

# AtCGL160 recruits chloroplast coupling factor 1

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Running title: AtCGL160 links CF<sub>1</sub> to a CF<sub>o</sub> assembly module

16 **Abstract**

17 ATP synthases couple the generation of chemical energy to a transmembrane electro-  
18 chemical potential. Like ATP synthases in bacteria and mitochondria, chloroplast ATP  
19 synthases consist of a membrane-spanning (CF<sub>0</sub>) and a soluble coupling factor (CF<sub>1</sub>).  
20 Accessory factors facilitate subunit production and orchestrate the assembly of the functional  
21 CF<sub>1</sub>-CF<sub>0</sub> complex. It was previously shown that the accessory factor CGL160 promotes the  
22 formation of plant CF<sub>0</sub> and performs a similar function in the assembly of its c-ring to that of  
23 the distantly related bacterial Atp1/Uncl protein. In this study, we show that the N-terminal  
24 portion of CGL160 (AtCGL160N), which is specific to the green lineage, is required for late  
25 steps in CF<sub>1</sub>-CF<sub>0</sub> assembly in *Arabidopsis thaliana*. In plants that lacked this stroma-exposed  
26 domain, photosynthesis was impaired, and amounts of CF<sub>1</sub>-CF<sub>0</sub> were reduced to about 65%  
27 of the wild-type level. Loss of AtCGL160N did not perturb c-ring formation, but led to a 10-fold  
28 increase in the numbers of CF<sub>1</sub> sub-complexes in the stroma relative to the wild type and the  
29 CF<sub>1</sub> assembly mutant *atcgld11-1*. Co-immunoprecipitation and protein crosslinking assays  
30 revealed an association of AtCGL160 with CF<sub>1</sub> subunits. Yeast two-hybrid assays localized the  
31 interaction to a stretch of AtCGL160N that binds to the thylakoid-proximal domain of CF<sub>1</sub>-β that  
32 includes the conserved DELSEED motif. We therefore propose that AtCGL160 has acquired  
33 an additional function in the recruitment of soluble CF<sub>1</sub> to a membrane-integral CF<sub>0</sub> sub-  
34 complex, which is critical for the modulation of CF<sub>1</sub>-CF<sub>0</sub> activity and photosynthesis in  
35 chloroplasts.

36

37 **Keywords:** chloroplast, photosynthesis, ATP synthase, thylakoid complex, assembly, CF<sub>1</sub>-  
38 CF<sub>0</sub>, Arabidopsis

## 39 Introduction

40 F-type ATP synthases, which utilize chemiosmotic membrane potentials to generate ATP, are  
41 central actors in the energy metabolism of bacteria, mitochondria and chloroplasts. These  
42 biological nanomotors share a largely conserved structure, consisting of a soluble F<sub>1</sub> and a  
43 membrane-bound F<sub>0</sub> moiety. Bacterial and chloroplast ATP synthases (CF<sub>1</sub>-CF<sub>0</sub>) are closely  
44 related with respect to size and subunit composition (Groth and Pohl, 2001; Vollmar et al.,  
45 2009; Hahn et al., 2018) and, in contrast to the multimeric mitochondrial ATP synthases, exist  
46 as monomers in thylakoid membranes (Daum et al., 2010). In the chloroplasts of higher plants,  
47 CF<sub>1</sub>-CF<sub>0</sub> complexes reside exclusively in stroma lamellae and grana-end membranes,  
48 because the ~16-nm stromal extension of CF<sub>1</sub> prevents its incorporation into the tightly packed  
49 grana stacks (Daum et al., 2010).

50 During photophosphorylation, CF<sub>1</sub>-CF<sub>0</sub> complexes couple the light-driven generation of the  
51 trans-thylakoid proton-motive force (*pmf*) to ADP phosphorylation. The membrane-embedded  
52 proteolipidic c<sub>14</sub>-ring, together with the non-covalently bound central stalk γε, form the motor  
53 unit, and drive rotary catalysis by CF<sub>1</sub>. The peripheral stator consists of the subunits a, b and  
54 b', and is connected to the (αβ)<sub>3</sub> unit by the δ subunit, which acts as a flexible hinge between  
55 CF<sub>1</sub> and CF<sub>0</sub> (Murphy et al., 2019). Protons are translocated from the luminal to the stromal  
56 side via two aqueous channels in the a subunit. During translocation, each proton enters the  
57 access channel and binds to a conserved glutamate residue in subunit c. The c<sub>14</sub> motor  
58 executes an almost complete rotation before releasing the proton into the stroma through the  
59 exit channel (Hahn et al., 2018). The counterclockwise rotation of the central stalk in the  
60 vicinity of the hexamer triggers alternating nucleotide-binding affinities in the β subunits that  
61 ultimately drive ATP generation (reviewed in von Ballmoos et al., 2009; Junge and Nelson,  
62 2015).

63 As a result of extensive organellar gene transfer during plant evolution, three CF<sub>1</sub>-CF<sub>0</sub> subunits  
64 (b', γ, δ) are encoded in the nuclear genome, while the remaining CF<sub>1</sub>-CF<sub>0</sub> genes are organized  
65 into two plastid operons. Consequently, two different gene-expression systems must be tightly  
66 coordinated with the chloroplast protein import machinery for efficient CF<sub>1</sub>-CF<sub>0</sub> biogenesis.  
67 Several CF<sub>1</sub>-CF<sub>0</sub> auxiliary factors involved in plastid gene expression have been identified,  
68 including proteins involved in mRNA processing (AEF1), mRNA stabilization (PPR10, BFA2)  
69 and translation initiation (ATP4, TDA1) (Pfalz et al., 2009; Eberhard et al., 2011; Zoschke et  
70 al., 2012; Yap et al., 2015; Zhang et al., 2019). Moreover, CF<sub>1</sub>-CF<sub>0</sub> assembly factors ensure  
71 correct complex stoichiometry, and prevent the accumulation of dead-end products or harmful  
72 intermediates that could lead to wasteful ATP hydrolysis or *pmf* dissipation.

73 As in the case of the bacterial assembly model, plastid CF<sub>1</sub>-CF<sub>0</sub> complexes are constructed  
74 from different intermediates or modules (reviewed in Rühle and Leister, 2015). CF<sub>1</sub> assembly  
75 was first examined using in-vitro reconstitution assays, and was shown to be initiated by  $\alpha/\beta$   
76 dimerization in a chaperone-assisted process (Chen and Jagendorf, 1994). CF<sub>1</sub> formation  
77 depends on CGLD11/BFA3, which is specific to green plants, interacts with the hydrophobic  
78 catalytic site of the  $\beta$ -subunit and may prevent aggregation or formation of unfavorable  
79 homodimers (Grahl et al., 2016; Zhang et al., 2016). Moreover, PAB (Mao et al., 2015) and  
80 BFA1 (Zhang et al., 2018) have been proposed to be required for efficient incorporation of the  
81  $\gamma$  subunit into CF<sub>1</sub>.

82 Less is known about CF<sub>0</sub> assembly, and only one accessory factor – CONSERVED ONLY IN  
83 THE GREEN LINEAGE 160 (CGL160) – has been identified so far (Rühle et al., 2014).  
84 Absence of CGL160 in the *Arabidopsis thaliana* mutant *atcgl160-1* is associated with a  
85 significant reduction (70-90%) in wild-type CF<sub>1</sub>-CF<sub>0</sub> levels, and CF<sub>0</sub>-c subunits accumulate as  
86 monomers. Moreover, split-ubiquitin assays have provided evidence that AtCGL160 interacts  
87 with CF<sub>0</sub>-c and CF<sub>0</sub>-b. It was therefore concluded that AtCGL160 is required for efficient  
88 formation of the c-ring in chloroplasts and shares this function with its distantly related bacterial  
89 counterpart Atp1/Uncl (Suzuki et al., 2007; Ozaki et al., 2008). Furthermore, AtCGL160 was  
90 suggested to participate in CF<sub>1</sub> assembly into the holo-complex, based on CF<sub>1</sub> subcomplex co-  
91 migration and crosslinking experiments using a putatively specific anti-AtCGL160 antibody  
92 (Fristedt et al., 2015).

93 In this study, the function of the N-terminal domain that is conserved in all CGL160 proteins  
94 from the green lineage was investigated in *Arabidopsis*. The results demonstrate that this  
95 domain (AtCGL160N) mediates the critical connection of CF<sub>1</sub> to CF<sub>0</sub> assembly modules by  
96 interacting with subunit  $\beta$ . Thus, CGL160 emerges as a key auxiliary factor that not only  
97 promotes CF<sub>0</sub> formation, but is also involved in late CF<sub>1</sub>-CF<sub>0</sub> assembly steps.

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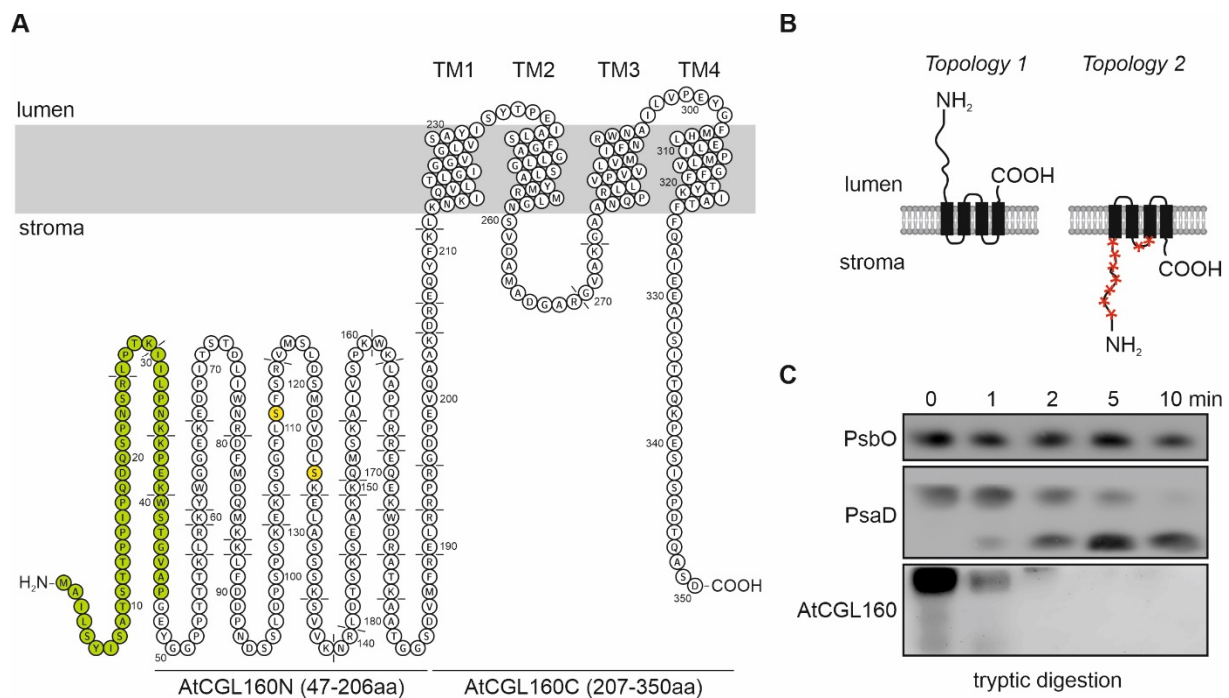
## 99 **Results**

100

### 101 The N-terminal moiety of AtCGL160 is required for efficient photosynthesis and CF<sub>1</sub>- 102 CF<sub>0</sub> functionality

103 CGL160 was identified based on its coregulation with photosynthetic genes in ATTED-II  
104 (Obayashi et al., 2009) and its affiliation to the GreenCut suite of proteins (Merchant et al.,  
105 2007; Karpowicz et al., 2011). The C-terminal transmembrane segment of CGL160 (~15 kDa)  
106 is distantly related to bacterial Atp1/Uncl (Rühle et al., 2014; Fristedt et al., 2015), whereas the  
107 larger N-terminal portion of the protein sequence is only conserved in algae, bryophytes, and

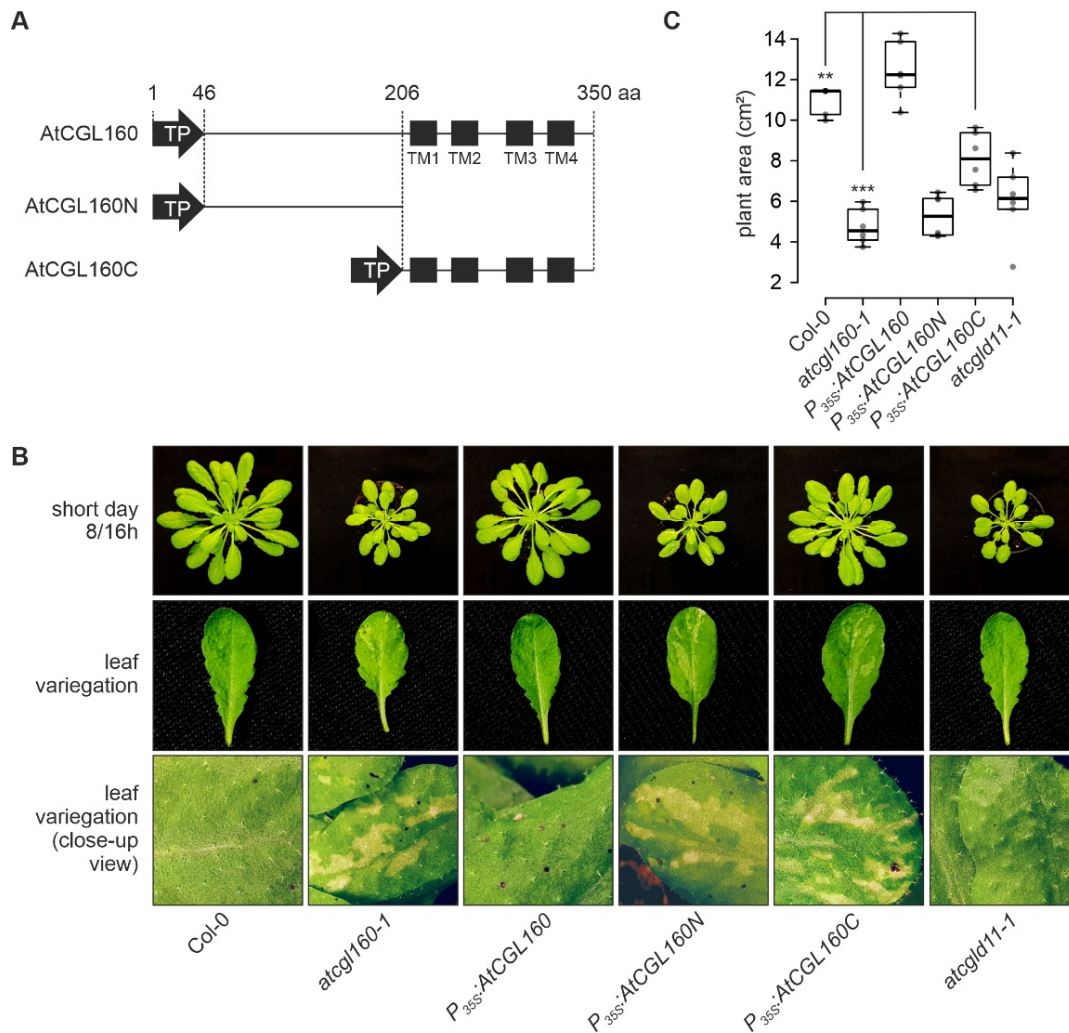
108 higher plants (Supplemental Fig. 1). This latter domain of ~200 amino acids (aa) in *Arabidopsis*  
 109 *thaliana* (AtCGL160N) includes a predicted N-terminal chloroplast transit peptide (cTP) of 46  
 110 aa (Emanuelsson et al., 1999), and mass spectrometry has identified several phosphorylated  
 111 peptides which are derived from positions 106-134 (Reiland et al., 2009; Reiland et al., 2011;  
 112 Roitinger et al., 2015). Indeed, two conserved putative phosphorylation sites were found in a  
 113 multiple sequence alignment of CGL160 homologs from species across the green lineage,  
 114 which correspond to positions S111 and S126 in AtCGL160 (Fig. 1, Supplemental Fig. 1).



115  
 116 **Figure 1. Topology of AtCGL160 and trypsin cleavage-site prediction.** **A**, Transmembrane  
 117 (TM) domain predictions were obtained from the AtCGL160 UniProt protein accession  
 118 O82279. Putative trypsin cleavage sites are highlighted in dashed lines and amino-acid  
 119 positions are indicated. The topology was drawn for the full-length sequence of AtCGL160  
 120 including the predicted transit peptide (green) with Protter (Omasits et al., 2014). Two  
 121 conserved serine residues (S111 and S126) are marked in yellow. **B**, Representation of two  
 122 putative AtCGL160 topologies. The four transmembrane domains are indicated as black  
 123 boxes. Accessible trypsin digestion sites are highlighted by red stars. **C**, Immunoblot of  
 124 thylakoid membranes of the WT (Col-0) fractionated by SDS-PAGE, untreated (0 min) or  
 125 treated with trypsin for 1, 2, 5 and 10 min. Blots were probed with antibodies against the lumen-  
 126 oriented PSII subunit PsbO, the stroma-exposed PSI subunit PsaD and AtCGL160.  
 127

128 Earlier studies have provided experimental evidence for the localization of AtCGL160 to the  
 129 thylakoid membrane (Rühle et al., 2014; Tomizioli et al., 2014; Fristedt et al., 2015). To gain  
 130 further insights into the topology of AtCGL160, a protease protection assay was carried out  
 131 (Fig. 1B, C). In the case of topology 1, all trypsin cleavage sites in AtCGL160 reside in the  
 132 lumen of the thylakoid and remain fully protected from proteolytic degradation (Fig. 1B).  
 133 Conversely, the stromal orientation of AtCGL160N predicted for topology 2 would expose  
 134 trypsin cleavage sites and lead to degradation products of less than 2 kD (Fig. 1A). To test the  
 135 accessibility of native AtCGL160N, wild-type thylakoids were isolated and treated with trypsin

136 for 10 min (Fig. 6B). As expected, the luminal PSII subunit PsbO was not affected by the  
 137 enzyme, whereas the stromally exposed PSI subunit PsaD was susceptible to the protease.  
 138 AtCGL160N was also efficiently digested, leaving no detectable proteolytic cleavage products,  
 139 which is consistent with protrusion of the entire N-terminal domain into the stroma, as shown  
 140 in topology 2 (Fig. 6A,B).



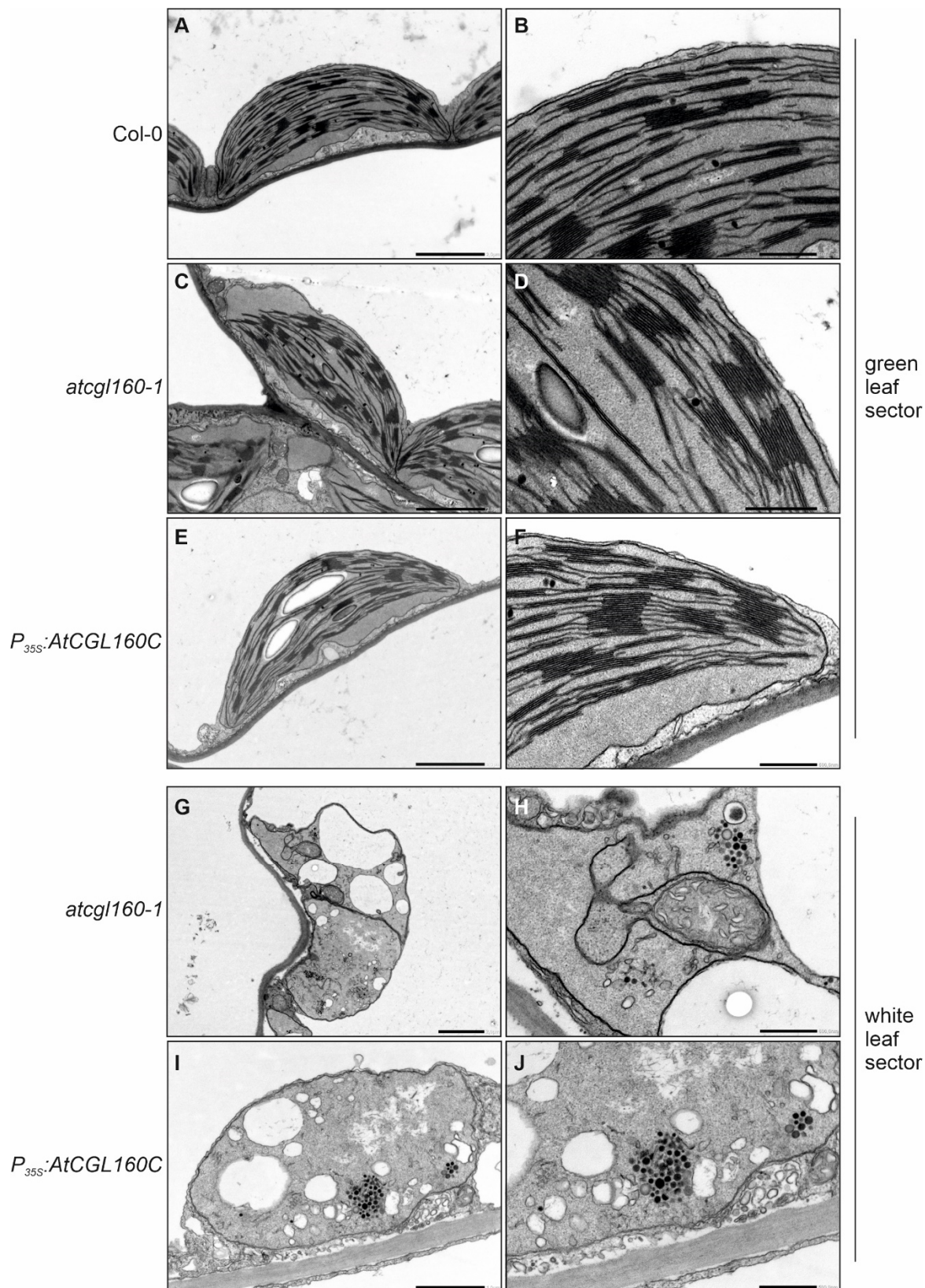
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142 **Figure 2. Growth phenotype and leaf variegation of  $P_{35S}::AtCGL160$ ,  $P_{35S}::AtCGL160N$  and**  
 143  **$P_{35S}::AtCGL160C$  plants under short-day conditions. **A**, Schematic representations of**  
 144 **reintroduced AtCGL160 coding sequences. Plants lacking AtCGL160 were transformed with**  
 145 **overexpressor constructs harboring the coding sequences for the full-length AtCGL160**  
 146 **( $P_{35S}::AtCGL160$ ) and its N- ( $P_{35S}::AtCGL160N$ ) and C-terminal ( $P_{35S}::AtCGL160C$ ) segments.**  
 147 **Transcription was under the control of the 35S CaMV promoter and targeting to the chloroplast**  
 148 **was mediated by the transit peptide of AtCGL160 (TP). Amino-acid positions are indicated and**  
 149 **predicted transmembrane domains (TM1-TM4) are schematically shown as black boxes. **B**,**  
 150 **Leaf morphology of Col-0, *atcgl160-1*,  $P_{35S}::AtCGL160$ ,  $P_{35S}::AtCGL160N$ ,  $P_{35S}::AtCGL160C$  and**  
 151 ***atcgld11-1* plants. **C**, Leaf areas of 6 individual plants per genotype were determined 4 weeks**  
 152 **after germination. The horizontal lines represent the median, and boxes indicate the 25th and**  
 153 **75th percentiles. Whiskers extend the interquartile range by a factor of 1.5 $\times$ , and outliers are**  
 154 **represented by dots. The effect of the deletion of AtCGL160N in  $P_{35S}::AtCGL160C$  plants on**  
 155 **growth under short-day conditions was tested by paired sample t-test (two-sided). Statistically**  
 156 **significant differences are marked with asterisks (\* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001).**  
 157

157

158 To dissect the function of the N-terminal portion of AtCGL160, three different constructs under  
159 control of the 35S promoter were cloned, transformed into the *atcgl160-1* background and  
160 screened for complementation (Fig. 2, Supplemental Fig. 2A). Plants that overexpressed the  
161 full-length coding sequence (CDS) of *AtCGL160* served as controls ( $P_{35S}:AtCGL160$ ), while  
162 the other two genotypes expressed either the CDS of the N-terminal ( $P_{35S}:AtCGL160N$ ) or the  
163 C-terminal segment ( $P_{35S}:AtCGL160C$ ) of the protein (Supplemental Fig. 2B, C). In the case of  
164  $P_{35S}:AtCGL160C$  plants, targeting of the truncated version to chloroplasts was achieved by  
165 fusing the CDS of the AtCGL160-derived cTP (1-46 aa) to that of AtCGL160C (Fig. 2A). As  
166 was previously demonstrated in complementation analyses with  $P_{35S}:AtCGL160-eGFP$  lines  
167 (Rühle et al., 2014), overexpression of the full-length *AtCGL160* rescued the *atcgl160-1*  
168 phenotype (Supplemental Fig. 2A), as indicated by wild-type-like growth and restored leaf  
169 morphology under short-day conditions (Fig. 2B, C).  $P_{35S}:AtCGL160N$  failed to complement  
170 the mutant phenotype (Fig. 2B, C, Supplemental Fig. 2A) and AtCGL160N could not be  
171 detected in either stromal or thylakoid extracts (Supplemental Fig. 3). Since *AtCGL160N*  
172 transcripts were present in WT-like amounts in  $P_{35S}:AtCGL160N$  plants (Supplemental Fig.  
173 2C), the lack of AtCGL160N is probably due to proteolytic degradation owing to its inability to  
174 associate correctly with thylakoids. Nevertheless,  $P_{35S}:AtCGL160N$  plants were retained and  
175 served as an additional AtCGL160 knockout control.  $P_{35S}:AtCGL160C$  plants with similar  
176 overexpression rates to  $P_{35S}:AtCGL160$  plants (Supplemental Fig. 2B, C) were characterized  
177 by a significant increase in leaf area compared to the mutant background *atcgl160-1*, but were  
178 growth-retarded with respect to the wild-type control. Interestingly, like *atcgl160-1*,  
179  $P_{35S}:AtCGL160C$  plants developed a variegated phenotype in old leaves, which was not found  
180 either in the wild type or in the CF<sub>1</sub> assembly mutant *atcgld11-1* (Grahl et al., 2016) under  
181 short-day conditions (Fig. 2B).

182 To analyze the leaf phenotype in more detail, we carried out electron microscopic analyses of  
183 Col-0, *atcgl160-1* and  $P_{35S}:AtCGL160C$  plants (Fig. 3). In these genotypes, the chloroplast  
184 ultrastructure in preparations from green leaf sections was unchanged with regard to thylakoid  
185 content, curvature and grana organization (Fig. 3 A-F). These observations in *atcgl160-1*,  
186 together with previous ultrastructural analyses of the CF<sub>1</sub> assembly mutant line *atcgld11-1*  
187 (Grahl et al., 2016) and spinach chloroplasts (Daum et al., 2010), support the idea that CF<sub>1</sub>-  
188 CF<sub>0</sub> complexes are not physically involved in thylakoid curvature formation. Examination of  
189 white leaf sections in *atcgl160-1* and  $P_{35S}:AtCGL160C$  revealed the absence of thylakoids in  
190 plastids, accompanied by the appearance of plastoglobuli in densely packed stromal clusters  
191 (Fig. 3 G-J). Furthermore, large vesicles were observed, which also point to increased  
192 catabolic activity and degradation processes in *atcgl160-1* and  $P_{35S}:AtCGL160C$  plastids.  
193 Another finding was the inclusion of mitochondria in degraded plastids, which was also  
194 observed, to a lesser extent, in white leaf sectors of  $P_{35S}:AtCGL160C$ .



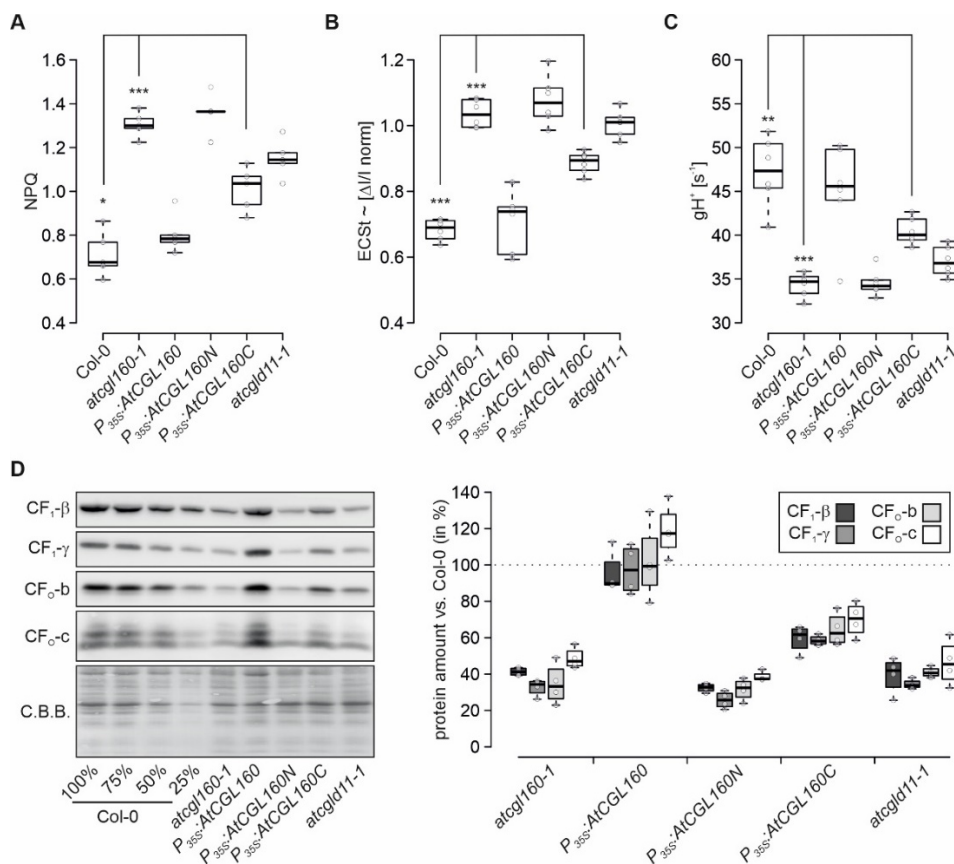
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196 **Figure 3. Plastid ultrastructure in white leaf sectors is altered in the absence of**  
197 **AtCGL160N under short-day growth conditions.** Electron micrographs of samples from  
198 green leaf sections obtained from Col-0 (A, B), *atcgl160-1* (C, D) and *P<sub>35S</sub>:AtCGL160C* (E, F)  
199 plants. The ultrastructure of chloroplasts was further examined in samples of white leaf  
200 sections obtained from *atcgl160-1* (G, H) and *P<sub>35S</sub>:AtCGL160C* (I, J) plants. The photos on the  
201 right show enlargements of the images on the left. The scale bar corresponds to 2 μm in A, C,  
202 E, and G, 1 μm in I and 0.5 μm in B, D, F, H and J.



203

204 To test whether disruption of AtCGL160N impairs photosynthesis and CF<sub>1</sub>-CF<sub>0</sub> activity,  
 205 measurements of chlorophyll *a* fluorescence and electrochromic shift (ECS) were carried out  
 206 on Col-0, *atcgl160-1*, *P<sub>35S</sub>:AtCGL160*, *P<sub>35S</sub>:AtCGL160N*, *P<sub>35S</sub>:AtCGL160C* and *atcgld11-1*  
 207 plants (Fig. 4A-C). As expected, the CF<sub>1</sub>-CF<sub>0</sub> assembly mutants *atcgl160-1* and *atcgld11-1*  
 208 showed higher heat dissipation (indicated as non-photochemical quenching, NPQ) and  
 209 increased proton-motive force (*pmf*), but lower proton conductivity (gH<sup>+</sup>) through the thylakoid  
 210 membrane compared to the wild-type control. *P<sub>35S</sub>:AtCGL160* and *P<sub>35S</sub>:AtCGL160N* plants  
 211 displayed similar levels of NPQ, *pmf* and gH<sup>+</sup> to the wild type and the CF<sub>1</sub>-CF<sub>0</sub> assembly  
 212 mutant *atcgld11-1*, respectively. Notably, photosynthetic parameters were only partially  
 213 restored in *P<sub>35S</sub>:AtCGL160C* lines.



214

215 **Figure 4. Lack of AtCGL160N perturbs photosynthesis and CF<sub>1</sub>-CF<sub>0</sub> integrity.** **A**, Heat  
 216 dissipation (non-photochemical quenching, NPQ) in Col-0, *atcgl160-1*, *P<sub>35S</sub>:AtCGL160*,  
 217 *P<sub>35S</sub>:AtCGL160N*, *P<sub>35S</sub>:AtCGL160C* and *atcgld11-1* plants grown under short-day conditions.  
 218 NPQ values from five plants per genotype were determined 105 s after light induction (145  
 219 μmol photons m<sup>-2</sup> s<sup>-1</sup>) using an Imaging-PAM system (Walz). **B**, Dark-interval relaxation  
 220 kinetics (DIRK) derived from ECS signals were recorded after 10 min of illumination from six  
 221 individual plants grown under short-day conditions. Total amplitude of the P515 differential  
 222 absorption signal was normalized to a single turnover flash 4 min after the ECS measurement.  
 223 **C**, Proton conductivity of the thylakoid membrane was determined from ECS signal relaxation  
 224 rates, which were fitted to a first-order decay function. Calculated rate constants were  
 225 expressed as gH<sup>+</sup> [s<sup>-1</sup>]. **D**, Steady-state levels of immunodetected CF<sub>1</sub>-CF<sub>0</sub> marker subunits.  
 226 After fractionation of thylakoid proteins on SDS-PAGE and transfer to PVDF membranes, blots  
 227 were probed with antibodies against CF<sub>1</sub>-β, CF<sub>1</sub>-γ, CF<sub>0</sub>-b, and CF<sub>0</sub>-c. Coomassie Brilliant Blue

228 (C.B.B.) staining is shown as loading control. For quantification, signals from four technical  
229 replicates of each marker subunit were normalized to signals detected in Col-0 samples.  
230 Horizontal lines represent the median, and boxes indicate the 25th and 75th percentiles.  
231 Whiskers extend the interquartile range by 1.5 $\times$ . The effect of the deletion of AtCGL160N on  
232 photosynthetic parameters of *P*<sub>35S</sub>:AtCGL160C plants shown in panels **A-C** was tested in  
233 paired-sample t-tests (two-sided). Statistically significant differences are marked with asterisks  
234 (\**P*<0.05, \*\**P*<0.01, and \*\*\**P*<0.001).  
235

236 To assess the integrity of the CF<sub>1</sub>-CF<sub>0</sub> complex in thylakoids, marker subunits were  
237 immunodetected in *atcgl160-1*, *P*<sub>35S</sub>:AtCGL160, *P*<sub>35S</sub>:AtCGL160N, *P*<sub>35S</sub>:AtCGL160C and  
238 *atcgl11-1* plants, and quantified relative to Col-0 samples (Fig. 4D). Levels of CF<sub>1</sub>- $\beta$ , CF<sub>1</sub>- $\gamma$ ,  
239 CF<sub>0</sub>-b and CF<sub>0</sub>-c were restored to normal in *P*<sub>35S</sub>:AtCGL160, but reduced to about 60-65% of  
240 wild-type amounts in *P*<sub>35S</sub>:AtCGL160C plants. Transformation with the *P*<sub>35S</sub>:AtCGL160N  
241 construct had no effect on CF<sub>1</sub>-CF<sub>0</sub> subunit levels in the *atcgl160-1* mutant.

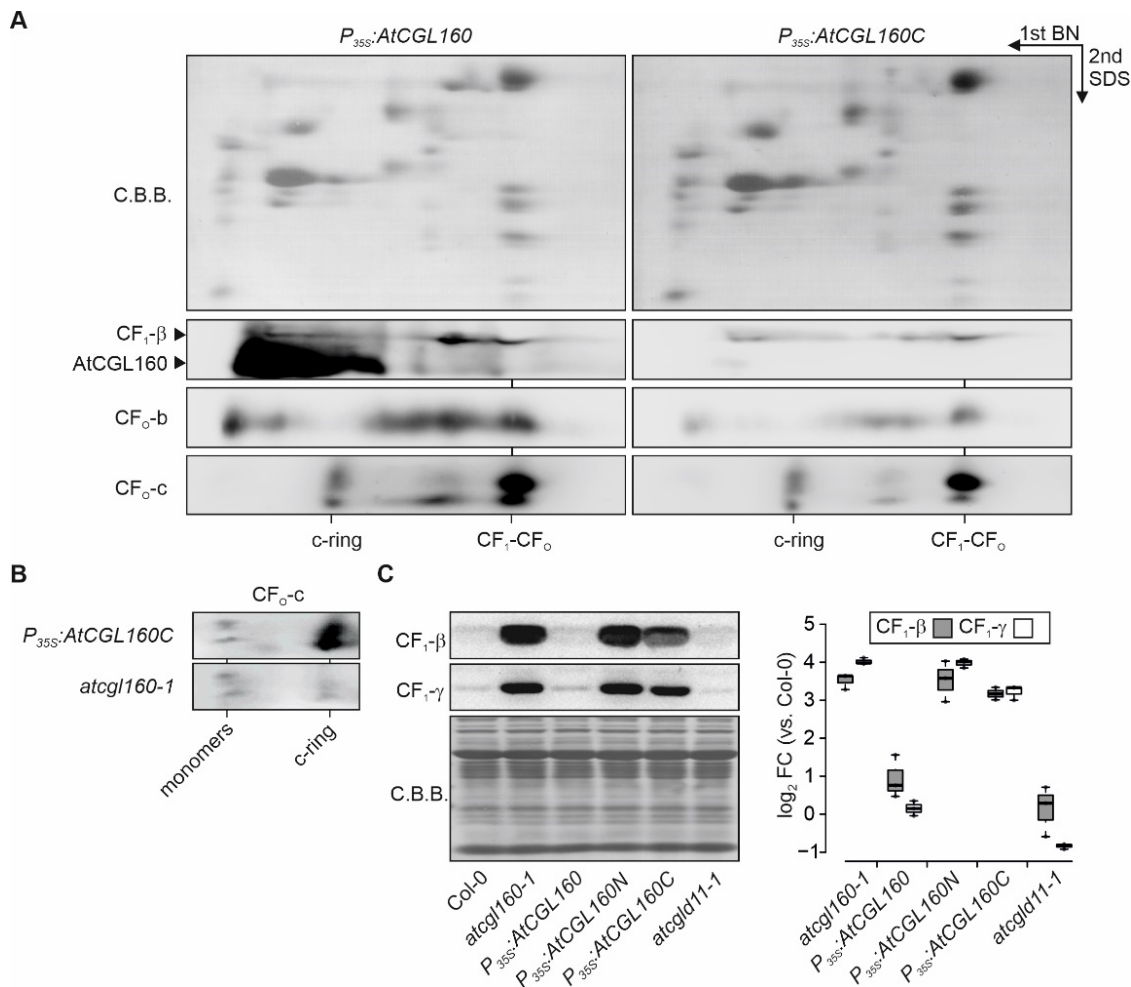
242 Overall, overexpression of the Atp1/Unc1-like AtCGL160 domain alone (AtCGL160C) in the  
243 *atcgl160-1* background only partially restored CF<sub>1</sub>-CF<sub>0</sub> amounts (Fig. 4D) and activity (Fig.  
244 4C). Consequently,  $\Delta$ pH-dependent quenching mechanisms (Fig. 4A) were more highly  
245 activated, resulting in downregulation of photosynthesis (Fig. 4B) and growth impairment of  
246 *P*<sub>35S</sub>:AtCGL160C plants. We deduced from these results that AtCGL160N might also be  
247 involved in CF<sub>1</sub>-CF<sub>0</sub> assembly at steps other than CF<sub>0</sub>-c ring formation.  
248

#### 249 Stromal accumulation of CF<sub>1</sub> in the absence of AtCGL160N

250 To investigate the effects of deletion of AtCGL160N on CF<sub>1</sub>-CF<sub>0</sub> assembly, we performed  
251 BN/SDS-PAGE (2D-PAGE) analysis on thylakoids isolated from *P*<sub>35S</sub>:AtCGL160 and  
252 *P*<sub>35S</sub>:AtCGL160C plants grown under short-day conditions. Consistent with the accumulation  
253 of CF<sub>1</sub>-CF<sub>0</sub> marker subunits in Fig. 4D, CF<sub>1</sub>- $\beta$ , CF<sub>0</sub>-b and CF<sub>0</sub>-c levels were reduced in  
254 *P*<sub>35S</sub>:AtCGL160C compared to plants that overexpressed the full-length CDS of AtCGL160  
255 (Fig. 5A). No accumulation of pre-complexes was observed, as amounts of free proteins, and  
256 components of the c-ring, CF<sub>1</sub> and the holo-complex were reduced uniformly. To assess the  
257 assembly status of the c-ring in more detail, we carried out 2D-PAGE with increased amounts  
258 of *atcgl160-1* and *P*<sub>35S</sub>:AtCGL160C thylakoids (Fig. 5B). C-ring levels were considerably higher  
259 in *P*<sub>35S</sub>:AtCGL160C than in the *atcgl160-1* mutant background. We also examined CF<sub>1</sub>  
260 accumulation in the stroma of Col-0, *P*<sub>35S</sub>:AtCGL160, *P*<sub>35S</sub>:AtCGL160N, *P*<sub>35S</sub>:AtCGL160C and  
261 *atcgl11-1* plants (Fig. 5C), since CF<sub>1</sub>-CF<sub>0</sub> assembly takes place in a modular fashion and  
262 involves distinct thylakoid-integral and soluble intermediates. Strikingly, CF<sub>1</sub>- $\beta$  and CF<sub>1</sub>- $\gamma$  were  
263 enriched about 10-fold in the stroma of *atcgl160-1*, *P*<sub>35S</sub>:AtCGL160N and *P*<sub>35S</sub>:AtCGL160C, but  
264 were detected in close to wild-type levels in *P*<sub>35S</sub>:AtCGL160 and *atcgl11-1* plants. In-depth  
265 2D-PAGE analysis of CF<sub>1</sub> intermediates in *atcgl160-1*, and comparison with results from the  
266 co-migration database for photosynthetic organisms (PCom-DB, Takabayashi et al., 2017),

267 revealed that in *atcgl160-1* stromal CF<sub>1</sub>-β and CF<sub>1</sub>-γ were predominantly present in an α<sub>3</sub>β<sub>3</sub>γε  
 268 complex that lacked subunit CF<sub>1</sub>-δ (Supplemental Fig. S4).

269 We concluded that re-introduction of the transmembrane Atp1/Unc1-like domain of AtCGL160  
 270 restores c-ring formation, but leads to an overall reduction in CF<sub>1</sub>-CF<sub>0</sub> levels due to a defect in  
 271 the attachment of CF<sub>1</sub> to a membrane-integral CF<sub>0</sub> intermediate.

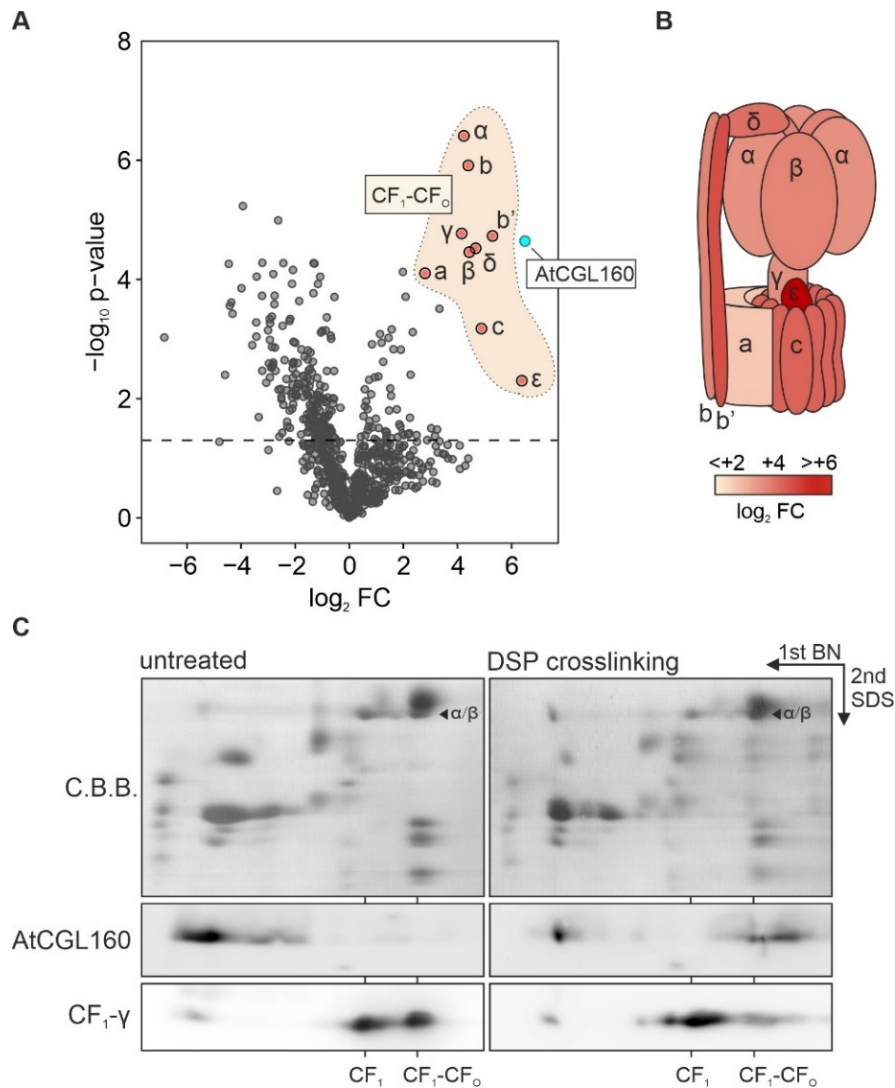


272

273 **Figure 5. CF<sub>1</sub>-CF<sub>0</sub> assembly is perturbed in the absence of AtCGL160N.** **A**, Thylakoid  
 274 complexes from *P<sub>35S</sub>::AtCGL160* and *P<sub>35S</sub>::AtCGL160C* plants were solubilized with *n*-dodecyl  
 275 β-D-maltoside (1% [w/v]) and further separated by Blue-Native (BN, 1<sup>st</sup> dimension) and SDS-  
 276 PAGE (SDS, 2<sup>nd</sup> dimension). After protein transfer, PVDF membranes were probed with  
 277 antibodies against CF<sub>1</sub>-β, CF<sub>0</sub>-b and CF<sub>0</sub>-c, and CF<sub>1</sub>-β blots were subsequently exposed to  
 278 anti-AtCGL160 antibodies. Positions of the ATP synthase holo-complex (CF<sub>1</sub>-CF<sub>0</sub>) and the c-  
 279 ring are indicated. Coomassie Brilliant Blue G-250 (C.B.B.) staining of PVDF membranes is  
 280 shown as loading control. **B**, C-ring assembly in *atcgl160-1* and *P<sub>35S</sub>::AtCGL160C* plants.  
 281 Increased amounts of thylakoid complexes (corresponding to 120 μg total chlorophyll) were  
 282 solubilized and fractionated by BN/SDS-PAGE. Blots were probed with an antibody against  
 283 CF<sub>0</sub>-c. Positions of free c-monomers and the assembled c-ring are indicated. **C**, CF<sub>1</sub>-β and  
 284 CF<sub>1</sub>-γ enrichment in stromal extract, which was isolated from Col-0, *atcgl160-1*,  
 285 *P<sub>35S</sub>::AtCGL160*, *P<sub>35S</sub>::AtCGL160N*, *P<sub>35S</sub>::AtCGL160C* and *atcgl11-1* rosette leaves. Signals of  
 286 three CF<sub>1</sub>-β and CF<sub>1</sub>-γ immunodetection assays were quantified and are shown on a  
 287 logarithmic scale. Horizontal lines represent the median, boxes indicate the 25th and 75th  
 288 percentiles and whiskers extend the interquartile range by a factor of 1.5×.

289

290 AtCGL160 interacts physically with CF<sub>1</sub>-containing complexes



291  
 292 **Figure 6. AtCGL160 association with CF<sub>1</sub> subunits.** **A**, Co-immunoprecipitation analyses  
 293 were carried out with solubilized thylakoids isolated from *P<sub>35S</sub>:AtCGL160*, while  
 294 *P<sub>35S</sub>:AtCGL160C* plants served as the negative control. Co-immunoprecipitated proteins were  
 295 further subjected to tryptic digestion, and peptides were analyzed by liquid chromatography  
 296 coupled to mass spectrometry. Data for differentially enriched proteins are presented in a  
 297 volcano plot. The relative abundance ( $\log_2$  fold change [ $\log_2$  FC]) of proteins co-  
 298 immunoprecipitated from *P<sub>35S</sub>:AtCGL160* samples is plotted against their statistically  
 299 significant enrichment as Benjamini-Hochberg corrected p-values ( $-\log_{10}$  p-value). The dashed  
 300 line indicates a negative  $\log_{10}$  p-value of 1.5, and was defined as the threshold for robust  
 301 reliability of differences in co-immunoprecipitation data. Blue and red dots highlight  
 302 quantification results for AtCGL160 and CF<sub>1</sub>-CF<sub>0</sub> subunits, respectively. **B**, Schematic  
 303 representation of differentially enriched subunits in a CF<sub>1</sub>-CF<sub>0</sub> cartoon. Relative amounts of  
 304 co-immunoprecipitated CF<sub>1</sub>-CF<sub>0</sub> subunits are shown in colors on a  $\log_2$  FC scale from white  
 305 ( $\log_2$  FC < 2) to red ( $\log_2$  FC > 6). Co-immunoprecipitation assays were carried out on three  
 306 independent biological replicates. **C**, Co-migration of AtCGL160 with CF<sub>1</sub>-CF<sub>0</sub> in crosslinking  
 307 experiments. Two-dimensional BN/SDS-PAGE analysis was used to compare untreated  
 308 thylakoid extracts of the WT (Col-0) with extracts that had been crosslinked with  
 309 dithiobis(succinimidyl propionate) (DSP). Blots of the second dimension were probed with  
 310 antibodies against AtCGL160 and CF<sub>0</sub>- $\gamma$ . The positions of CF<sub>1</sub>-CF<sub>0</sub>, the CF<sub>1</sub> intermediate, and  
 311 the free protein fraction are indicated based on the mobility of  $\alpha/\beta$  on the C.B.B. stained gel.

312

313 To pinpoint the role of AtCGL160 in the recruitment of CF<sub>1</sub> to a membrane-integral CF<sub>O</sub>  
314 intermediate, protein interactions were assessed in co-immunoprecipitation (co-IP) assays  
315 (Fig. 6B). Quantitative data for precipitated proteins were obtained by tryptic digestion and  
316 subsequent peptide-fragment analysis using liquid chromatography coupled to mass  
317 spectrometry. Since the commercially available AtCGL160 antibody (Agrisera AS12 1853)  
318 displayed non-specific binding to either CF<sub>1</sub>- $\alpha$  or CF<sub>1</sub>- $\beta$  (Supplemental Fig. 3), an AtCGL160  
319 antibody with no significant cross-reactions to other thylakoid proteins was generated  
320 (Supplemental Fig. 3). In the first step of antibody production, the N-terminal part of AtCGL160  
321 (AtCGL160<sub>29-206aa</sub>) was fused to the maltose-binding protein and injected into rabbits. In the  
322 second step, antibodies specific for AtCGL160<sub>29-206aa</sub> were affinity-purified from rabbit antisera  
323 using an immobilized fusion protein consisting of AtCGL160<sub>29-206aa</sub> and glutathione S-  
324 transferase. As expected, when the resulting antibody fraction was tested in immunodetection  
325 assays, it showed only one distinct signal in the WT sample, which was enriched in extracts of  
326 *P<sub>35S</sub>:AtCGL160*, but was absent in both the *atcgl160-1* mutant and in *P<sub>35S</sub>:AtCGL160C* samples  
327 (Supplemental Fig. 3).

328 Next, NP40-solubilized thylakoid proteins from *P<sub>35S</sub>:AtCGL160* plants grown under short-day  
329 conditions were chosen as co-IP input and pulled-down protein amounts were compared to  
330 those recovered in co-IP experiments carried out on thylakoid protein extracts of  
331 *P<sub>35S</sub>:AtCGL160C*. Plants devoid of AtCGL160 were not considered for use as negative  
332 controls, since the reduction in CF<sub>1</sub>-CF<sub>O</sub> levels observed in *atcgl160-1* (and *P<sub>35S</sub>:AtCGL160N*)  
333 (Fig. 4D) might lead to misinterpretation of differential co-IP experiments. As expected,  
334 AtCGL160 was pulled down efficiently from *P<sub>35S</sub>:AtCGL160C* extracts (log<sub>2</sub> FC ~6.5).  
335 Moreover, all CF<sub>1</sub>-CF<sub>O</sub> subunits were identified in co-IPs (Fig. 6A,B) with high differential  
336 enrichment levels for the subunits  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , b, b' and c (log<sub>2</sub> FC > 4.4). Subunit CF<sub>O</sub>-a was  
337 co-immunoprecipitated at lower levels (log<sub>2</sub> FC ~2.8). The pull-down of CF<sub>1</sub> subunits was  
338 confirmed by immunodetection assays of the two marker subunits CF<sub>1</sub>- $\beta$  and CF<sub>1</sub>- $\gamma$ , which  
339 were only detectable in co-IP output fractions obtained from *P<sub>35S</sub>:AtCGL160* samples  
340 (Supplemental Fig. 5). Other known CF<sub>1</sub>-CF<sub>O</sub> assembly factors were not co-  
341 immunoprecipitated (Supplemental Table 3), indicating that AtCGL160 is associated with a  
342 late CF<sub>1</sub>-CF<sub>O</sub> assembly stage or the fully assembled complex from which other auxiliary factors  
343 had already dissociated.

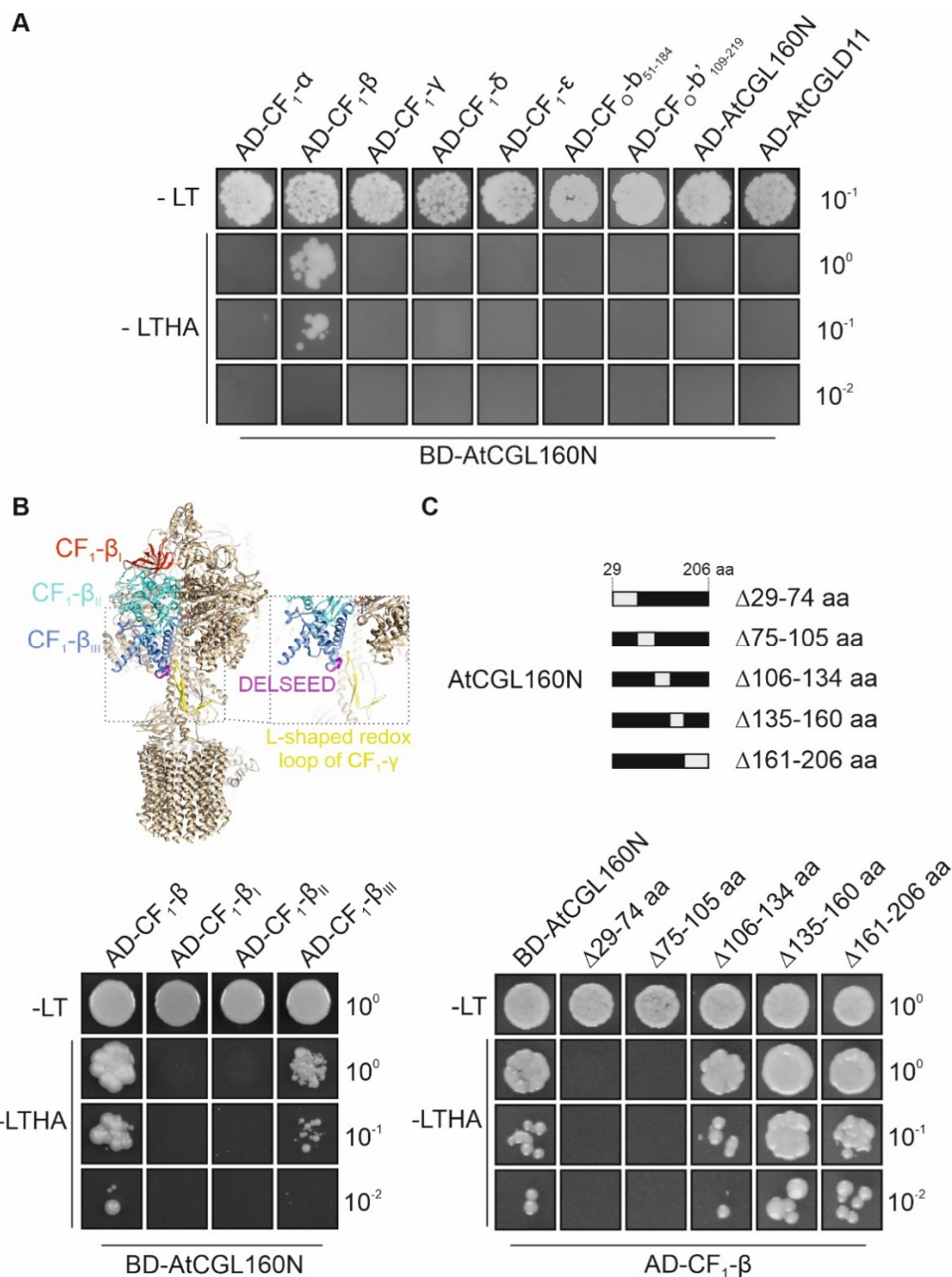
344 To confirm the association of AtCGL160 with CF<sub>1</sub>-containing complexes, crosslinking  
345 experiments were also carried out (Fig. 6C). To this end, thylakoid membranes of wild-type  
346 plants were treated with the crosslinker dithiobis(succinimidyl propionate) (DSP), and  
347 subsequently subjected to 2D-PAGE and immunodetection of AtCGL160 and CF<sub>1</sub>-CF<sub>O</sub> marker  
348 subunits. In analyses with untreated thylakoid samples, AtCGL160 migrated predominantly in

349 the monomer fraction. After crosslinking, AtCGL160 could be detected at a molecular mass  
350 range which corresponded to that of the CF<sub>1</sub>-CF<sub>0</sub> holo-complex.

351 In summary, co-IP of all CF<sub>1</sub>-CF<sub>0</sub> subunits with an AtCGL160-specific antibody, together with  
352 the observation that AtCGL160 co-migrated with the CF<sub>1</sub>-CF<sub>0</sub> holo-complex after DSP cross-  
353 linking, corroborates the involvement of AtCGL160 in the functional integration of CF<sub>1</sub> into the  
354 holo-complex at a late step in CF<sub>1</sub>-CF<sub>0</sub> assembly.

355

### 356 AtCGL160N interacts with CF<sub>1</sub>-β in yeast two-hybrid assays



357

358 **Figure 7. AtCGL160N interaction studies in yeast two-hybrid assays.** **A**, Interactions of  
359 AtCGL160 with CF<sub>1</sub>-CF<sub>0</sub> structural components exposed on the stromal side of thylakoids were  
360 tested by transformation of a construct that fuses AtCGL160N to the GAL4 DNA-binding

361 domain (BD-AtCGL160). Cells were then co-transformed with constructs coding for GAL4  
362 activation domain (AD) fused to CF<sub>1</sub>- $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , CF<sub>O</sub>-b<sub>51-184</sub> or CF<sub>O</sub>-b'<sub>109-219</sub>, as well as  
363 AtCGL160N or AtCGLD11. **B**, Interaction of AtCGL160N with structural domains of CF<sub>1</sub>- $\beta$ .  
364 Yeast cells carrying a construct coding for BD-AtCGL160 were transformed with constructs  
365 coding for AD-CF<sub>1</sub>- $\beta$ <sub>I</sub>, AD-CF<sub>1</sub>- $\beta$ <sub>II</sub>, and AD-CF<sub>1</sub>- $\beta$ <sub>III</sub>. Structural domains of the CF<sub>1</sub>- $\beta$  are colored  
366 in red (domain I), turquoise (domain II), and blue (domain III). The conserved DELSEED motif  
367 is shown in purple, and the L-shaped redox loop of CF<sub>1</sub>- $\gamma$  in yellow. The atomic model of CF<sub>1</sub>-  
368 CF<sub>O</sub> was obtained from the PDB database (ID: 6fkh, Hahn et al. (2018)) and formatted with  
369 ChimeraX (Pettersen et al., 2021). **C**, Mapping of the AtCGL160N interaction site. Consecutive  
370 regions (grey boxes) coding for segments of the soluble AtCGL160 domain were omitted from  
371 the BD-AtCGL160N vector and co-transformed with AD-CF<sub>1</sub>- $\beta$  into competent yeast cells.  
372 Transformations were verified by plating on permissive medium lacking Leu and Trp (-LT).  
373 Interactions were then tested on selective medium (-Leu/-Trp/-His/-Ade, [-LTHA]) by plating  
374 equal numbers of yeast cells in serial dilutions (10<sup>0</sup>, 10<sup>-1</sup>, and 10<sup>-2</sup>).

375  
376 Interactions between the stroma-oriented AtCGL160N domain and individual CF<sub>1</sub> subunits  
377 were further examined by yeast two-hybrid experiments (Fig. 7). A construct coding for a  
378 fusion of AtCGL160N<sub>29-206aa</sub> to the GAL4-binding domain (BD) was co-transformed into yeast  
379 cells together with constructs coding for GAL4 activation domain (AD) fusions to all CF<sub>1</sub>  
380 subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ). Moreover, BD-AtCGL160N interaction was tested with AD fusions to  
381 the soluble parts of the stator subunits b and b', AtCGL160N, and CF<sub>1</sub> assembly factor  
382 AtCGLD11. As a result, only yeast cells carrying constructs for AD-CF<sub>1</sub>- $\beta$  and BD-AtCGL160N  
383 could grow on selective medium (Fig. 7A). To narrow down the CF<sub>1</sub>- $\beta$  interaction site, additional  
384 AD fusion constructs were cloned that encoded three different CF<sub>1</sub>- $\beta$  subdomains (Fig. 7B)  
385 defined in earlier studies (Groth and Pohl, 2001; Zhang et al., 2016). Domain I comprises a  
386 thylakoid-distal  $\beta$ -barrel structure and interacts with CF<sub>1</sub>- $\delta$ . Domain II harbors the catalytic site  
387 involved in ATP generation or hydrolysis. The thylakoid-proximal domain III contains the  
388 conserved "DELSEED" motif, which is required for CF<sub>1</sub>- $\gamma$ / $\epsilon$ -dependent regulation of ATP  
389 hydrolysis and synthase activity (Kanazawa et al., 2017; Hahn et al., 2018). When tested on  
390 restrictive medium, only cells harboring AD-CF<sub>1</sub>- $\beta$ <sub>III</sub> together with BD-AtCGL160N could grow.  
391 In a reciprocal approach, coding sequences of AtCGL160N were deleted successively from  
392 the BD-AtCGL160N construct ( $\Delta$ 29-74,  $\Delta$ 75-105,  $\Delta$ 106-134,  $\Delta$ 135-160 and  $\Delta$ 161-206 aa) and  
393 tested for AD-CF<sub>1</sub>- $\beta$  interaction in yeast cells (Fig. 7C). Only the  $\Delta$ 29-74 and  $\Delta$ 75-105 deletions  
394 resulted in an absence of growth, while yeast strains with deletion constructs of  $\Delta$ 106-134,  
395  $\Delta$ 135-160 and  $\Delta$ 161-206 aa were able to proliferate on selective medium (Fig. 6B). Thus, the  
396 interaction between AtCGL160 and CF<sub>1</sub> involves AtCGL160<sub>29-105</sub> and the thylakoid-proximal  
397 domain of CF<sub>1</sub>- $\beta$ <sub>III</sub>, while the phosphorylation hotspot identified in the protein segment 106-134  
398 aa (Fig. 1A) is dispensable for the interaction.

399

## 400 Discussion

401

### 402 AtCGL160N recruits a stromal $\alpha_3\beta_3\gamma\epsilon$ complex for late CF<sub>1</sub>-CF<sub>0</sub> assembly steps

403 Despite structural similarities and comparable subunit compositions, the number of known  
404 assembly factors for ATP synthases is markedly higher in chloroplast than in bacterial systems  
405 (reviewed in Zhang et al., 2020). Moreover, in plants the Atp1/Uncl-related CGL160 assembly  
406 factor has acquired an N-terminal domain that is specific for the green lineage. Thus, the  
407 expanded molecular inventory for CF<sub>1</sub>-CF<sub>0</sub> assembly in chloroplasts might reflect the need for  
408 tight post-translational control of CF<sub>1</sub>-CF<sub>0</sub> formation, since the complex plays a central role in  
409 *pmf* utilization and regulation of photosynthesis (reviewed in Avenson et al., 2005). In this  
410 context, an important finding of previous studies was that disruption of full-length AtCGL160  
411 (Rühle et al., 2014; Fristedt et al., 2015) was more detrimental to levels of functional ATP  
412 synthase than the loss of Atp1/Uncl in bacteria (Gay, 1984; Liu et al., 2013). Furthermore, we  
413 show here that expression of *P*<sub>35S</sub>:*AtCGL160C* in plants that lack AtCGL160N only partially  
414 restores CF<sub>1</sub>-CF<sub>0</sub> levels and activity (Fig. 4). These observations prompted us to investigate  
415 the molecular function of the green-lineage-specific AtCGL160N in the CF<sub>1</sub>-CF<sub>0</sub> assembly  
416 process in more detail.

417 Several lines of evidence suggest that the N-terminal domain of AtCGL160 recruits a stromal  
418 CF<sub>1</sub> intermediate, while the C-terminal segment participates in *c*<sub>14</sub>-ring assembly: (i)  
419 AtCGL160N protrudes into the stroma, as deduced from protease protection assays (Fig. 1);  
420 (ii) formation of the *c*<sub>14</sub> ring is restored in the presence of AtCGL160C alone, but CF<sub>1</sub>  
421 accumulates strongly in the stroma in the absence of AtCGL160N (Fig. 5), (iii) CF<sub>1</sub> subunits  
422 are differentially enriched in co-IP analyses performed with solubilized thylakoids isolated from  
423 *P*<sub>35S</sub>:*AtCGL160* plants (Fig. 6 A,B), (iv) AtCGL160 co-migrates with a large complex after DSP-  
424 mediated crosslinking (Fig. 6C) and (v) AtCGL160N interacts with CF<sub>1</sub>- $\beta$  in yeast two-hybrid  
425 experiments (Fig. 7).

426 A role for AtCGL160 in the incorporation of CF<sub>1</sub> into the holocomplex was previously proposed  
427 by Fristedt et al. (2015). This assumption was based on the observations that AtCGL160 co-  
428 migrated with CF<sub>1</sub> subcomplexes in BN/SDS-PAGE analyses and could be cross-linked to CF<sub>1</sub>  
429 subunits in wild-type protein samples. However, we detected AtCGL160 predominantly in the  
430 monomer fraction in untreated thylakoid preparations in this study (Fig. 6C), as well as in  
431 previous work (Rühle et al., 2014) – and co-migration of AtCGL160 with high-molecular-mass  
432 complexes was only observed after thylakoid proteins had been crosslinked with DSP (Fig.  
433 6C). Furthermore, the commercially available AtCGL160 antibody (AS12 1853, Agrisera)  
434 employed in the study of Fristedt et al. (2015) was found here to cross-react strongly with CF<sub>1</sub>-  
435  $\alpha$  or CF<sub>1</sub>- $\beta$  (Supplemental Fig. 3), which complicates the interpretation of one-dimensional co-  
436 migration and crosslinking experiments in the absence of appropriate controls. Therefore, a



437 new antibody was generated that does not cross-react with CF<sub>1</sub>-CF<sub>0</sub> subunits and thus  
438 provides a reliable means of probing the molecular interactions of AtCGL160 (Supplemental  
439 Fig. S3).

440 Besides CGL160, ALB4 – a member of the bacterial ALB3/Oxa1/YidC protein insertase family  
441 – was previously proposed to participate in the linkage of a CF<sub>1</sub> to a CF<sub>0</sub> assembly module  
442 (Benz et al., 2009). Another study provided evidence that ALB4 and its paralog ALB3 physically  
443 interact with each other, and show significant functional overlap in the membrane insertion of  
444 subunits of the Cyt *b<sub>6</sub>f* complex (Trosch et al., 2015). Moreover, alleles of *ALB4* (*STIC1*) have  
445 been identified as suppressors of the chloroplast protein import mutant *tic40* (Bedard et al.,  
446 2017), and ALB4/STIC1 and STIC2 were shown to act together in thylakoid protein targeting  
447 in a pathway that also involves cpSRP54 and cpFtsY. In our study, we did not identify  
448 ALB4/STIC1 in co-IP experiments with anti-AtCGL160 antibodies (Fig. 6, Supplemental Table  
449 S1) and amounts of thylakoid-associated CF<sub>1</sub>-β in *atalb4-1* mutants (SALK\_136199C) grown  
450 under short-day conditions were unaltered (Supplemental Fig. S6). Thus, ALB4/STIC1 does  
451 not act in concert with CGL160 in late stages of CF<sub>1</sub>-CF<sub>0</sub> assembly, but serves as a general  
452 thylakoid protein biogenesis factor involved in folding or assembly of a specific subset of  
453 transmembrane proteins (Bedard et al., 2017).

454

#### 455 AtCGL160 is critical for chloroplast development in the dark

456 It has long been thought that the hydrolytic activity of CF<sub>1</sub>-CF<sub>0</sub> needs to be inactivated in the  
457 dark to prevent futile ATP depletion (Ort and Oxborough, 1992). However, analysis of the  
458 constitutively redox-activated γ-subunit mutant *gamera*, in which a 'dark *pmf*' is maintained,  
459 revealed increased stability of photosynthetic complexes upon prolonged darkness,  
460 suggesting that a certain degree of ATPase activity may be beneficial during the night  
461 (Kohzuma et al., 2017). Concomitantly, several processes have been proposed to depend on  
462 the maintenance of a dark *pmf*. These include thylakoid protein transport via the Tat- and Sec-  
463 dependent pathways, modulation of protease activity and ion homeostasis in the chloroplast.  
464 In this regard, a remarkable influence of AtCGL160 disruption on leaf variegation (Fig. 2) and  
465 chloroplast development (Fig. 3) was observed exclusively under short-day conditions.  
466 Surprisingly, this phenotype was not detectable in *atcgl11-1* plants with a defect in CF<sub>1</sub>  
467 assembly and reduced amounts of CF<sub>1</sub>-CF<sub>0</sub> comparable to those in *atcgl160-1* (Fig. 4D).  
468 However, the leaf phenotype correlated with the accumulation of a CF<sub>1</sub> intermediate in the  
469 stroma (Fig. 5C). Thus, AtCGL160-mediated CF<sub>1</sub> recruitment might also be critical in  
470 preserving the dark *pmf* at night. Alternatively, stroma-enriched CF<sub>1</sub> complexes (Fig. 5C) could  
471 alter the chloroplast ATP/ADP ratio by excessive hydrolytic activity, and disturb ATP-  
472 dependent nocturnal processes that ultimately lead to premature chloroplast degradation (Fig.  
473 3).

474

475 AtCGL160 is a central CF<sub>1</sub>-CF<sub>0</sub> assembly factor with multiple functions

476 Assembly of membrane-embedded ATP synthase modules and their subsequent association  
477 with F<sub>1</sub> subcomplexes are critical steps in bacterial and organellar ATP synthase biogenesis,  
478 as premature formation of the proton-translocating channel between the c-ring and the a-  
479 subunit (equivalent to the ATP9 ring and the ATP6 subunit in mitochondria) can lead to  
480 uncontrolled dissipation of the *pmf* (Birkenhäger et al., 1999; Franklin et al., 2004), and only  
481 efficient integration of F<sub>1</sub> triggers ATP production. In this context, molecular aspects of the  
482 assembly processes were recently elucidated for bacterial (reviewed in Deckers-Hebestreit,  
483 2013), as well as yeast and human mitochondrial ATP synthases (reviewed in Song et al.,  
484 2018). One significant outcome was that, while ATP synthase assembly pathways and the  
485 repertoire of auxiliary factors differ among these systems, formation of the proton-translocating  
486 unit during the final assembly steps is common to all of them.

487 Intriguingly, our data revealed a dual involvement of AtCGL160 in CF<sub>1</sub>-CF<sub>0</sub> assembly, namely  
488 in c-ring formation and the recruitment of a CF<sub>1</sub> intermediate. In fact, these two events were  
489 suggested to proceed sequentially in the assembly of bacterial ATP synthases (Deckers-  
490 Hebestreit, 2013). Since an *E. coli* strain lacking subunit  $\delta$  accumulates a c<sub>10</sub> $\alpha_3\beta_3\gamma\epsilon$   
491 subcomplex, it is assumed that cytoplasmic F<sub>1</sub> first binds to the c<sub>10</sub> ring, and c<sub>10</sub> $\alpha_3\beta_3\gamma\epsilon$   
492 associates with the ab<sub>2</sub> module in a  $\delta$ -dependent manner in the final assembly step (Hilbers et  
493 al., 2013). By analogy with the bacterial assembly pathway, AtCGL160 may facilitate the  
494 integration of a stator assembly module into the holo-complex. Indeed, the interaction of  
495 AtCGL160C with CF<sub>0</sub>-b has been demonstrated in split-ubiquitin assays (Rühle et al., 2014).  
496 Moreover, CF<sub>0</sub>-a was less highly enriched in co-IP analyses than other CF<sub>1</sub>-CF<sub>0</sub> subunits (Fig.  
497 6A, B), which might argue for the release of AtCGL160 after functional incorporation of CF<sub>0</sub>-a  
498 in the final steps of CF<sub>1</sub>-CF<sub>0</sub> assembly. In this scenario, AtCGL160 could act as a placeholder  
499 to prevent the premature formation of proton-translocating intermediates. A similar function  
500 has been described for the INA complex in yeast mitochondria, which binds to the c-ring, but  
501 also to a distinct assembly intermediate consisting of ATP6, ATP8, ATP10, ATP23, peripheral  
502 stalk subunits and the F<sub>1</sub> domain (Naumenko et al., 2017). This ensures that the c-ring and  
503 subunit ATP6 are assembled into the proton-conducting unit in a controlled manner. However,  
504 due to a generally low turnover rate of CF<sub>1</sub>-CF<sub>0</sub> assembly (reviewed in Schöttler et al., 2014)  
505 and inefficient detection of distinct thylakoid-integral intermediates, a robust CF<sub>0</sub> assembly  
506 map is still lacking, and 'true' stator-containing assembly modules have not been described so  
507 far.

508 Nevertheless, a straightforward assembly mechanism for the recruitment of CF<sub>1</sub> can be derived  
509 from our study. After AtCGL160-assisted ring formation (Rühle et al., 2014), the stromally  
510 oriented AtCGL160N (Fig. 1) binds to a CF<sub>1</sub> intermediate consisting of  $\alpha_3\beta_3\gamma\epsilon$  but not subunit

511  $\delta$  (Fig. 5C, Supplemental Fig. S4). Recruitment is mediated through interaction of AtCGL160<sub>29-</sub>  
512 <sub>105</sub> with subunit CF<sub>1</sub>- $\beta$ ; thus, the phosphorylatable AtCGL160 segment is dispensable for the  
513 interaction (Fig. 7). Since AtCGL160 can be cross-linked to high-molecular-mass complexes  
514 that are larger than CF<sub>1</sub> (Fig. 6C), AtCGL160 might remain attached to a putative c<sub>14</sub> $\alpha_3\beta_3\gamma\epsilon$  or  
515 bb'c<sub>14</sub> $\alpha_3\beta_3\gamma\epsilon$  intermediate. Its release could then be triggered by the incorporation of subunit  
516 CF<sub>0</sub>-a or CF<sub>1</sub>- $\delta$  in the final assembly steps.

517 At this stage, we cannot rule out the possibility that AtCGL160N might have regulatory  
518 functions beyond CF<sub>1</sub> recruitment, as it interacts with the thylakoid-proximal domain III of CF<sub>1</sub>-  
519  $\beta$ , which contains the conserved DELSEED motif (Fig. 7B). Several regulatory mechanisms  
520 have been elucidated in which the subunit  $\beta$  and the DELSEED motif are implicated. For  
521 instance, the autoinhibitory subunit  $\epsilon$  interacts with the DELSEED motif in bacteria (Tanigawara  
522 et al., 2012; Sobti et al., 2016), whereas in bovine (Cabezón et al., 2003) and yeast  
523 mitochondria (Robinson et al., 2013), the small protein IF<sub>1</sub> inhibits ATPase activity by binding  
524 at the  $\alpha/\beta$  interface. In plants, a regulatory mechanism controls CF<sub>1</sub>-CF<sub>0</sub> activity involving also  
525 the DELSEED and an L-shaped, two  $\beta$ -hairpin containing motif with two conserved redox-  
526 sensitive cysteines in the CF<sub>1</sub>- $\gamma$  subunit (Hahn et al., 2018). By analogy with the role of IF<sub>1</sub>,  
527 which was shown to inhibit ATPase activity during the assembly of human mitochondrial ATP  
528 synthases (He et al., 2018), AtCGL160N may regulate ATPase activity during CF<sub>1</sub>-CF<sub>0</sub>  
529 assembly via an as yet unknown mechanism.

## 530 **Methods**

531

### 532 Bioinformatics Sources

533 Protein and gene sequences were downloaded from the Arabidopsis Information Resource  
534 server (TAIR; <http://www.arabidopsis.org>), Phytozome  
535 (<https://phytozome.jgi.doe.gov/pz/portal.html>) and the National Center for Biotechnology  
536 Information server (NCBI; <http://www.ncbi.nlm.nih.gov/>). Transit peptides were predicted by  
537 ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>) (Emanuelsson et al., 1999). Structural data  
538 was obtained from the PDB homepage (<https://www.rcsb.org/>) and processed with ChimeraX  
539 (<https://www.cgl.ucsf.edu/chimerax/>) (Pettersen et al., 2021). Multiple sequence alignments  
540 were generated with the CLC workbench software (v8.1) and protein features were visualized  
541 with Protter (<https://wlab.ethz.ch/protter/start/>) (Omasits et al., 2014). Co-migration of stromal  
542 proteins was examined with the online tool PCom-DB  
543 (<http://pcomdb.lowtem.hokudai.ac.jp/proteins/top>) (Takabayashi et al., 2017). Boxplots were  
544 drawn with BoxPlotR (<http://shiny.chemgrid.org/boxplotr/>) (Spitzer et al., 2014).

545

### 546 Plant Material and Growth Conditions

547 T-DNA lines for *atcgl160-1* (SALK\_057229, Col-0 background), *atcgl11-1* (SALK\_019326C,  
548 Col-0 background) and *atalb4-1* (SALK\_136199C) were obtained from the SALK collection  
549 (Alonso et al., 2003). Plants were grown on potting soil (A210, Stender, Schermbeck,  
550 Germany) under controlled greenhouse conditions (70-90  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 16/8 h  
551 light/dark cycles), or in climate chambers on an 8h light/16h dark cycle for biochemical and  
552 physiological analyses. Fertilizer was added to plants grown under greenhouse conditions  
553 according to the manufacturer's recommendations (Osmocote Plus; 15% nitrogen [w/v], 11%  
554 [w/v]  $\text{P}_2\text{O}_5$ , 13% [w/v]  $\text{K}_2\text{O}$ , and 2% [w/v]  $\text{MgO}$ ; Scotts, Germany). For domain-specific  
555 complementation assays, either the complete coding region of *AtCGL160* ( $P_{35S}:AtCGL160$ ) or  
556 parts of the CDS corresponding to amino acids 1-206 ( $P_{35S}:AtCGL160N$ ) and 207-350  
557 ( $P_{35S}:AtCGL160C$ ) were cloned into the binary Gateway vector pB2GW7 (Karimi et al., 2002),  
558 placing the genes under control of the 35S CaMV promoter. The transit peptide coding  
559 sequence (for amino acids 1-46) was fused to the *AtCGL160C* CDS in the case of the  
560  $P_{35S}:AtCGL160C$  vector. The constructs were first transformed into *Agrobacterium*  
561 *tumefaciens* strain GV3101, and then into *atcgl160-1* plants by the floral-dip method (Clough  
562 and Bent, 1998). T1 plants were selected by screening for Basta resistance. Basta positives  
563 were screened for equal amounts of the *AtCGL160* transcript by RNA gel-blot hybridization as  
564 described below.

565

#### 566 Transmission electron microscopy

567 Leaf pieces of about 1.5 × 1.0 mm were cut with a new double edge razor blade (Feather,  
568 Osaka, Japan) and immediately immersed in fixation buffer (0.1 M sodium phosphate buffer,  
569 pH 7.4, 2.5% [v/v] glutaraldehyde, 4% [v/v] formaldehyde) at room temperature. A mild vacuum  
570 (about 20 mbar) was applied until the leaf pieces did sink, the fixation buffer replaced with fresh  
571 one and the samples fixed overnight at 4 °C. After three 10-min washes in sodium phosphate  
572 buffer (pH 7.4), the samples were osmicated with 1% osmium tetroxide and 1.5% potassium  
573 ferricyanide in 0.1 M sodium phosphate buffer (pH 7.4) for 60 min at 4°C. The samples were  
574 rinsed three times for 10 min each in distilled water and incubated in 1% uranyl acetate (in  
575 distilled water) at 4°C overnight. After three washes of 10 min each in distilled water the  
576 samples were dehydrated using increasing concentrations of ethanol and infiltrated, with  
577 propylene oxide as intermediate solvent, in glycid ether 100 (formerly Epon 812; Serva,  
578 Heidelberg, Germany) following standard procedures. Polymerization was carried out for 40 -  
579 48 h at 65 °C. Ultrathin sections (~60 nm) were cut with a diamond knife (type ultra 35°;  
580 Diatome, Biel, Suisse) on an EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany)  
581 and mounted on single-slot Pioloform-coated copper grids (Plano, Wetzlar, Germany). The  
582 sections were stained using uranylacetate and lead citrate (Reynolds, 1963) and viewed with  
583 a JEM-1400 Plus transmission electron microscope (JEOL, Tokyo, Japan) operated at 80kV.  
584 Micrographs were taken using a 3.296 x 2.472 pixels charge-coupled device camera (Ruby,  
585 JEOL).

586

#### 587 Chl a Fluorescence Measurements

588 *In vivo* Chl a fluorescence of whole plants was measured using an imaging Chl fluorometer  
589 (Imaging PAM, Walz, Effeltrich, Germany). Plants were dark-adapted for 20 min and exposed  
590 to a pulsed, blue measuring beam (4 Hz, intensity 3, gain 3, damping 2; F<sub>0</sub>) and a saturating  
591 light flash (intensity 10) to calculate F<sub>v</sub>/F<sub>M</sub>. If not indicated otherwise, transient NPQ induction  
592 was measured at 145 μmol photons m<sup>-2</sup> s<sup>-1</sup>.

593

#### 594 ECS Measurements

595 ECS measurements were performed using the Dual-PAM-100 (Walz, Effeltrich, Germany)  
596 equipped with a P515/535 emitter-detector module (Schreiber and Klughammer, 2008). The  
597 measurement was carried out at 23°C under ambient CO<sub>2</sub> conditions. Plants grown in short-

598 day conditions for four weeks were light-adapted, and detached leaves were illuminated for at  
599 least 10 min with  $129 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  red light. After illumination, dark-interval relaxation  
600 kinetics (DIRK) were measured in the ms to s range. Values for *pmf* (ECSt), and proton  
601 conductivity ( $\text{gH}^+$ ) were calculated as described (Cruz et al., 2001; Schreiber and Klughammer,  
602 2008). Briefly, the maximum amplitude of the inverse electrochromic band-shift kinetic was  
603 measured in the second range, and normalized to a single saturating P515 pulse measured  
604 after 4 minutes of dark incubation. For proton conductivity, electrochromic band-shift kinetics  
605 were recorded in the millisecond range for 5 consecutive periods of 2 sec, separated by dark  
606 intervals of 30 sec. The combined signals were fitted to a single exponential decay function  
607 and the reciprocal value of the lifetime was used to estimate the proton conductivity (Kanazawa  
608 and Kramer, 2002).

609

#### 610 AtCGL160 Antibody Generation

611 Rabbit antibodies were generated against AtCGL160 that had been heterologously expressed  
612 in *Escherichia coli*, and then purified. To this end, the coding sequence corresponding to  
613 AtCGL160<sub>29-206</sub> was cloned into the pMal-c5x vector (New England Biolabs) and purified on  
614 amylose columns (New England Biolabs) according to the manufacturer's instructions. The  
615 protein was injected into rabbits for antibody production (Pineda, Berlin, Germany). To reduce  
616 epitope cross-reactions, the antiserum was purified on a column crosslinked with  
617 heterologously expressed AtCGL160<sub>29-206</sub> fused to the glutathione-S-transferase (GST) tag.  
618 Purified antibody was employed at a dilution of 1:1000. Signals were detected by enhanced  
619 chemiluminescence (Pierce™ ECL Western Blotting Substrate, Thermo Scientific) using an  
620 ECL reader system (Fusion FX7; PeqLab, Erlangen, Germany).

621

#### 622 Nucleic Acid Analysis

623 Total RNA from snap-frozen leaves was extracted with the RNeasy Plant Mini Kit (Qiagen)  
624 according to the supplier's instructions. Samples equivalent to 8 or 20  $\mu\text{g}$  total RNA were  
625 fractionated by electrophoresis in formaldehyde-containing agarose gels (1.2%), blotted onto  
626 nylon membranes (Hybond-N+, Amersham Bioscience) and fixed by UV radiation  
627 (Stratalinker® UV Crosslinker 1800). To control for equal loading, abundant RNAs on nylon  
628 membranes were stained with methylene blue solution (0.02% [w/v] methylene blue, 0.3 M  
629 sodium acetate pH 5.5). To detect gene-specific transcripts, DNA fragments amplified from  
630 cDNA were labelled with radioactive [ $\alpha$ -<sup>32</sup>P]dCTP and subsequently used as probes in  
631 hybridization experiments (see Supplemental Table S2 for primer information). Signals were  
632 detected with the Typhoon Phosphor Imager System (GE Healthcare).

633

## 634 Protein Analysis

635 Leaves from 4-week-old plants grown under short-day conditions were harvested shortly after  
636 the onset of the light period, and thylakoid membrane-enriched samples were isolated  
637 according to Rühle et al. (2014). Crosslinking of thylakoids was performed by incubation with  
638 2.5 mM dithiobis(succinimidyl propionate) (DSP, Thermo Scientific). After incubation for 20 min  
639 on ice, crosslinking was quenched with 60 mM Tris/HCl (pH 7.5). Chl concentrations were  
640 determined as described in Porra et al. (1989). For immunotitrations, thylakoid membrane  
641 pellets were resuspended in loading buffer (100 mM Tris/HCl pH 6.8, 50 mM dithiothreitol  
642 [DTT], 8% [w/v] SDS, 24% [w/v] glycerol and 0.02% [w/v] bromophenol blue). Denaturation for  
643 5 min at 70°C and protein fractionation on Tricine-SDS-PAGE gels (10% gels supplemented  
644 with 4M Urea) was carried out according to Schägger (2006). Immunodetections were  
645 performed as described below. Sample preparation for BN-PAGE was performed with freshly  
646 prepared thylakoids as described in Peng et al. (2008). First, membranes were washed twice  
647 in wash buffer (20% glycerol, 25 mM BisTris/HCl pH 7.0). Then, samples were treated with  
648 wash buffer containing 1% (w/v) n-dodecyl  $\beta$ -D-maltoside and adjusted to 1 ml mg<sup>-1</sup> Chl for 10  
649 min on ice. After centrifugation (16,000g, 20 min, 4°C), supernatants were supplemented with  
650 1/10 volume of BN sample buffer (100 mM BisTris/HCl pH 7.0, 750 mM  $\epsilon$ -aminocaproic acid,  
651 5% [w/v] Coomassie G-250). BN-PAGE gels (4-12% gradient) were prepared as described in  
652 Schägger et al. (1994). Solubilized samples corresponding to 60  $\mu$ g Chl were loaded per lane  
653 and gels were run at 4°C overnight. To separate complexes into their subunits, BN-PAGE  
654 strips were treated with denaturing buffer (0.2 M Na<sub>2</sub>CO<sub>3</sub>, 5% [w/v] SDS, 50 mM DTT) for 30  
655 min at room temperature and loaded on Tricine-SDS-PAGE gels. Gels were subsequently  
656 subjected to immunoblot analysis with antibodies against CF<sub>1</sub>-CF<sub>0</sub> subunits and AtCGL160,  
657 as described below.

658 For analysis of the stromal CF<sub>1</sub> intermediate, intact chloroplasts from 4-week-old plants were  
659 isolated according to Rühle et al. (2021). After lysis in 25 mM HEPES/KOH (pH 7.5) containing  
660 5 mM MgCl<sub>2</sub> for 30 min on ice, the stromal fraction was separated from membranes by  
661 centrifugation at 35,000g for 30 min (4 °C). Protein concentration was measured using the  
662 Bradford Protein Assay (Bio-Rad). Stromal BN analysis was performed according to Reiter et  
663 al. (2020). In brief, chloroplast-enriched pellets were resuspended in BN washing buffer and  
664 mechanically disrupted by passage through an 0.45-mm syringe. The stromal fraction was  
665 separated from membranes by centrifugation at 35,000g for 30 min (at 4°C). 100  $\mu$ g of total  
666 soluble protein was mixed with 1/10 volume of BN sample buffer before fractionation in the first  
667 dimension as described above.

668

669 Immunoblot Analyses

670 Proteins fractionated by gel electrophoresis were transferred to polyvinylidene difluoride  
671 membranes (PVDF) (Immobilon®-P, Millipore) using a semi-dry blotting system (Biorad) as  
672 described in the supplier's instructions. After blocking with TBST (10 mM Tris/HCl pH 8.0, 150  
673 mM NaCl and 0.1% [v/v] Tween-20) supplemented with 3% (w/v) skim milk powder, the  
674 membranes were incubated with antibodies at 4°C overnight. Antibodies used in this study  
675 were obtained from Agrisera (CF<sub>1</sub>-β: AS05 085, 1:5000; CF<sub>1</sub>-γ: AS08 312, 1:5000; CF<sub>0</sub>-b: AS10  
676 1604, 1:5000; CF<sub>0</sub>-c: AS09 591, 1:3000; and AtCGL160: AS12 1853, 1:1000).

677

678 Yeast-two-Hybrid Experiments

679 Yeast two-hybrid assays were carried out using the Matchmaker Two-Hybrid System Kit  
680 (Clontech). The *AtCGL160* CDS without the signal peptide (see Supplemental Table S2 for  
681 primer information) was cloned into the bait vector pGBKT7 (BD-AtCGL160), whereas the  
682 coding sequences of CF<sub>1</sub>-α, -β, -γ, -δ, -ε, the soluble domains of CF<sub>0</sub>-b (51-184 aa) and b'  
683 (109-219 aa), AtCGL160 and the CF<sub>1</sub> assembly factor AtCGLD11 were cloned into the prey  
684 vector pGADT7 (named AD-CF<sub>1</sub>-α, AD-CF<sub>1</sub>-β, AD-CF<sub>1</sub>-γ, AD-CF<sub>1</sub>-δ, AD-CF<sub>1</sub>-ε, AD-CF<sub>0</sub>-b, AD-  
685 CF<sub>0</sub>-b', AD-AtCGL160 and AD-AtCGLD11). As in the case of AtCGL160, signal peptide  
686 sequences were omitted from the nucleus-encoded subunits CF<sub>1</sub>-γ, CF<sub>1</sub>-δ, CF<sub>0</sub>-b' and  
687 AtCGLD11. For binding-domain analysis of CF<sub>1</sub>-β, the respective CDS was sub-divided into  
688 three parts, according to Groth and Pohl (2001) and cloned into pGADT7. In the case of  
689 AtCGL160N binding-site analysis, sequences coding for 29-74, 75-105, 106-134, 135-160 and  
690 161-206 aa were deleted from the BD-AtCGL160 vector using a site-directed mutagenesis kit  
691 (NEB). Primers are listed in Supplemental Table S2. Bait and prey vectors were co-  
692 transformed into AH109 yeast strains (Clontech) following manufacturer's instructions. Co-  
693 transformants were selected on synthetic dropout (SD) medium (Clontech) lacking leucine and  
694 tryptophan (-LT). In order to identify protein interactions, double transformants were grown on  
695 SD medium lacking leucine, tryptophan, histidine, and adenine (-LTHA).

696

697 Co-immunoprecipitation

698 Freshly extracted thylakoids corresponding to ~10 mg chlorophyll were resuspended in 500 μl  
699 extraction buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 5% [w/v] glycerol, 1%  
700 [v/v] Nonidet P40 [NP40], 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and solubilized for 30  
701 min on ice. After centrifugation at 35,000g for 30 min and 4°C, the supernatant was added to  
702 20 μl Dynabeads (Thermo Scientific), equilibrated with equilibration buffer (50 mM Tris/HCl pH  
703 7.5, 150 mM NaCl, 5% [w/v] glycerol, 0.05% [v/v] NP40) and labelled with AtCGL160 antibody



704 according to the manufacturer's instructions. The suspension was incubated with rotation for  
705 3 h at 4°C, washed three times with equilibration buffer, and twice with the same buffer but  
706 omitting NP40. Proteins were eluted with 100 µl 0.1 M glycine pH 2.0 for 10 min and neutralized  
707 with 100 µl 0.1 M ammonium bicarbonate. After treatment with 10 µl of 45 mM DTT and 10 µl  
708 of 0.1 M iodoacetamide, samples were digested with 1.5 µg of trypsin at 37°C overnight.  
709 Peptides were desalted with home-made C18 stage tips (Rappsilber et al., 2003), vacuum-  
710 dried to near dryness and stored at -80°C. LC MS/MS run and data analysis were performed  
711 as described in Reiter et al. (2020).

712

### 713 **Author Contributions**

714 B.R. and T.R. designed research. B.R., L.R., G.M., S.G. and T.R. carried out experiments.  
715 B.R., D.L. and T.R. prepared the article. T.R. supervised the whole study.

716

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721

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970 **Supplemental tables**

971 **Supplemental Table S1. AtCGL160 co-immunoprecipitation experiments.** Differential  
 972 enriched proteins in *P<sub>35S</sub>:AtCGL160* versus *P<sub>35S</sub>:AtCGL160C* samples sorted by log<sub>2</sub> fold  
 973 change (-log<sub>10</sub> *P*-value > 1.5). Nucleus-encoded genes are written in capital letters.

Protein IDs (Uniprot)	Gene names	log <sub>2</sub> FC	-log <sub>10</sub> p-value
O82279	<i>AtCGL160</i>	6.495	4.644
P09468	<i>atpE</i>	6.38	2.301
Q42139	<i>ATPG</i>	5.231	4.747
P56760	<i>atpH</i>	4.886	3.176
Q9SSS9	<i>ATPD</i>	4.672	4.523
P19366	<i>atpB</i>	4.437	4.459
P56759	<i>atpF</i>	4.399	5.913
P56757	<i>atpA</i>	4.235	6.41
Q01908	<i>ATPC1</i>	4.156	4.772
Q2HIU0	<i>At3g15110</i>	3.333	3.508
P56758	<i>atpl</i>	2.799	4.104
O49445	<i>LECRK72</i>	2.346	3.115
Q67XC4	<i>TBL40</i>	2.268	2.402
Q8LCQ4	<i>LHCA6</i>	2.082	3.71
A0A1P8B288, Q39099	<i>XTH4</i>	1.983	4.126
Q41963	<i>TIP1-2</i>	1.907	2.821
O22957	<i>At2g34040</i>	1.632	2.829
Q9SRL2, Q9M9X0, F4J8G2, Q9SRL7, Q9S9U3	<i>RLP32, RLP33, RLP34, RLP35, RLP53</i>	1.564	3.162
P38418, A0A1I9LPH1	<i>LOX2</i>	1.544	2.047
Q8LBV4	<i>At1g78140</i>	1.515	2.171
F4IUJ0, F4IUI9	<i>At2g26340</i>	1.451	3.021
Q9SF53, A0A1I9LSB4, Q9M3D2	<i>RPL35A, RPL35C</i>	1.254	2.619
A0A1P8B6D0, Q9SUI4	<i>PSAL</i>	1.193	2.938
Q9FFW9, F4KBJ3	<i>At5g38520</i>	1.136	3.084
Q96242	<i>CYP74A</i>	1.078	2.371
Q9SYW8, F4K8I1	<i>Lhca2</i>	0.941	2.356
Q9SR92	<i>STR10</i>	0.839	2.913
P56777	<i>psbB</i>	0.807	2.072
Q9LHA6	<i>At3g28220</i>	0.731	2.315
Q9S7N7	<i>PSAG</i>	0.575	2.157

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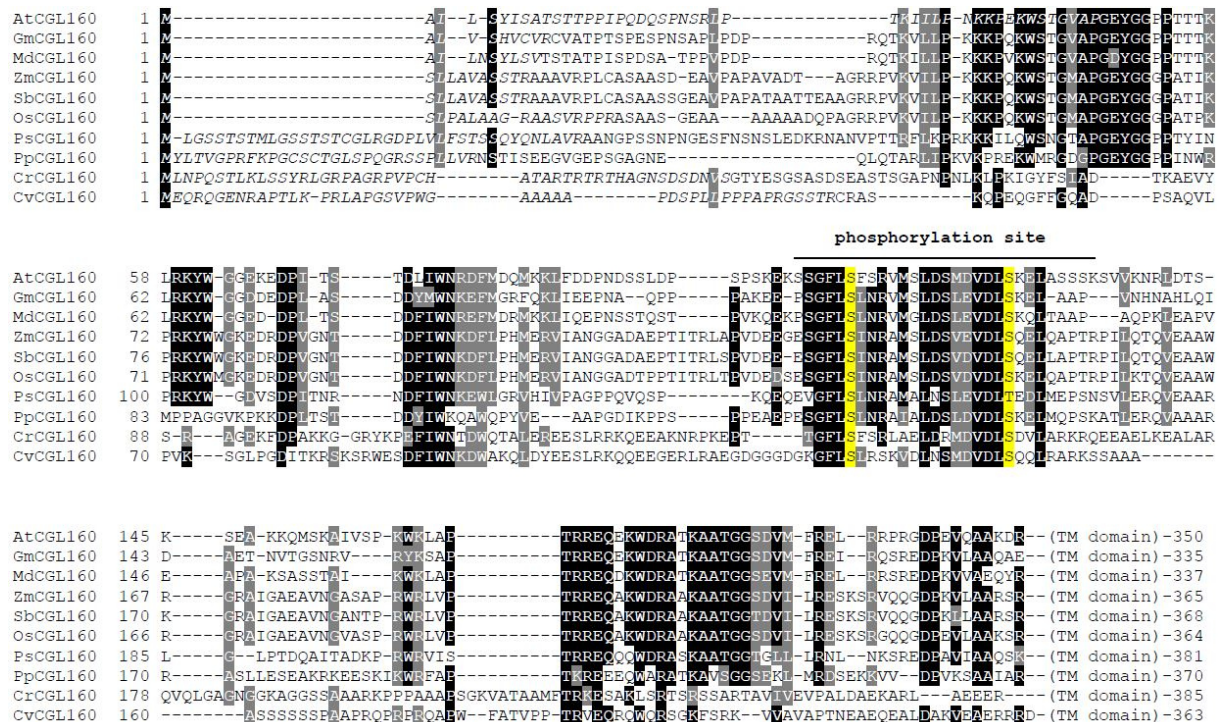
975 **Supplemental Table S2. Primers used in this study.**

Primer name	Primer sequence 5' to 3'	Comment
oeAtCGL160_s	GGGGACAAGTTTGTACAAAAAAGCAGG CTCAATGGCGATTCTTAGTTACAT	Gateway primer
oeAtCGL160_as	GGGGACCACTTTGTACAAGAAAGCTGG GTTTAATCACTGGCCTGTGTGT	Gateway primer
oeAtCGL160C_s	GGGGACAAGTTTGTACAAAAAAGCAGG CTCAatgGAACAATATTTTAAGCTGAAAA	Gateway primer
TP-GC9C_fus_s	GGTCCACCGGAGTTGCTCCCGAACAAT ATTTTAAGCTGAA	Fusion PCR
TP-Gc9C_fus_as	TTCAGCTTAAAAATATTGTTTCGGGAGCAA CTCCGGTGGACC	Fusion PCR
CGL160-MBP_s	AAAATCATTCTACCCAATAA	MBP cloning primer
CGL160N-MBP_as	GGTCCTGAATTCTTACCTGTCTTTAGCA GCTTGTA	MBP cloning primer
GST-CGL160N-s	GGGGACAAGTTTGTACAAAAAAGCAGG CTCAAAAATCATTCTACCCAATAAGAAA CCTGA	Gateway primer
GST-CGL160N-as	GGGGACCACTTTGTACAAGAAAGCTGG GTCTTACCTGTCTTTAGCAGCTTGTAC	Gateway primer
cgl160cTP_probe_s	ATGGCGATTCTTAGTTACATCTCAGC	Northern-probe
cgl160cTP_probe_as	GGGAGCAACTCCGGTG	Northern-probe
pGBKT7-CGL160N_s	GGTGGTCATATGAAAATCATTCTACCCA ATAAGA	Y2H cloning primer
pGBKT7-CGL160N_as	GGTCCTGAATTCTTACCTGTCTTTAGCA GCTTGTA	Y2H cloning primer
pGADT7-alpha_s	GGTGGTCATATGGTAACCATTAGAGCC GACGA	Y2H cloning primer
pGADT7-alpha_as	GGTCCTGAATTCTTATACTTTCTCCTGA AGTA	Y2H cloning primer
pGADT7-beta_s	GGTGGTCATATGAGAACAAATCCTACTA CTTC	Y2H cloning primer
pGADT7-beta_as	GGTCCTGAATTCTCATTCTTCAATTTA CTCT	Y2H cloning primer
pGADT7-gamma_s	GGTGGTCATATGGCTTCTCTGTTTCAC CACT	Y2H cloning primer
pGADT7-gamma_as	GGTCCTGAATTCTCAAACCTGTGCATTA GCTC	Y2H cloning primer
pGADT7-delta_s	GGTGGTCATATGGCCACCGCAGCATCA AGCTA	Y2H cloning primer
pGADT7-delta_as	GGTCCTGAATTCTCAAGTAGCTAATTGA ATCT	Y2H cloning primer

pGADT7-epsilon_s	GGTGGTCATATGACCTTAAATCTTTGTG TACTGACTC	Y2H cloning primer
pGADT7-epsilon_as	GGTCCTGAATTCTCAAATCGTATTGAGA GCCT	Y2H cloning primer
pGADT7-AtCGL160_s	GGTGGTCATATGAAAATCATTCTACCCA ATAAGA	Y2H cloning primer
pGADT7-AtCGL160_as	GGTCCTGAATTCTTACCTGTCTTTAGCA GCTTGTA	Y2H cloning primer
pGADT7-AtCGLD11_s	GGTGGTCATATGTCTTCGAGTCTATGG AAGCT	Y2H cloning primer
pGADT7-AtCGLD11_as	GGTCCTGAATTCTTAACCCTGGAGTAAT TTCA	Y2H cloning primer
pGADT7-atpFsoluble_s	GGTGGTCATATGGATTTATTAGATAACC GAAAG	Y2H cloning primer
pGADT7-atpFsoluble_as	GGTCCTGAATTCTTAATCAGTTATTTCT TTCATCG	Y2H cloning primer
pGADT7-atpGsoluble_s	GGTGGTCATATGCCGCTTGGTAACTTC ATGG	Y2H cloning primer
pGADT7-atpGsoluble_as	GGTCCTGAATTCTTAAGAAGGAAGAAC CTTCTTGAC	Y2H cloning primer
pGADT7_AtpBI-AD_s	CGCGAATTCATGAGAACAATCCTAC	Y2H cloning primer
pGADT7_AtpBI-AD_as	ACTCTCGAGTCAATTTCCCATATCAACC AC	Y2H cloning primer
pGADT7_AtpBII-AD_s	ATGGAATTCCTCTAAGTGTTCAG	Y2H cloning primer
pGADT7_AtpBII-AD_as	AACCTCGAGTCAAGGTTGTAGCATAGT TG	Y2H cloning primer
pGADT7_AtpBIII-AD_s	CTAGAATTCGAATCGTTGGCGAG	Y2H cloning primer
pGADT7_AtpBIII-AD_as	GCGCTCGAGTCATTTCTTCAATTTACTC	Y2H cloning primer
pGBKT7_CGL160N_del29_74_s	GACTTAATCTGGAACAGAGATTTTATGG	Y2H cloning primer
pGBKT7_CGL160N_del29_74_as	CATATGCAGGTCCTCCTCT	Y2H cloning primer
pGBKT7_CGL160N_del75_105_s	GTCTTCTGGGTTTCTGAG	Y2H cloning primer
pGBKT7_CGL160N_del75_105_as	GTGGAAGTAATGGGATCTTC	Y2H cloning primer
pGBKT7_CGL160N_del106_134_s	CGTTGTGAAAAATCGTCTTGACAC	Y2H cloning primer
pGBKT7_CGL160N_del106_134_as	GACTTTTCCTTTGAAGGAGATGG	Y2H cloning primer
pGBKT7_CGL160N_del135_160_s	GAAGCTGGCACCTACACG	Y2H cloning primer
pGBKT7_CGL160N_del135_160_as	CATTTAGAAGACGATGCAAGCTCTTTAC TTAAATC	Y2H cloning primer
pGBKT7_CGL160N_del161_206_s	GAATTCCTGGGGATCCG	Y2H cloning primer
pGBKT7_CGL160N_del161_206_as	CTATTTAGGAGACACAATAGCCTTACTC ATTTG	Y2H cloning primer

## 977 Supplemental Figures

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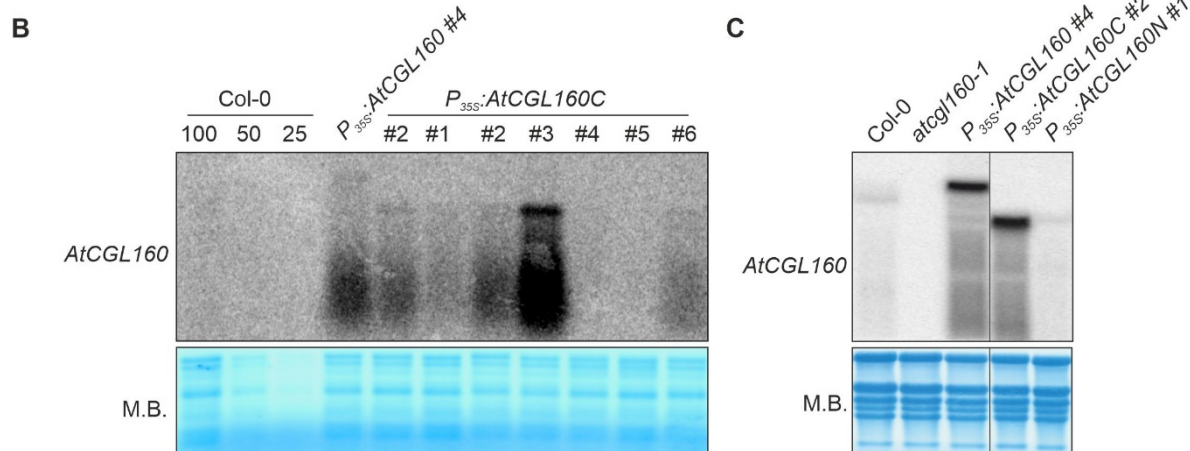
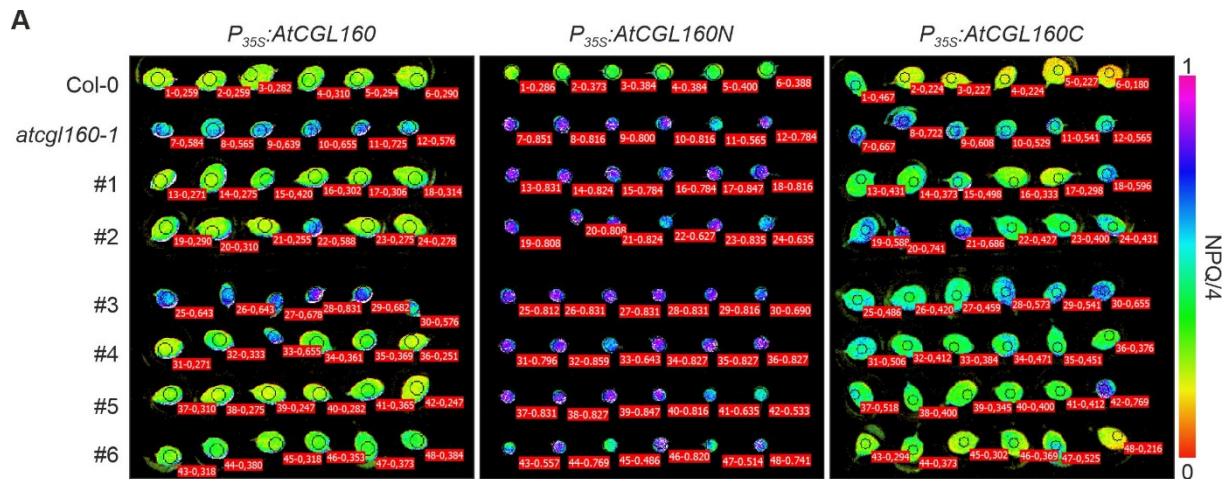
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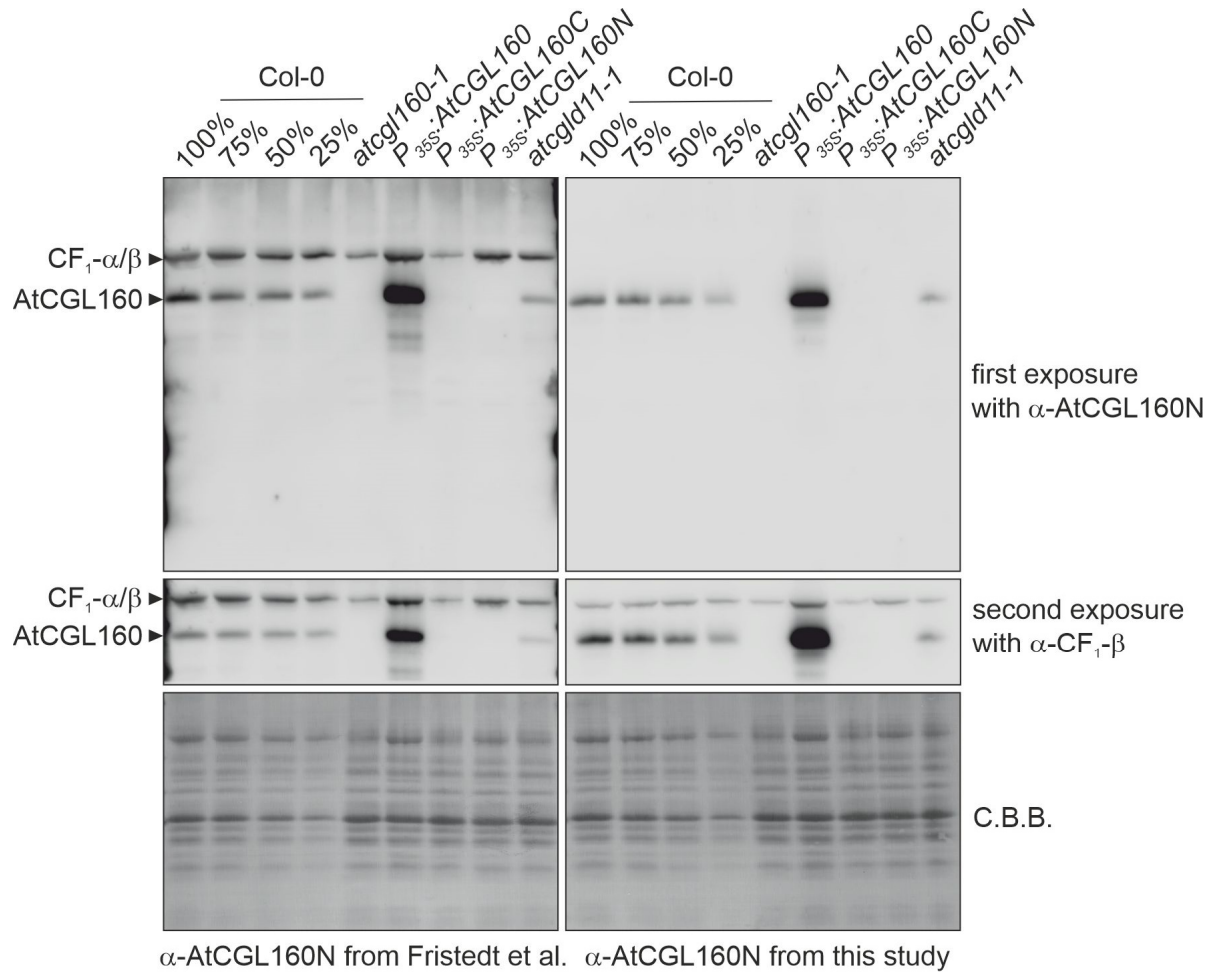
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**Supplemental Figure S1. Multiple alignment of the N-terminal portions of CGL160 sequences identified in species belonging to the green lineage.** Chloroplast transit peptides predicted by ChloroP are depicted in italics. Similar and identical amino acids conserved in 70% of the sequences are highlighted in grey and black, respectively. The region that includes several identified phosphopeptides in AtCGL160 is indicated and two conserved S/T residues are shown in yellow. Note that CGL160 transmembrane (TM) domains were omitted from the alignment. Sequence identifiers for CGL160 homologs are as follows: *Arabidopsis thaliana* (AtCGL160, NP\_565711), *Glycine max* (GmCGL160, XP\_006582279.1), *Malus domestica* (MdCGL160, XP\_008353735.1), *Zea mays* (ZmCGL160, NP\_001170362.2), *Sorghum bicolor* (SmCGL160, XP\_021312638.1), *Oryza sativa* Japonica group (OsCGL160, XP\_015619276.1), *Picea sitchensis* (PsCGL160, ABR16992.1), *Physcomitrella patens* (PpCGL160, XP\_024381807.1), *Chlamydomonas reinhardtii* (CrCGL160, XP\_001690237.1) and *Chlorella variabilis* (CvCGL160, XP\_005844436.1).



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 995 **Supplemental Figure S2. Screening of  $P_{35S}::AtCGL160$ ,  $P_{35S}::AtCGL160N$  and**  
 996  **$P_{35S}::AtCGL160C$  plants. A,** After transformation of *atcgl160-1*, T2 offspring of independent  
 997 T1 plants (#1-#6) were examined using an Imaging-PAM (Walz, Effeltrich, Germany) system.  
 998 Non-photochemical quenching (NPQ/4) was measured in light induction experiments on  
 999 detached leaves after 8 min of irradiation at 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and is indicated on a  
 1000 false-color scale from 0 to 1. Col-0 and *atcgl160-1* leaves served as controls.  $P_{35S}::AtCGL160$   
 1001 lines #1, #2, #4, #5 and #6 rescued the *atcgl160-1* phenotype. Transformation of *atcgl160-1*  
 1002 plants with the  $P_{35S}::AtCGL160N$  and  $P_{35S}::AtCGL160C$  constructs resulted in no  
 1003 complementation and partial complementation, respectively. **B,** *AtCGL160* transcript levels in  
 1004  $P_{35S}::AtCGL160C$  plants determined by Northern analysis. Note that RNA samples (8  $\mu\text{g}$ ) of  
 1005 line #2 were loaded twice for direct comparison of transcript levels with line #1  
 1006 ( $P_{35S}::AtCGL160$ ). **C,** Northern analyses of selected, homozygous lines (T3 generation). Total  
 1007 RNA (20  $\mu\text{g}$ ) from 4-week-old Col-0, *atcgl160-1*,  $P_{35S}::AtCGL160$ ,  $P_{35S}::AtCGL160N$  and  
 1008  $P_{35S}::AtCGL160C$  plants was size-fractionated on a denaturing formaldehyde gel and blotted  
 1009 onto a nylon membrane. Hybridization was carried out with a radioactive probe specific for the  
 1010 *AtCGL160* chloroplast transit-peptide coding region. Line #4 ( $P_{35S}::AtCGL160$ ) and line #2  
 1011 ( $P_{35S}::AtCGL160C$ ) were selected for further experiments due to their similar transcript levels.  
 1012 Methylene blue (M.B.) staining of the nylon membrane served as an RNA loading control in **B**  
 1013 and **C**.

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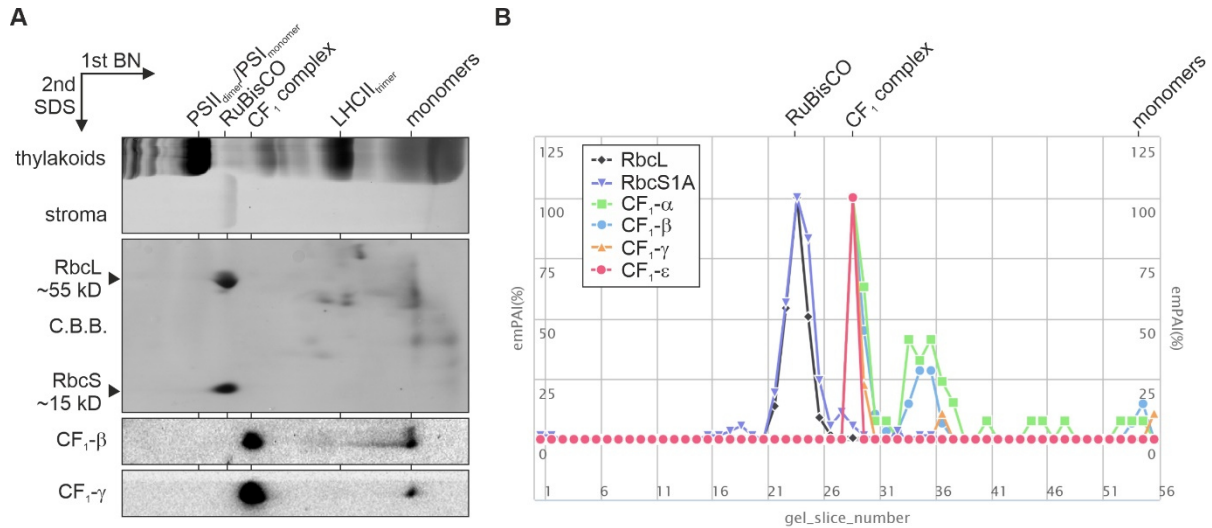


1015

$\alpha$ -AtCGL160N from Fristedt et al.  $\alpha$ -AtCGL160N from this study

1016 **Supplemental Figure S3.** Immunodetection of AtCGL160 in Col-0, *atcg1160-1*,  
 1017 *P<sub>35S</sub>::AtCGL160*, *P<sub>35S</sub>::AtCGL160N*, *P<sub>35S</sub>::AtCGL160C* and *atcgld11-1* plants. Thylakoid proteins  
 1018 were separated by denaturing SDS-PAGE and blotted onto PVDF membranes. Membranes  
 1019 were first probed with antibodies against AtCGL160N. After signal detection, membranes were  
 1020 re-probed with an antibody against CF<sub>1</sub>- $\beta$ . Coomassie brilliant blue staining (C.B.B.) of PVDF  
 1021 membranes is shown as a loading control. On the left, immunodetection analyses are shown  
 1022 for an AtCGL160 antibody (AS12 1853) which is commercially available from Agrisera and was  
 1023 employed in Fristedt et al. (2015). A side-by-side comparison with the newly generated  
 1024 antibody against the N-terminal part of AtCGL160 is provided in the right panel. Note that  
 1025 antibody AS12 1853 from Agrisera binds nonspecifically to CF<sub>1</sub>- $\alpha$  or CF<sub>1</sub>- $\beta$  and was therefore  
 1026 not considered for use in co-immunoprecipitation, cross-linking or 2D native/SDS-PAGE  
 1027 experiments.

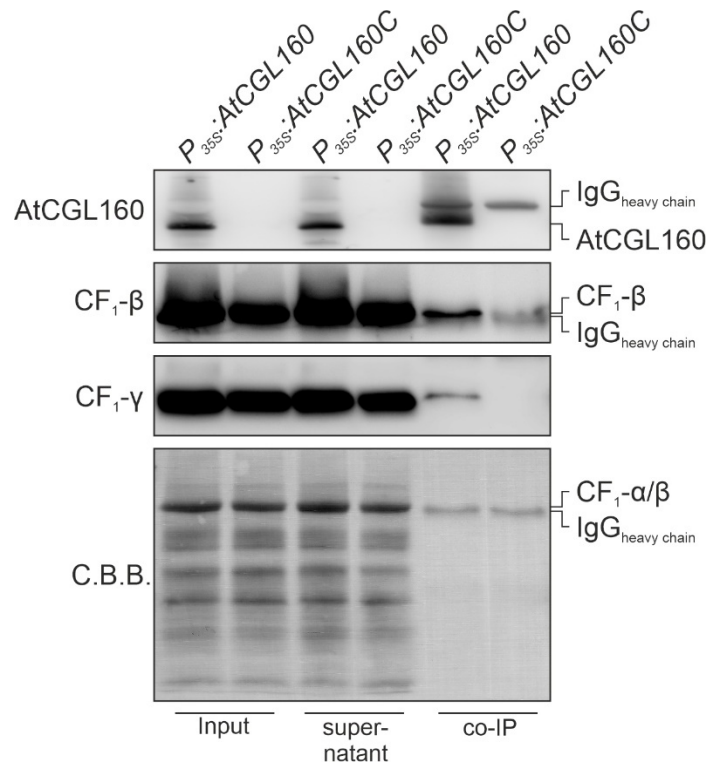
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1029

1030 **Supplemental Figure S4. Characterization of the stromal CF<sub>1</sub> complex in *atcg160-1***  
 1031 **plants. A**, A stromal protein extract of *atcg160-1* plants was subjected to 2D gel  
 1032 electrophoresis (Blue Native- and SDS-PAGE) and immunodetection of CF<sub>1</sub>-β and CF<sub>1</sub>-γ.  
 1033 Coomassie brilliant blue (G-250) staining of the PVDF membrane after transfer visualized  
 1034 abundant stromal complexes such as RuBisCO, which is composed of RbcL and RbcS.  
 1035 Prominent thylakoid complexes of *P<sub>35S</sub>:AtCGL160C* plants served as molecular mass  
 1036 standards. **B**, Composition of the stromal CF<sub>1</sub> sub-complex in Arabidopsis according to the  
 1037 Protein Co-migration Database for photosynthetic organisms (PCom-DB,  
 1038 <http://pcomdb.lowtem.hokudai.ac.jp/proteins/top>). Co-migration of RbcL (black diamonds) and  
 1039 RbcS (purple triangles) is provided for better comparison between PCom-DB results and the  
 1040 2D gel analyses presented in panel **A**. Subunit content is quantified according to the  
 1041 exponentially modified protein abundance index (emPAI) method and is normalized for each  
 1042 individual subunit to the maximal emPAI identified in a gel slice (Ishihama et al., 2005). The  
 1043 maximal RuBisCO and CF<sub>1</sub> content was detected in gel slices 24 and 29, respectively. Note  
 1044 that CF<sub>1</sub>-δ was not identified in the stromal CF<sub>1</sub> subcomplex.

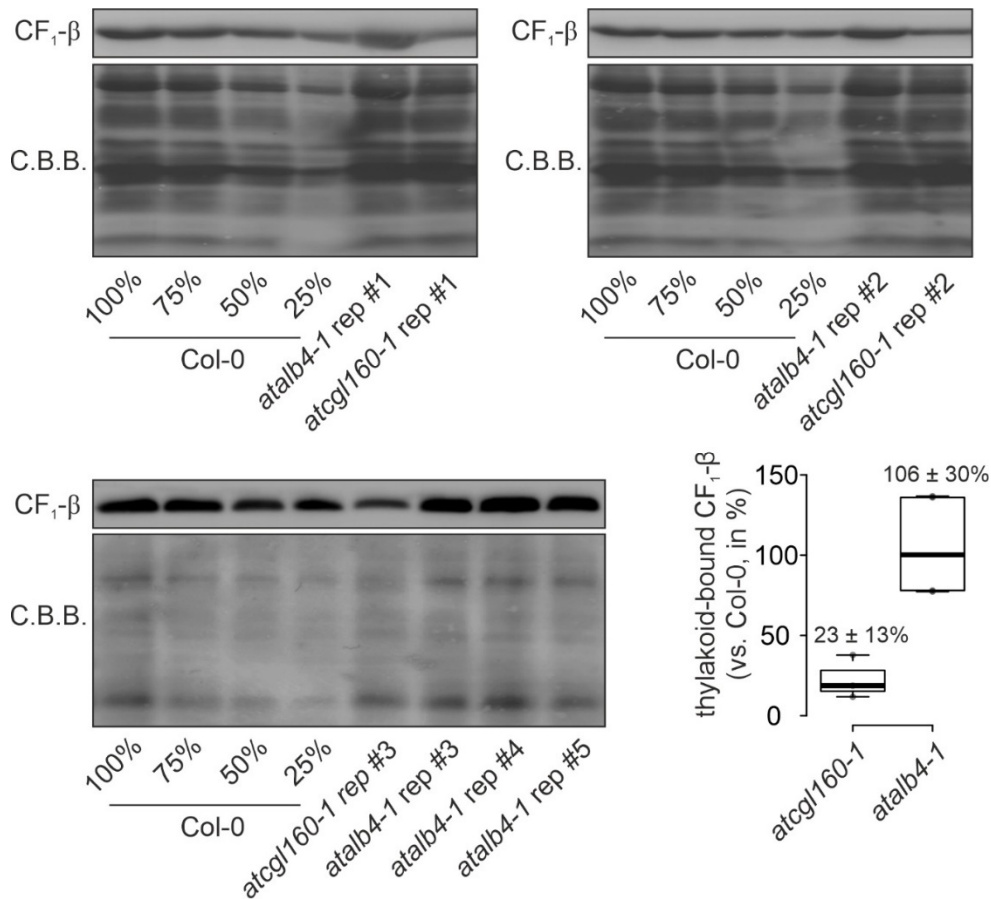
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1046

1047 **Supplemental Figure S5. Immunoblot analysis of AtCGL160 co-immunoprecipitation**  
1048 **assays.** A, Co-immunoprecipitation with NP40-solubilized thylakoids of oeAtCGL160 and  
1049 oeAtCGL160C was repeated using reduced amounts of the AtCGL160 antibody. Protein A-  
1050 coupled magnetic beads (Dynabeads, Thermo) coated with AtCGL160 antibody and co-  
1051 immunoprecipitated proteins (IP) were boiled in SDS loading buffer, separated by denaturing  
1052 SDS-PAGE and blotted onto PVDF membranes. Samples of NP40-solubilized thylakoids  
1053 before (Input) and after (Flow) incubation with AtCGL160 antibody were loaded as controls.  
1054 Membranes were probed separately with antibodies against AtCGL160N and CF<sub>1</sub>-β/CF<sub>1</sub>-γ. The  
1055 positions of the heavy chain of the AtCGL160 antibody are indicated (IgG). Coomassie brilliant  
1056 blue staining (C.B.B.) is shown as loading control, and the positions of CF<sub>1</sub>-α/β are indicated.





1057

1058 **Supplemental Figure S6. Quantification of thylakoid-bound CF<sub>1</sub>-β subunits in *atalb4-1***  
1059 ***Arabidopsis* mutant lines.** Thylakoid proteins were isolated from Col-0, *atcgl160-1* and  
1060 *atalb4-1* (SALK\_136199C) plants grown under short-day conditions, fractionated on SDS-  
1061 PAGE, transferred to PVDF membranes and probed with CF<sub>1</sub>-β antibodies. Membranes were  
1062 stained with Coomassie brilliant blue G-250 (C.B.B.) as loading control. Signals were  
1063 quantified relative to signals detected in the wild-type sample using the Bio-1D software  
1064 (version 15.03, Vilber Lourmat, Eberhardzell, Germany) and are provided as percentages.  
1065 Horizontal lines represent the median, and boxes indicate the 25th and 75th percentiles.  
1066 Whiskers extend the interquartile range by a factor of 1.5×. Means ± standard deviations are  
1067 provided above the boxes. Quantification is based on three and five replicates (rep) for  
1068 *atcgl160-1* and *atalb4-1* samples, respectively.

1069

## 1070 Additional references in Supplemental Figures

1071 **Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., and Mann, M.**  
1072 (2005). Exponentially modified protein abundance index (emPAI) for estimation of  
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