96 Tris-acetate-phosphate (TAP) medium for maintenance. For physiological and biochemical
97 experiments, a 5 mL volume of cells were grown in liquid TAP medium for pre-cultivation,
98 and diluted with modified high-salt medium supplemented with 20 mM 3-(N-
99 Morpholino)propanesulfonic acid (pH 7.0) to an OD₇₃₀ of ~0.05 ~~for photoautotrophic growth.~~
100 Then, the cells were grown under HC (5% v/v) conditions at 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ until
101 midlog phase with OD₇₃₀ of 0.3 to 0.5. For LC induction, HC-acclimated cells were
102 centrifuged at 600 \times g, and pellets were resuspended in 50 mL of fresh HSM medium, and
103 cultured in LC (0.04% v/v) conditions at 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for indicated time

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

107 **Plasmid construction and transformation**

108 The genomic sequence of *CAS* was amplified by PCR with PrimeSTAR GXL (Takara Bio)
109 using genomic DNA extracted from strain C-9 as a template with forward primer TP-clover-F
110 (5'-TTTGCAGGATGCATATGCAGCTTGCTAACGCTCCT-3') and reverse primer gCAS-
111 clover-R (5'-CGATGACGTCAGATCTCGAGCGGGGGCGGGCAG-3'). The PCR
112 products were purified and cloned into pOptimized Clover vector (Lauersen et al. 2015)
113 digesting with *NdeI* and *BglIII* using a SLiCE cloning method (Motohashi 2015). For the
114 introduction of a flexible amino acid linker between CAS and Clover, two synthetic oligo
115 nucleotides, gCAS_clover_linker-F (5'-
116 CCCCGCTCGAGATCTGGCGGCGCGGCCGCGGGCAGATCTGACGTCATCG-3') and
117 gCAS_clover_linker-R (5'-
118 CGATGACGTCAGATCTGCCCGCGGCCGCGCCGCCAGATCTCGAGCGGGGG-3')
119 was annealed and then cloned into the above plasmid digesting with *BglIII* using a SLiCE
120 cloning method (18-bp nucleotide sequences encoding flexible linker are shown by
121 underlines). This expression plasmid of CAS-Clover was transformed into the H82 mutant
122 (Wang et al. 2014) by electroporation using a NEPA-21 electroporator (NEPAGENE), as
123 described previously (Yamano et al. 2013). The transformants were incubated at 25°C for 24
124 h with gentle shaking and illumination of less than 1.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and spread over
125 TAP plates containing 30 $\mu\text{g mL}^{-1}$ hygromycin.

127 **Immunoblotting analyses**

128 Extracted total proteins suspended in SDS loading buffer containing 50 mM Tris HCl (pH
129 8.0), 25% (vol/vol) glycerol, 2% (wt/vol) SDS, and 0.1 M DTT were incubated at 37°C for
130 30 min and subsequently centrifuged at 13,000 $\times g$ for 5 min. The supernatant was loaded
131 onto an SDS-polyacrylamide gel electrophoresis (SDS/PAGE) gel for the separation of
132 proteins. Next, proteins were transferred to polyvinylidene fluoride (Pall Life Science)
133 membranes using a semidry blotting system. Membranes were blocked with 5% (wt/vol)
134 skim milk powder (Wako) in phosphate-buffered saline (PBS). Blocked membranes were
135 washed with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T) and treated with anti-CAS
136 (1:5,000 dilution) or anti-Histone H3 (1:20,000 dilution) antibodies. To recognize the
137 primary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Life

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

142

143 **Photosynthetic oxygen evolution**

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

149

150 **Capture of high-resolution fluorescence images**

151 To reduce *Chlamydomonas* cell movement, $2.5 \mu\text{L}$ cells were placed between a coverslip and
152 a thin agarose pad (Skinner et al. 2013), and then 16-bit digital fluorescence images were
153 acquired with oil immersion objective lens (HC PL APO $63\times/1.40$; Leica) using an inverted
154 laser-scanning confocal fluorescence microscope TCS SP8 (Leica) equipped with a sensitive
155 hybrid detector (HyD). CAS-Clover was excited at 488 nm and emission was detected at
156 500–520 nm. Image scanning was performed with pinhole size of 0.6 Airy units, with z-stack
157 distance of the scan at 150 nm, at a pixel size of 25 nm, and with a line scan speed of 200 Hz.
158 Huygens Essential software (Scientific Volume Imaging B.V.) was used for data processing.
159 Deconvolution of confocal datasets was performed using the point-spread function (PSF)
160 theoretically calculated from the microscopic parameters attached to the data and classic
161 maximum likelihood estimation (CMLE) algorithm (settings: maximum iterations: 100;
162 signal-to-noise: 20; quality criterion: 0.05).

163

164 **Results**

165 **Isolation of transgenic lines expressing CAS-Clover**

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

172 transformants showing paromomycin resistance were obtained, and nine transformants
173 designated as CL-1–CL-9 showing fluorescence signals derived from CAS-Clover inside the
174 pyrenoid were screened. By immunoblotting analysis using an anti-CAS antibody, a band of
175 approximately 63 kDa corresponding to the predicted size of the CAS-Clover fusion protein
176 was detected (Fig. 1b). Among these transformants, strain CL-2 showed the strongest
177 fluorescence signal and was selected for further analyses. The values of maximum O₂-
178 evolving activity (V_{\max}) and $K_{0.5}$ (Ci), the Ci concentration required for half V_{\max} , of CL-2
179 were similar to those of wild-type (WT) cells (Fig. 1c), indicating that decreased
180 photosynthetic Ci-affinity of H82 was complemented by expressing the CAS-Clover.

181 182 **High-resolution suborganellar localization of CAS-Clover *in vivo***

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

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

206 pyrenoid tubules (Fig. 3e*d*). In LC conditions after 12 h, the wheel-like structure had almost
207 disappeared, and CAS-Clover was localized to the center of the pyrenoid (Fig. 3f*e*).
208 Considering that part of the thylakoid membrane, termed the pyrenoid tubules, penetrates into
209 the pyrenoid and fuses at the center of the pyrenoid, forming a knotted core (Engel et al.
210 2015; Meyer et al. 2016) and that relocation of CAS was not associated with *de novo* protein
211 synthesis (Wang et al. 2016), dispersed CAS-Clover in the chloroplast in HC conditions
212 could move and gather into the pyrenoid along the thylakoid membranes during CCM
213 induction.

214 215 **Discussion**

216 In this study, we determined suborganellar localization of CAS based on fluorescence images
217 of functional CAS-Clover *in vivo* at high resolution. CAS showed distinct different
218 localization patterns between HC and LC conditions. Dispersed localization of CAS-Clover
219 in HC conditions changed to a wheel-like structure in LC conditions at 2 h and aggregated
220 inside the pyrenoid at 12 h. In particular, this wheel-like localization of CAS-Clover was
221 clearly observed for the first time in this study, strengthening the hypothesis that CAS gathers
222 inside the pyrenoid along the pyrenoid tubules during the operation of the CCM (Wang et al.
223 2016). Although the relocation of CAS in the chloroplast and its importance for regulation of
224 the CCM has been proposed, it remains unclear how CAS moves along thylakoid membranes.

225 One possible mechanism is posttranslational modification. Other CCM-related
226 proteins, such as LCIB and CAH3, also change their localization in response to CO₂
227 availability and undergo phosphorylation when CO₂ availability is limiting (Blanco-Rivero et
228 al. 2012; Yamano et al. 2010). LCIB is an indispensable factor in the CCM and is observed
229 as dispersed speckles in the chloroplast in HC conditions, but changes its localization as a
230 ring-like structure in the vicinity of the pyrenoid in the LC-adapted cells (Yamano et al.
231 2010), which is distinctly different from the CAS localization pattern. Because *de novo*
232 protein synthesis inhibits the relocation of LCIB (Yamano et al. 2014), but does not affect
233 that of CAS (Wang et al. 2016), the regulatory mechanism of relocation could be different
234 between these proteins. An α -type carbonic anhydrase, CAH3, is shown to be associated with
235 dehydration of HCO₃⁻ to CO₂ within the lumen of pyrenoid tubules (Karlsson et al. 1998).
236 Although CAH3 is associated with the donor side of PSII in the stroma of thylakoid
237 membranes in HC conditions, CAH3 is partly concentrated in the pyrenoid tubules, which
238 does not contain PSII, to provide CO₂ to Rubisco in LC conditions (Blanco-Rivero et al.
239 2012). Moreover, LCI5/EPYC1 was the first reported protein phosphorylated in response to

240 CO₂-limiting conditions (Turkina et al. 2006). LCI5/EPYC1 is colocalized with Rubisco in
241 the pyrenoid matrix and assists in the formation of the pyrenoid and the packing of Rubisco
242 in the pyrenoid in LC conditions by linking with Rubisco (Mackinder et al. 2016). In
243 *Arabidopsis*, it is reported that a light-dependent thylakoid protein kinase STN8
244 phosphorylates a stroma-exposed Thr380 residue of CAS (flanking sequence is SGTKFLP
245 and phosphorylated Threonine is underlined; Vainonen et al. 2008), which is also conserved
246 as Thr370 (flanking sequence is TSTRRLP and putative phosphorylated Threonine is
247 underlined) in *Chlamydomonas* CAS. Based on these results, phosphorylation could be an
248 important factor to regulate the relocation and/or function of CCM-related proteins.
249 Identifying kinases, phosphorylation sites, and obtaining high-resolution images of these
250 proteins could lead to a better understanding of the regulatory mechanism of suborganellar
251 protein relocation.

252 Another possible mechanism is the structural dynamics of thylakoid membranes. CAS
253 has a hydrophobic sequence that separates the protein sequence into an N-terminus with a
254 Ca²⁺-binding region and a C-terminus with a rhodanese-like domain, and it is thought that
255 CAS anchors to the thylakoid membrane via the hydrophobic sequence (Wang et al. 2016). A
256 recent study revealed that both the structural stability and flexibility of thylakoid membranes
257 is essential for dynamic protein reorganization (Iwai et al. 2014). It is possible that CAS also
258 moves along with the membrane dynamics, although directional movement of the thylakoid
259 membrane from dispersed chloroplast region into the pyrenoid and *vice versa* is unknown.

260 Recently, CAS was detected in a purified fraction of the *Chlamydomonas* eyespot and
261 also involved in regulating the positive phototactic response under continuous illumination
262 (Trippens et al. 2017). Consistent with this result, we first observed that the fluorescence
263 signal of CAS-Clover overlapped with the eyespot *in vivo*. Ca²⁺ influx through the channel
264 rhodopsins in the eyespot region play an important role for the regulation of phototactic
265 behavior, but the primary Ca²⁺ sensing mechanism is unknown. Using our knockout mutant
266 H82, the regulatory roles of CAS associated with the positive phototactic response could be
267 more clarified.

268 It has become clear that the pyrenoid is important not only for CO₂ fixation but also
269 for the regulation of the CCM (Meyer et al. 2017; Mitchell et al. 2017). So far, hundreds of
270 proteins with unknown function have been identified in the purified pyrenoid (Mackinder et
271 al. 2016), and there could be other CCM-related proteins that could relocate in the chloroplast
272 in response to the CO₂ availability as reported previously (Yamano et al. 2010). Further
273 screening of mutants showing aberrant localization patterns of these proteins could lead to

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

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395 **Figure legends**

396 **Fig. 1** Isolation of transgenic lines expressing CAS-Clover. (a) A schematic illustration of the
397 expression plasmid of CAS-Clover. The plasmid was constructed based on the pOptimized
398 Clover vector (Lauersen et al. 2015). Translation start (ATG) and stop (TAA) sites are shown.
399 The genomic sequence of *CAS* is placed at the downstream of *P_{A/R}*, *HSP70A/RBCS2* tandem
400 promoter, which is followed by first intron of *RBCS2*. The nucleotide acid sequence
401 GGCGGCGCGGCCGCGGGC encoding the amino acid sequence Gly-Gly-Ala-Ala-Ala-Gly
402 represents a synthetic flexible linker between CAS and Clover. The expression of CAS-
403 Clover is terminated by the *T_{RBCS2}*, 3'-untranslated region of *RBCS2*. Restriction enzyme sites
404 for cloning of *CAS* (*NdeI* and *BgIII*) and for insertion check of the flexible linker (*NotI*) are
405 shown. (b) Accumulation of CAS and CAS-Clover fusion protein in wild-type (WT), H82,
406 and transformants (CL strains). Cells were grown in low-CO₂ (LC) conditions for 12 h.
407 Histone H3 was used as a loading control. (c) Maximum photosynthetic O₂-evolving activity
408 (V_{max} ; left) and inorganic carbon (Ci) affinity (right) of WT, H82, and CL-2 cells grown in
409 LC conditions for 12 h. Photosynthetic O₂-evolving activity was measured in externally
410 dissolved Ci concentrations at pH 7.8, and the $K_{0.5}$ (Ci), the Ci concentrations required for
411 half V_{max} , were calculated. Data in all experiments are mean values \pm standard deviation from
412 three biological replicates. * $P < 0.001$ by Student's t test.

413
414 **Fig. 2** Fluorescence signals derived from CAS-Clover in high-CO₂ (HC) conditions. (a) CL-2
415 cells were adapted to HC conditions. Defocused images +1.0 μm from the focal plane are
416 shown in the bottom row. Each image is placed with the flagella facing upward on the panel.
417 DIC, differential interference contrast image. Scale bar, 2 μm . (b) Enlarged fluorescence
418 images of the white boxed area in (a) obtained by defocusing the sample from -0.6 to +0.8
419 μm from the focal plane. Scale bar, 400 nm.

420
421 **Fig. 3** Fluorescence signals derived from CAS-Clover in low-CO₂ (LC) conditions. (a) CL-2
422 cells grown in high-CO₂ conditions were transferred to LC conditions for 2 h. Defocused
423 images +1.0 μm from the focal plane are shown in the bottom row. Each image is placed with
424 the flagella facing upward on the panel. White arrowheads indicate the eyespot. DIC,
425 differential interference contrast image. Scale bar, 2 μm . (b) Autofluorescence image of wild-
426 type (WT) cells grown in LC conditions for 2 h. White arrowheads indicate the eyespot
427 region. Scale bar, 2 μm . (bc) Enlarged fluorescence images of the pyrenoid region by
428 defocusing the sample from -0.6 to +0.8 μm from the focal plane. Scale bar, 400 nm. (ed)

429 Different images of CAS-Clover merged with chlorophyll grown in LC conditions for 2 h.

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2 430 (~~e~~) High-contrast image of chlorophyll autofluorescence in the pyrenoid region. Scale bar,

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4 431 400 nm. (~~e~~) Fluorescence images derived from CAS-Clover in LC conditions for 12 h. Scale

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6 432 bar, 2 μ m.

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Conflict of Interest:

The authors declare that they have no conflict of interest.





