

## **Generating and characterising single- and multi-gene mutants of the Rubisco small subunit family in Arabidopsis**

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Highlight: A CRISPR/Cas9 approach combined with available T-DNA insertion lines to generate and characterise a suite of single and novel multiple gene knockout mutants of the small subunit family in Arabidopsis.

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1 **Abstract**

2 The primary CO<sub>2</sub>-fixing enzyme Rubisco limits the productivity of plants. The small subunit  
3 of Rubisco (SSU) can influence overall Rubisco levels and catalytic efficiency, and is now  
4 receiving increasing attention as a potential engineering target to improve the performance of  
5 Rubisco. However, SSUs are encoded for by a family of nuclear *rbcS* genes in plants, which  
6 makes them challenging to engineer and study. Here we have used CRISPR/Cas9 and T-  
7 DNA insertion lines to generate a suite of single and multiple gene knockout mutants for the  
8 four members of the *rbcS* family in *Arabidopsis*, including two novel mutants *2b3b* and  
9 *1a2b3b*. *1a2b3b* contained very low levels of Rubisco (ca. 3% relative to WT) and is the first  
10 example of a mutant with a homogenous Rubisco pool consisting of a single SSU isoform  
11 (1B). Growth under near-outdoor levels of light demonstrated Rubisco-limited growth  
12 phenotypes for several SSU mutants and the importance of the 1A and 3B isoforms. We also  
13 identified *1a1b* as a likely lethal mutation, suggesting a key contributory role for the least  
14 expressed 1B isoform during early development. The successful use of CRISPR/Cas here  
15 suggests this is a viable approach for exploring the functional roles of SSU isoforms in plants.

16 Key words: *Arabidopsis thaliana*, chloroplast, CRISPR/Cas9, photosynthesis, protoplasts,  
17 Rubisco, SpCas9, T-DNA

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## 20 **Introduction**

21 Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) catalyses the  
22 reaction between CO<sub>2</sub> and ribulose-1,5-bisphosphate (RuBP) and is responsible for net  
23 carbon gain in all oxygenic photosynthetic organisms including plants. Despite its  
24 importance, Rubisco is a relatively slow and error-prone enzyme that limits the efficiency of  
25 photosynthesis. This phenomenon is particularly evident in C3 crop plants, where increasing  
26 the operating efficiency of Rubisco and reducing photorespiration are considered promising  
27 strategies for improving growth and productivity (Rae et al., 2017; Kubis and Bar-Even,  
28 2019; Simkin et al., 2019; South et al., 2019). In plants, Rubisco (Form IB) is composed of  
29 eight large subunits (LSUs) and eight small subunits (SSUs) that form an L<sub>8</sub>S<sub>8</sub> complex  
30 (Bracher et al., 2017). The LSU is encoded for by a single, highly conserved gene on the  
31 chloroplast genome (*rbcL*), while SSUs are encoded by a family of nuclear *rbcS* genes that  
32 show significantly more diversity between species compared to *rbcL*. The size of the SSU  
33 family also differs between species, with up to twenty isoforms reported in cultivated  
34 polyploid wheat varieties (Sasanuma, 2001).

35 In Form I Rubisco, the eight LSUs form dimers that carry two active sites per dimer.  
36 The SSUs are located distal to the active sites and are not required for carboxylation, but are  
37 essential to maximise Rubisco activity and are thought to provide structural stability to the  
38 L<sub>8</sub>S<sub>8</sub> complex (Andersson and Backlund, 2008; van Lun et al., 2011). A substantial body of  
39 work in the green alga *Chlamydomonas reinhardtii*, cyanobacteria and higher plants has  
40 demonstrated that directed mutagenesis of the SSU, or expression of heterologous SSUs, can  
41 significantly modify the catalytic properties of Rubisco, including the turnover rate ( $k_{cat}$ ),  
42 CO<sub>2</sub>/O<sub>2</sub> specificity ( $S_{c/o}$ ) and the ability to assemble the L<sub>8</sub>S<sub>8</sub> complex (Schneider et al., 1990;  
43 Paul et al., 1991; Read and Tabita, 1992; Kostov et al., 1997; Getzoff et al., 1998; Spreitzer et  
44 al., 2005; Genkov and Spreitzer, 2009; Ishikawa et al., 2011; Esquivel et al., 2013; Whitney  
45 et al., 2015; Atkinson et al., 2017; Fukayama et al., 2019; Orr et al., 2019). More recently,  
46 several native, specialised SSU isoforms have been shown to enhance the catalytic properties  
47 of Rubisco in plants (Morita et al., 2014; Laterre et al., 2017; Pottier et al., 2018; Lin et al.,  
48 2019). This suggests that SSUs naturally play a role in modifying Rubisco performance and  
49 thus could be an important target for crop improvement.

50 The expression levels of SSUs also play a key role in regulating Rubisco abundance,  
51 and thus affect whole plant nitrogen and carbon partitioning, and the overall capacity for

52 carbon uptake (Rodermeil, 1999). Previous work has shown that reducing SSU content by  
53 antisense RNA or T-DNA insertion results in a decrease in *rbcL* mRNA translation, and  
54 subsequently, a decrease in Rubisco content (Rodermeil et al., 1996; Wostrikoff and Stern,  
55 2007; Izumi et al., 2012; Ogawa et al., 2012; Wostrikoff et al., 2012; Atkinson et al., 2017).  
56 The transcript abundances of SSU families have been explored in detail only for a small  
57 number of plant species, such as tomato, wheat, rice and *Arabidopsis* (Wanner and Gruissem,  
58 1991; Galili et al., 1992; Suzuki et al., 2009; Izumi et al., 2012). Within a species, the  
59 strength of promoters for each SSU can vary significantly, resulting in a range of expression  
60 levels between different SSU isoforms. Furthermore, expression levels of individual SSUs  
61 can vary depending on tissue type and developmental stage, and in response to the growth  
62 environment (Wehmeyer et al., 1990; Meier et al., 1995; Ewing et al., 1998; Day et al., 2000;  
63 Morita et al., 2014; Laterre et al., 2017). Functional studies for individual SSUs in a species  
64 are challenging due to the high sequence homology between SSU isoforms within a family  
65 (Yamada et al., 2019). Nevertheless, a better understanding of how the expression of different  
66 SSU isoforms is co-ordinated in response to the environment could lead to novel strategies to  
67 improve plant growth performances (Cavanagh and Kubien, 2014).

68 The model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) has four SSU genes,  
69 *rbcS1A*, *rbcS1B*, *rbcS2B* and *rbcS3B* (hereafter *1A*, *1B*, *2B* and *3B*, respectively), which are  
70 divided into A and B subfamilies based on linkage and sequence similarities (Krebbes et al.,  
71 1988; Schwarte et al., 2011). *1A* and *3B* are typically reported as the dominant SSU  
72 isoforms, while *1B* and *2B* are expressed at lower levels (Izumi et al., 2012; Klepikova et al.,  
73 2016). The *Arabidopsis* SSU family show signs of spatially overlapping and distinct  
74 expression during early leaf development (Sawchuk et al., 2008). Although the response of  
75 *Arabidopsis* SSUs to environmental stimuli is relatively well studied (Dedonder et al., 1993;  
76 Cheng et al., 1998; Yoon et al., 2001; Sawchuk et al., 2008), a clear understanding of their  
77 impact on growth and performance is still lacking.

78 Gene knockout (KO) mutants generated by T-DNA insertion have been useful tools  
79 for functional studies in *Arabidopsis* (Izumi et al., 2012; Atkinson et al., 2017), but this  
80 approach does have limitations. Firstly, T-DNA insertion sites are prone to small deletions,  
81 duplications, and filler sequence of unknown origin, while T-DNA lines have been shown to  
82 contain chromosomal translocations, and in some cases significant chromosomal  
83 rearrangements (Nacry et al., 1998; Clark and Krysan, 2010). Thus, multiple T-DNA  
84 insertion lines for a given gene are typically required to verify experimental findings.

85 However, in some cases only a limited number of T-DNA lines are available for a given  
86 locus. Secondly, generating multiple gene knockout lines is time consuming and not feasible  
87 for genes with loci in close proximity, as is the case for the three SSU B subfamily gene  
88 which, in Arabidopsis, are in a tandem array on chromosome 5 (Krebbers et al., 1988; Niwa  
89 et al., 1997).

90 To overcome this challenge, we have utilised a pooled clustered regularly interspaced  
91 short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) approach and  
92 available T-DNA insertion lines to generate a novel suite of *rbcs* mutants. We produced new  
93 single *rbcs* mutants for all four *rbcs* genes, a double *rbcs* mutant (*2b3b*) and a triple *rbcs*  
94 mutant (*1a2b3b*). Molecular characterisations were performed to examine the impact of  
95 specific SSU mutations on protein and Rubisco contents, while physiological analysis under  
96 near-outdoor light levels (1,000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) provided novel insights into the  
97 contributions of different SSU isoforms to growth performance. This study serves as a proof  
98 of principle for future studies to examine the roles of different SSU isoforms in other species.

## 100 **Materials and Methods**

### 101 *Plant material and growth conditions*

102 *Arabidopsis* (*Arabidopsis thaliana* (L.) Heyn. Col-0) seeds were sown on soil and stratified  
103 for 3d at 4 °C and grown at 22 °C, ambient CO<sub>2</sub>, 70% relative humidity and a photosynthetic  
104 photon flux (PPFD) of 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (standard lab conditions) or 1,000  $\mu\text{mol}$   
105  $\text{photons m}^{-2} \text{s}^{-1}$  (high light) supplied by cool white fluorescent lamps (Percival SE-41AR2,  
106 Clf Plantclimatics GmbH) in 12:12 light:dark. For comparison of different genotypes, plants  
107 were grown from seeds of the same age and storage history, harvested from plants grown in  
108 the same environmental conditions. *Arabidopsis* T-DNA insertion lines *1a* [GABI\_608F01  
109 (At1g67090)], *1b* [SAIL\_755\_D09 (At5g38430)], *2b* [GABI\_324A03 (At5g38420)] and *3b*  
110 [SALK\_117835 (At5g38410)] were sourced from the Nottingham Arabidopsis Stock Centre  
111 (NASC). The *1a2b* mutant (GABI\_608F01; GABI\_324A03) generated previously (Atkinson  
112 et al., 2017) was backcrossed with a wild-type plant to remove potential background  
113 mutations. The *1a3b* mutant (GABI\_608F01; SALK\_117835) was provided by Hiroyuki  
114 Ishida, Department of Applied Plant Science, Tohoku University, Japan. Homozygous T3  
115 seed stocks for mutants generated via CRISPR/Cas9 in this study can be obtained through the

116 Nottingham Arabidopsis Stock Centre (<http://arabidopsis.info>) (NASC IDs N2109789 –  
117 N2109802).

118

### 119 Construction of CRISPR/Cas9 vectors

120 Plasmid vectors were assembled using the Plant MoClo Golden Gate modular cloning kit  
121 (Engler et al., 2014). New Level 0 parts were made according to Patron et al. (2015). Level 0  
122 vectors (100 ng each) carrying the UBI10 promoter, the SpCas9 coding sequence (Parry et  
123 al., 2016) or the heat shock protein (HSP) terminator (Nagaya et al., 2010) were assembled  
124 into the Level 1 Position 2 (L1P2) acceptor vector in a 20 µl assembly reaction (BsaI  
125 (ThermoFisher Scientific, UK) (10U), 1X Buffer G, T4 DNA ligase (ThermoFisher  
126 Scientific) (400 U), 20 nmol ATP] as in Vasudevan et al. (2019). PCR amplicons of each  
127 complete gRNA (the spacer fused to the RNA scaffold) were combined with a Level 0 vector  
128 carrying the U6 promoter for assembly (Table S1). Each pair of gRNA expression cassettes  
129 were constructed in L1P3 and L1P4, respectively, as described in Raitskin et al. (2019). Four  
130 Level 1 transcriptional units (the pFAST selection marker (Shimada et al., 2010) in L1P1,  
131 L1P2, L1P3 and L1P4) were then assembled into a Level 2 acceptor vector in a 20 µl  
132 assembly reaction [BpiI (ThermoFisher Scientific) (10U), 1 X Buffer G (ThermoFisher  
133 Scientific), T4 DNA ligase (ThermoFisher Scientific) (400 U) and 20 nmol of ATP] (see  
134 Data S1) as in Vasudevan et al. (2019).

135

### 136 DNA and RNA extraction, PCR and RT-qPCR

137 DNA was extracted from a mature leaf as described in Li and Chory (1998). PCR reactions  
138 were performed as in McCormick and Kruger (2015) using gene-specific primers (Table S2).  
139 Total RNA was isolated from leaves using the RNeasy plant mini kit (Qiagen, USA). Isolated  
140 RNA was treated with DNase (Qiagen) and reverse transcribed with random primers  
141 (Promega, USA). Gene-specific primers amplifying the unique 3' region of the transcript  
142 were used for RT-qPCR (Izumi et al., 2012). A DNA fragment containing regions matching  
143 the target loci of the *rbcS* for RT-qPCR primers was synthesised (Gblock, IDT) (Fig. S1).  
144 RT-qPCR calibration curves were constructed using known concentrations of the standard to  
145 quantify mRNA levels for each *rbcS* transcript pool. For quantitative analysis, an aliquot of



146 cDNA derived from 4 ng of RNA was used (total volume 20 µl) with SYBR Green Master  
147 Mix (Eurogentec, Belgium).

148

#### 149 *CRISPR/Cas9 cassettes in protoplasts*

150 Leaves from 4-week-old plants were cut vertically into 1 mm strips and digested in 10 ml  
151 maceration glycine glucose (MGG) digestion solution as in Chupeau et al. (2013) containing  
152 cellulase “Onozuka” R-10 (1.5% [w/v]) and Macerozyme R-10 (0.4% ([w/v]) (Yakult  
153 Pharmaceutical, Japan) for 3 h. Released protoplasts were filtered from the digestate using a  
154 70 µm cell strainer and washed three times with MGG not containing enzymes to remove  
155 traces of the enzyme solution and cell debris. Protoplasts were resuspended in MMM solution  
156 (0.4 M mannitol, 15 mM MgCl<sub>2</sub>, 0.1% [w/v] MES [pH 8]) to a concentration of 5 x 10<sup>5</sup> cells  
157 ml<sup>-1</sup> in a 5 ml glass test tube. For protoplast transformations, 8 µl DNA (4 µg total) was added  
158 to 75 µl of the protoplast suspension, followed by addition of 83 µl of PEG solution (0.4 M  
159 mannitol, 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 40% [w/v] PEG 4000 [pH 8]). Following a 1 min  
160 incubation, 2 ml of MGG solution was added. Following a further 1 hr incubation at RT, the  
161 protoplasts were centrifuged at 70 g for 5 min and the supernatant removed. Fresh MGG  
162 solution was added (100 µl) and transfected protoplasts were incubated in the dark for 18 h at  
163 RT. The target loci of each CRISPR/Cas9 vector was analysed by PCR of protoplast DNA  
164 extracts (Table S2).

165

#### 166 *Expression of Cas9 and gRNA in Arabidopsis*

167 Binary vectors (Level 2) were transformed into *Agrobacterium tumefaciens* (AGL1) for  
168 stable insertion in Arabidopsis by floral dipping (Clough and Bent, 1999). T1 plants were  
169 screened for the presence of the transgene by the pFAST selectable marker (Shimada et al.,  
170 2010), and for the presence of CRISPR/Cas9-mediated mutations by PCR and Sanger  
171 sequencing (Table S2). Stable mutations in transgene-free T2 plants were confirmed by  
172 Sanger sequencing.

173

174



175 *Protein quantification and Rubisco content*

176 Leaf samples (20-40 mg) were collected from 35-d-old plants, snap frozen and stored at -80  
177 °C prior to extraction. Samples were ground rapidly in an ice-cold mortar and pestle in 200 µl  
178 of protein extraction buffer (50 mM Bicine-NaOH pH 8.2, 20 mM MgCl<sub>2</sub>, 1 mM EDTA, 2  
179 mM benzamidine, 5 mM ε-aminocaproic acid, 50 mM 2-mercaptoethanol, 10 mM  
180 dithiothriitol, 1% [v/v] protease inhibitor cocktail [Sigma-Aldrich, USA], and 1 mM  
181 phenylmethylsulphonyl fluoride) for *ca.* 1 min followed by centrifugation at 14,700 g at 4 °C  
182 for 1 min. Supernatant (90 µl) was then mixed with 100 µl of carboxyarabintol-1,5-  
183 bisphosphate (CABP) binding buffer (100 mM Bicine-NaOH [pH 8.2], 20 mM MgCl<sub>2</sub>, 20  
184 mM NaHCO<sub>3</sub>, 1.2 mM [37 kBq/µmol] [<sup>14</sup>C]CABP), incubated at RT for 25 min, and Rubisco  
185 content determined via [<sup>14</sup>C]CABP binding (Sharwood et al., 2016). Bradford assay was used  
186 to determine total soluble protein in the same supernatant as prepared for Rubisco content  
187 analysis (Bradford, 1976).

188 Extracts were subjected to SDS-PAGE on a 4-12% (w/v) polyacrylamide gel (Bolt®  
189 Bis-Tris Plus Gel) (ThermoFisher Scientific, UK), transferred to PVDF membrane then  
190 probed with rabbit serum raised against wheat Rubisco at 1:10,000 dilution (Howe et al.,  
191 1982) followed by LI-COR IRDye® 800CW goat anti-rabbit IgG (LI-COR Biosciences,  
192 USA) at 1:10,000 dilution, then viewed on an LI-COR Odyssey CLx Imager. The relative  
193 abundance of LSU and SSUs were estimated densitrometrically using Image Studio Lite (LI-  
194 COR Biosciences) and the values were means ± SE based on three immunoblots as in  
195 Atkinson et al. (2017)

196  
197 *Chlorophyll quantification*

198 Leaf discs (20 mm<sup>2</sup> in total) were frozen in liquid N<sub>2</sub>, powdered, and then mixed with 1 ml of  
199 ice-cold 80% (v/v) acetone, 10 mM Tris-HCl. Following centrifugation at 17,200 g for 10  
200 min, chlorophyll was quantified according to Porra et al. (1989).

201

202

203 *Measurement of photosynthetic parameters*

204 Gas exchange and chlorophyll fluorescence were determined using a LI-COR LI-6400  
205 portable infra-red gas analyser (LI-COR Biosciences) with a LI6400-40 leaf chamber (2 cm<sup>2</sup>  
206 area) on either the sixth or seventh leaf of 35- to 45-d-old rosettes grown under 200 μmol  
207 photons m<sup>-2</sup> s<sup>-1</sup> in large pots to generate leaf area sufficient for gas exchange measurements  
208 (Atkinson et al., 2017). For all gas exchange experiments, leaf temperature and chamber  
209 relative humidity were 25 °C and *ca.* 65%, respectively. The response of *A* to the intercellular  
210 CO<sub>2</sub> concentration (*C<sub>i</sub>*) was measured at various CO<sub>2</sub> concentrations (50, 100, 150, 200, 250,  
211 300, 350, 400, 500, 700, 900 and 1,200 μmol mol<sup>-1</sup>) under saturating light (1,800 μmol  
212 photons m<sup>-2</sup> s<sup>-1</sup>) (Fig. S2). Gas exchange data were corrected for CO<sub>2</sub> diffusion from the  
213 measuring chamber as in Bellasio et al. (2016). To calculate the maximum rate of Rubisco  
214 carboxylation (*V<sub>cmax</sub>*), the *A/C<sub>i</sub>* data were fitted to the C<sub>3</sub> photosynthesis model as in Ethier  
215 and Livingston (2004) using the catalytic parameters *K<sub>c</sub><sup>air</sup>* and affinity for O<sub>2</sub> (*K<sub>o</sub>*) values for  
216 wild-type *Arabidopsis* Rubisco at 25 °C from Atkinson et al. (2017). Estimates of the light-  
217 and CO<sub>2</sub>-saturated photosynthetic electron transport rate (*J<sub>max</sub>*) were not included as several  
218 of the SSU mutants were likely Rubisco-limited even at high CO<sub>2</sub> concentrations. Maximum  
219 quantum yield of photosystem II (PSII) (*F<sub>v</sub>/F<sub>m</sub>*) was measured using a Hansatech Handy PEA  
220 continuous excitation chlorophyll fluorimeter (Hansatech Instruments, UK) (Maxwell and  
221 Johnson, 2000).

222

223 *Rosette area and biomass*

224 Rosettes were imaged daily during growth experiments. Rosette area was calculated using  
225 iDiel Plant software (Dobrescu et al., 2017). For biomass measurements, aerial rosette tissue  
226 was removed and weighed to determine fresh weight. Samples were then dried in an oven (80  
227 °C for 3 d) and weighed to determine dry weight.

228

229

230 Statistical analysis

231 Significant differences between sample groups were assessed by one-way analysis of  
232 variance (ANOVA) followed by Tukey's honest significant difference (HSD) post hoc test  
233 (IBM SPSS Statistics Ver. 26.0, USA) for individual parameters. Difference in growth  
234 performance (as measured by rosettes area) were assessed by repeated measures ANOVA  
235 followed by Tukey's HSD post hoc test.

236

237 **Results**

238 Identification of T-DNA insertion lines and double knockout mutants

239 We initially performed a search for available Arabidopsis mutant lines on T-DNA Express  
240 (<http://signal.salk.edu/cgi-bin/tdnaexpress>) that carry a single homozygous T-DNA insertion,  
241 ideally located in exonic regions for members of the SSU gene family. We identified only  
242 one such line for 1A (GABI 608F01) and 2B (GABI 324A03) (Fig. 1A). The only available  
243 homozygous mutants for 1B (SAIL 755 D09) and 3B (SALK 117835) had insertion sites  
244 located in the 5' UTR. For the latter, 3B expression is reportedly reduced but not absent  
245 (Izumi et al., 2012).

246 Double mutants 1a2b and 1a3b were generated previously by crossing available T-  
247 DNA lines (Izumi et al., 2012; Atkinson et al., 2017). Upon further characterisation of 1a2b,  
248 we observed that the mutant had a defective silique phenotype, with a reduced silique size  
249 and seed count per silique (Fig. S3A, S3B). Backcrossing with a wild-type (WT) plant and  
250 then re-segregating 1a2b in the F2 generation removed the observed silique phenotype,  
251 indicating that this trait was not attributable to the absence of 1A and 2B. Furthermore, the  
252 new 1a2b showed a more robust growth phenotype compared to the original double mutant  
253 (Fig. S3C). Single *rbcS* mutant lines for 1a or 2b did not show a silique phenotype. We  
254 identified plants in the segregating F2 population with the silique phenotype or reduced  
255 growth, but with no T-DNA insertions in either 1A or 2B, indicating that these traits were  
256 possibly linked to a recessive heterozygous mutation in either the 1a or 2b parental line. The  
257 new 1a2b double mutant was used for the remainder of this study. We also attempted to  
258 generate a 1a1b double mutant by crossing the T-DNA insertion mutants for 1a and 1b, and  
259 were successful in generating a heterozygote F1 (1A1a1B1b) line (Fig. S4). We were not able  
260 to recover a 1a1b mutant after screening 125 F2 plants, but did identify a 1A1a1b1b line.

261 Analysis of 15 F3 progeny of the latter line failed to show a Mendelian distribution for the *IA*  
262 locus (8: 7: 0; WT: heterozygous: homozygous KO), suggesting that *ialb* may be lethal. The  
263 germination rates of seeds recovered from the mutant lines were comparable to WT plants  
264 (WT = 93%, 1A1a1B1b = 93%, 1A1a1b1b = 96% of ca. 100 seeds), indicating that selection  
265 against a *ialb* genotype occurred prior to seed development.

266

### 267 *gRNA targeting strategy and transient expression of Cas9/gRNAs in protoplasts*

268 To generate new *rbcS* mutants for individual members of the Arabidopsis SSU family using  
269 the CRISPR/Cas9 method, we initially designed two unique pairs of guide RNAs (gRNAs) to  
270 target specific regions of each of the four *rbcS* genes, for a total of eight gRNA pairs (Fig.  
271 1A; Table S1). Furthermore, to knock out *1B*, *2B* and *3B* simultaneously we designed a  
272 promiscuous pair of gRNAs to target homologous regions in the *1B-3B* locus (Fig. 1B; Table  
273 S1). A paired gRNA approach was chosen for two reasons: i) to increase the probability of  
274 generating mutations and ii) the generation of larger deletions between the two gRNAs could  
275 potentially be screened more easily and cheaply (e.g. by PCR). Each gRNA pair was  
276 assembled using the Plant MoClo system as an individual expression cassette in a Level 2  
277 binary vector containing a Cas9 expression cassette (Data S1) (Engler et al., 2014).

278 Vectors with each *rbcS* specific gRNA pair were initially tested using a novel  
279 Arabidopsis protoplast transient expression system to estimate the efficiency of generating  
280 deletion events (Fig. 2A). Following transfection of the gRNA pairs targeting *1A* or *1B*,  
281 amplification of the respective gene loci produced the expected WT band and a second lower  
282 band indicating a deletion event based on the target sites of the gRNA pair. Pairs 1AP2 and  
283 1BP2 most consistently produced prominent “deletion bands” for *1A* and *1B*, respectively,  
284 and thus were selected for *in planta* expression. Only one gRNA pair each for *2B* (2BP2) and  
285 *3B* (3BP1) produced a deletion band. Sequence analysis of the deletion bands indicated  
286 cleavage 3-4 bp downstream of the gRNA PAM sites, consistent with the activity of SpCas9,  
287 and deletions ranging from 96 bp to 180 bp (Fig. 2B).

288

289

290 Expression of CRISPR/Cas9 in planta to generate stable mutants for each *rbcS* isoform

291 Arabidopsis plants were stably transformed with CRISPR/Cas9 binary vectors containing  
292 gRNA pairs 1AP2, 1BP2, 2BP2, 3BP1 and the promiscuous pair 1B3B. Transformed T1  
293 seeds were visually selected using the pFAST red fluorescent seed coat marker for the  
294 presence of Cas9 and gRNAs (Shimada et al., 2010). The loci of the five gRNA pairs were  
295 initially screened by PCR for large deletions, which were detected in transformants for 1AP1,  
296 2BP2 and 3BP1 but not for 1BP2 (Fig. S5A). The deletion bands were consistent with those  
297 observed in protoplasts (Fig. 2A). Several deletion bands were detected for 1B3B that were  
298 consistent with predicted amplicon sizes following multiple cleavage events within the *1B-3B*  
299 locus (Fig. S5B). Sequencing of each amplicon showed the expected cleavage position  
300 downstream of the PAM sites.

301 The efficiency with which deletions were induced by gRNA pairs ranged from 1-14%  
302 (Table 1). However, all deletions that were detected were accompanied by a significantly  
303 brighter WT band, indicating chimeric rather than heritable genomic mutations (i.e.  
304 homozygous or heterozygous) (Feng et al., 2014; Pauwels et al., 2018). T1 transformants  
305 were also screened by Sanger sequencing to detect potential small indel mutations at each  
306 gRNA target site (Fig. S5C). Indels were generally detected at a higher frequency than large  
307 deletions (3-31%).

308 Screening for heritable mutations in the T2 generation was performed on segregated  
309 non-red seed progeny (i.e. containing no CRISPR/Cas9 insertion). Eight progeny from each  
310 T1 line that showed large deletions were screened by PCR (Table 1). No heritable large  
311 deletions were detected for any gRNA pair, confirming that the observed deletions in T1  
312 were chimeric mutations. Sanger sequencing was then performed on T2 lines that contained  
313 large deletions or indels in T1. Stable homozygous indels (i.e. frame-shift mutations in exonic  
314 regions that produced early stop codons) were identified for all four *rbcS* genes targeted by  
315 specific gRNA pairs (Table S3; Fig. S6). For the promiscuous gRNA pair 1B3B, each of the  
316 B subfamily genes was sequenced in 64 non-red T2 plants from eight T1 lines showing large  
317 deletions. No mutations were observed in *1B* in any of the lines tested. However, we did  
318 identify a 147 bp deletion in *3B* common to the progeny of a single T1 line. Sequencing of  
319 the eight progeny revealed an additional 4 bp indel deletion in *2B* in a single plant, thus  
320 producing a *2b3b* double mutant. For *2b3b*, both indels led to frame-shift mutations that  
321 produced early stop codons in *2B* and *3B*. For all *rbcS* lines, T2 plants containing

322 homozygous mutations were selected and seeds were collected for subsequent molecular and  
323 physiological characterisations in the T3 generation.

324

### 325 Generating triple *rbcS* mutants using the *1a2b* T-DNA insertion mutant

326 We next introduced CRISPR/Cas9 binary vectors containing gRNA pairs 3BP1 or 1BP2 into  
327 the new *1a2b* double T-DNA insertion mutant to attempt to produce the novel triple mutants  
328 *1a2b3b* or *1a1b2b*. Out of 30 T1 *1a2b* transformants for 3BP1, three showed a slow-growth,  
329 pale leaf phenotype compared to the *1a2b* phenotype (Table 1; Fig. 3A). Sanger sequencing  
330 of the *3B* locus confirmed the presence of a homozygous and biallelic frame-shift mutation in  
331 all three T1 plants (Fig. S6). The heritability of those mutations was confirmed in non-red  
332 seed progeny of the T2 generation for each line. In contrast, 1B is a minor SSU isoform, so  
333 no growth phenotype was expected in T1 *1a2b* transformants for 1BP2. Sanger sequencing of  
334 the *1B* locus in 33 T1 plants showed mutations in *1B* for eight plants (*ca.* 25%) indicating that  
335 the CRISPR/Cas9 was functional and efficient. However, in all cases only single bp changes  
336 (i.e. a single codon substitution) or silent substitutions were observed (Fig. S7).

337

### 338 Molecular characterisation of *rbcS* mutants

339 The expression profiles of the *rbcS* family and *rbcL* were quantified in T3 plants for each  
340 *rbcS* mutant line. Transcript abundances were generally reduced for *rbcS* genes in lines  
341 targeted by specific T-DNA insertions or CRISPR/Cas9 editing (Table S4). Consistent with  
342 previous observations, the relative expression of *rbcL* was more repressed in mutants with  
343 greater reductions in overall *rbcS* expression (i.e. *1a3b* and *1a2b3b*) (Wostrikoff and Stern,  
344 2007; Izumi et al., 2012; Wostrikoff et al., 2012; Atkinson et al., 2017).

345 The total soluble protein content in leaves of all single mutants, and double mutants  
346 *1a2b* and *2b3b*, was similar to that of WT plants, but was significantly reduced by 20% and  
347 82% for *1a3b* and *1a2b3b*, respectively (Fig. 3B, Table S5). Leaf Rubisco content was  
348 generally decreased in single *rbcS* mutants compared to WT plants, and was significantly  
349 reduced in all double mutants and *1a2b3b* (Fig. 3C). Specifically, *1a2b* and *2b3b* had  
350 Rubisco levels reduced by 42% and 38%, respectively, *1a3b* by 61% and *1a2b3b* by 97%  
351 relative to WT plants. Disruption of *1A* led to an absence of the lower 1A SSU (14.7 kDa)



352 band in *1a*, *1a2b*, *1a3b* and *1a2b3b* mutants as detected by Western blot (Fig. 3D). Similarly,  
353 disruption of *3B* resulted in a reduction in the intensity of the upper SSU band of the B-  
354 subfamily genes (14.8 kDa) in *3b*, *2b3b*, *1a3b* and *1a2b3b* mutants. In contrast, no observable  
355 reductions in upper band intensity were observed with mutants of the less expressed isoforms  
356 *1B* and *2B*. Nevertheless, the SSU band intensity of *1a2b3b* was 30-fold lower than that of  
357 the overall intensity of the WT SSU bands.

358 \_\_\_\_\_

### 359 *Characterisation of *rbcS* mutants under normal light and high light*

360 Growth assays were performed under standard lab conditions for Arabidopsis plants (i.e.  
361 PPFD = 200  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) and under near-outdoor levels of light (i.e. PPFD = 1,000  
362  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) to characterise the growth performance of the *rbcS* mutants. Under  
363 standard lab conditions, the gene knockout mutants for individual *rbcS* genes generated by  
364 CRISPR/Cas9 or T-DNA insertion (i.e. *1a*, *1b*, *2b* and *3b*), as well as double mutants *1a2b*  
365 and *2b3b*, did not differ from WT plants in terms of rosette expansion rate, fresh weight  
366 (FW), dry weight (DW), specific leaf area (SLA), maximum potential quantum efficiency of  
367 PSII ( $F_v/F_m$ ), or chlorophyll content (Fig. 4; Table S6). In contrast, *1a3b* and *1a2b3b* showed  
368 significant reductions in area, FW, DW, SLA, chlorophyll content, and  $F_v/F_m$ . *1a2b3b* was  
369 more slow-growing than *1a3b*, but eventually developed to a similar level under standard lab  
370 conditions and produced viable seeds (Fig. 3A).

371 Under high light, *1a* and all three double mutants had significantly lower area, FW,  
372 DW, and chlorophyll content compared to WT. A significant reduction in FW and DW was  
373 also observed for *3b* mutants, while rosette area remained similar to WT. As a result, *3b*  
374 mutants also had a significantly higher SLA than WT. High light was lethal to the *1a2b3b*  
375 triple mutant, which did not survive past 15 days after germination.

376 \_\_\_\_\_ The response of  $A$  to  $C_i$  under saturating light ( $A/C_i$  curves) was measured for all *rbcS*  
377 mutant lines grown under standard lab conditions (Fig. 5) as well as key photosynthetic  
378 variables (Table 2). Stomatal conductance to  $\text{CO}_2$  at ambient  $\text{CO}_2$  ( $g_s$ ) and respiration rates in  
379 the dark ( $R_d$ ) were the same in all lines, consistent with previous work showing that  
380 reductions in Rubisco do not affect stomatal behaviour or mitochondrial respiration in  
381 Arabidopsis (Atkinson et al., 2017). Single *rbcS* mutants for *1A* and *3B* showed significant  
382 decreases in the maximum rate of Rubisco carboxylation ( $V_{\text{cmax}}$ ) compared to WT plants. In



383 contrast, *1b* and *2b* mutants were similar to WT. All three double mutants (*1a2b*, *2b3b* and  
384 *1a3b*) had decreased  $V_{\text{cmax}}$ , and *1a3b* also showed an increased sub-stomatal  $\text{CO}_2$   
385 compensation point ( $\Gamma$ ). However, *1a2b3b* was significantly different from all other plant  
386 lines, with a four-fold higher  $\Gamma$  value, and  $V_{\text{cmax}}$  at 10% of WT values.

387

## 388 **Discussion**

389 Although the uptake of CRISPR/Cas9 in plant biology has increased dramatically in recent  
390 years, many aspects concerning efficiency are still unclear, especially as established  
391 guidelines for CRISPR/Cas9 in other biological systems are not necessarily applicable to  
392 plants (Liang et al., 2016; Hahn and Nekrasov, 2019). As the generation of stable plant  
393 transformants is time-consuming, we adopted a more rapid, transient expression approach  
394 using Arabidopsis protoplasts to test the efficiency of different gRNA pairs and select robust  
395 candidates. All selected gRNA pairs were active in Arabidopsis and resulted in stable *rbcS*  
396 mutants. Thus, in agreement with previous studies (e.g. Li et al., 2013; Durr et al., 2018),  
397 screening gRNAs in protoplasts appears to be a reliable method for selecting functional  
398 gRNAs *in planta*. Overall, CRISPR/Cas appeared a viable approach to examine the impact  
399 of mutating *rbcS* isoforms and exploring their functional roles in plants.

400 CRISPR/Cas9 editing can be utilised to edit closely linked genes, which is not  
401 technically feasible through crossing T-DNA insertion lines such as genes in the *rbcS* B-  
402 subfamily. Here, the *2b3b* double *rbcS* mutant was successfully generated by targeting  
403 homologous regions by the gRNA pair 1B3B. In addition, the triple mutant *1a2b3b* was  
404 generated by targeting 3B in the *1a2b* T-DNA line. Interestingly, disruption of 1B in  
405 conjunction with other *rbcS* genes was not accomplished using T-DNA insertion or  
406 CRISPR/Cas9 approaches despite successful generation of *1b* mutants with both methods. 1B  
407 is the least expressed *rbcS* isoform and the mature peptide differs from 2B and 3B by only  
408 two amino acid residues (Izumi et al., 2012). Thus, it is unlikely that the structure of 1B has a  
409 unique impact on Rubisco activity (Valegård et al., 2018). Nevertheless, the temporal and/or  
410 spatial expression of 1B may contribute a specialised functional role that is critical for  
411 Arabidopsis development or fitness (i.e. during procreation). For example, previous work has  
412 shown that 1B localises exclusively to the abaxial side of primordia and young leaves  
413 (Sawchuk et al., 2008). It would be interesting to further examine 1B localisation in  
414 reproductive tissues (e.g. siliques or flowers). As a product of gene duplication, 1B is subject

415 to selection pressure and is not retained in all accessions of Arabidopsis, although loss was  
416 correlated with disruption of the promoter (Schwarte and Tiedemann, 2011). Retention of 1B  
417 may be linked to sublocalisation (Qiu et al., 2019). Differential expression patterns of *rbcS*  
418 genes in different organs have also been observed in other plant species including tomato,  
419 maize, tobacco and rice (Wanner and Gruissem, 1991; Ewing et al., 1998; Morita, et al.,  
420 2014; Laterre et al., 2017). However, the extent of how important these organ-specific SSUs  
421 are has not yet been explored.

422 Gene editing via CRISPR/Cas9 resulted in a general decrease in mRNA abundance of  
423 target genes and significant reductions in Rubisco content for all *1a*, *1a2b*, *1a3b* and *1a2b3b*  
424 mutants. The observed reduction in mRNA levels was likely due to the presence of early stop  
425 codons that emerged from frame-shift mutations, which consequently led to the degradation  
426 of mRNA through the nonsense-mediated decay process (Hug et al., 2015). Reductions in  
427 *rbcS* transcripts led to a reduction in the *rbcL* transcript but not to the same extent, as  
428 Arabidopsis *rbcL* transcript is controlled post-transcriptionally at the translation initiation  
429 process (Rodermel et al., 1996). Similar to Arabidopsis, *rbcL* synthesis in tobacco was shown  
430 to be partially independent of the *rbcS* transcript level and LSU production was subject to  
431 assembly state-dependent regulation that operated at the translational level (Wostrikoff and  
432 Stern, 2007).

433 *rbcS* mutants with a relatively small reduction in Rubisco content (i.e. above < 40% of  
434 WT levels), showed no change in growth rate and biomass accumulation relative to WT  
435 plants under standard lab growth conditions (i.e. PPFD =  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). This was  
436 not unexpected as reductions in Rubisco content have previously been shown to be  
437 compensated by an increase in activation state of the remaining Rubisco pool under  
438 conditions that are non-limiting for Rubisco (Quick et al., 1991). However, under high light  
439 conditions (i.e. PPFD =  $1,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), biomass accumulation (i.e. FW and  
440 DW) was significantly decreased in *1a* and *3b* mutants and all three double mutants (i.e.  
441 *2b3b*, *1a2b* and *1a3b*). This demonstrates that under near-outdoor levels of light, both 1A and  
442 3B are critical for normal growth.

443 Differential expression of the Arabidopsis *rbcS* genes has been observed previously  
444 (e.g. under changing temperatures, light qualities and CO<sub>2</sub> concentrations (Dedonder et al.,  
445 1993; Cheng et al., 1998; Yoon et al., 2001; Sawchuk et al., 2008)) but the impact of these  
446 differences on growth remains unclear. One potential use of our *rbcS* mutant suite could be to

447 further examine the importance and interaction of specific SSU isoforms during development.  
448 All SSUs with the exception of *1B* are increased in response to specific wavelengths of light  
449 (i.e. blue, red and far-red light), but at differing relative gene expression levels (Dedonder et  
450 al., 1993; Sawchuk et al., 2008). Furthermore, *1A* is the major isoform below 20 °C, whereas  
451 *3B* expression is dominant at 30 °C (Yoon et al., 2001; Izumi et al., 2012).

452 The rosette area of *3b* mutants was not decreased under high light compared to WT  
453 plants. However, the observed reduction in biomass resulted in a significant increase in SLA  
454 and suggested that leaves were thinner in those lines. Furthermore, gas exchange  
455 measurements demonstrated a decrease in  $V_{\text{cmax}}$  for *3b* mutants. Similar increases in SLA and  
456 reductions in photosynthetic capacity were observed for *1a* and all three double mutants.  
457 These observations are in line with previous growth analyses of Arabidopsis Rubisco activase  
458 antisense lines under high light (Eckardt et al., 1997), where a reallocation of resources to  
459 expand leaf area and reduce thickness was observed when photosynthetic capacity was  
460 limiting (Hoshino et al., 2019).

461 For the most severely Rubisco-limited mutants, *1a3b* and *1a2b3b*,  $F_v/F_m$  was reduced  
462 under standard light. However,  $F_v/F_m$  for *1a3b* was similar to WT under high light, indicating  
463 that a reduction in Rubisco has less impact on the operating efficiency of the light reactions  
464 under high light. Thus, failure of *1a2b3b* to grow under high light could indicate an inability  
465 of the light reactions to co-ordinate product utilisation (i.e. ATP and NADPH) with the  
466 extremely low Rubisco content of the triple mutant.

467 CRISPR/Cas9 is a versatile tool that has been successfully used for genetic editing  
468 and the enhancement of breeding strategies in a wide variety of plant and crop species  
469 (Khumsupan et al., 2019; Wolter et al., 2019). This study has shown that CRISPR/Cas is a  
470 viable approach for characterizing the roles of SSUs in plant species and that Arabidopsis  
471 mutants lacking SSU isoforms are useful platforms for the study of functional roles of SSUs.  
472 In particular, the triple mutant *1a2b3b* is potentially a powerful resource for studying the  
473 impact of heterologous SSU expression that has previously been studied in the *1a3b*  
474 background (Atkinson et al., 2017). Unlike *1a3b*, which has a Rubisco content of ca. 35%  
475 relative to WT (Izumi et al., 2012; Atkinson et al., 2017), *1a2b3b* retained only 3% under the  
476 conditions tested, and is the first example of a plant line with a homogenous Rubisco pool  
477 consisting of single SSU and a single LSU isoform. Following complementation, further  
478 disruption of the remaining *1B* isoform (e.g. by CRISPR/Cas9 editing using gRNA pair

479 1BP2) could be used to generate a true hybrid Rubisco pool comprised of only heterologous  
480 SSU(s) and the native LSU. In addition, as the contribution of individual SSUs to the Rubisco  
481 enzyme is still unclear, the triple mutant could be exploited as a model to knock in native  
482 SSUs tagged with different fluorescent probes (Ishida et al., 2008). This method would allow  
483 for the visualisation of composition of each hexadecamer in the Rubisco enzyme.

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745 **Figure Legends**

746 **Figure 1.** The Rubisco small subunit gene family in *Arabidopsis thaliana*. (A) The sites of  
747 the T-DNA insertions for mutant lines used in this study are shown in blue with the  
748 orientation of the left border (LB) indicated. The locations targeted by CRISPR-Cas9 are  
749 shown, with the names of the targeting gRNAs in green boxes (see Table S1 and vector maps  
750 in Data S1 for gRNA sequences). The 5' and 3' untranslated regions (UTR) are shown for  
751 each gene. (B) The B subfamily is located in tandem in the 1B-3B locus. A promiscuous pair  
752 of gRNAs targeting the three small subunits in the 1B-3B locus is indicated.

753

754 **Figure 2.** Targeted mutagenesis of Rubisco small subunits in protoplasts of *Arabidopsis*  
755 *thaliana* using vectors encoding Cas9 and gRNA pairs. (A) Detection of mutations using  
756 PCR. Lane 1, DNA marker; Lanes 2 - 9, PCR products of genomic DNA from protoplasts  
757 transfected with vectors carrying gRNA pairs targeting Rubisco small subunit (SSU) genes  
758 *1A* (1AP1, 1AP2), *1B* (1BP1, 1BP2), *2B* (2BP1, 2BP2) and *3B* (3BP1, 3BP2) (see Table S2  
759 for primer details). (B) Sequencing results of the lower “deletion band” showed deletions  
760 events 3-4 bp upstream of the PAM site (underlined). The gRNA target sequences are shown  
761 in bold.

762

763 **Figure 3.** Total soluble protein and Rubisco contents in *rbcS* mutants of *Arabidopsis*  
764 *thaliana*. (A) The *1a2b3b* mutant compared to *1a3b* and WT plants grown under standard  
765 conditions. All images represent 28-d-old rosettes unless otherwise stated. (B) Total soluble  
766 protein content shown for 35-d-old plants. (C) Rubisco content determined by <sup>14</sup>C-CABP  
767 binding and subunit ratios estimated by immunoblotting. Values are means ± SE of five  
768 measurements. (D) Representative immunoblots of *rbcS* mutants probed with a serum  
769 containing polyclonal antibodies against Rubisco to illustrate the reduction or absence of each  
770 SSU subfamily. The LSU (55 kDa) and SSU (14.7 kDa for 1A and 14.8 kDa for the B  
771 subfamily) are shown. See Table S5 for further results and statistical analysis.

772

773

774

775 **Figure 4.** Growth phenotypes of *rbcS* mutants of *Arabidopsis thaliana* grown under standard  
776 and high light conditions. (A) Rosette area expansion of *rbcS* single T-DNA and CRISPR/Cas9  
777 (CC) mutants, (B) rosette area expansion of double and triple mutants (the insert shows the  
778 expansion of *1a2b3b* at appropriate scale), and (C) fresh and dry weights of 28-d-old rosettes  
779 of all mutant lines grown under standard conditions. (D) Rosette area expansion of *1b*, *2b* and  
780 *3b* T-DNA and CRISPR/Cas9 mutants, (E) rosette area expansion of *1a*, *2b3b*, *1a2b* and  
781 *1a3b* mutants, and (F) fresh and dry weights of 28-d-old rosettes for all mutant lines grown  
782 under high light conditions. (G) Representative examples of 20-d-old rosettes of plants grown  
783 standard conditions and (H) under high light conditions. Letters in the legends for (B) and  
784 (E) indicate significant difference between plant lines ( $P < 0.05$ ) as determined by repeated  
785 measures ANOVA followed by Tukey's HSD tests. See Table S6 for further results and  
786 statistical analysis.

787

788 **Figure 5.** Photosynthetic CO<sub>2</sub> response curves of *rbcS* mutants of *Arabidopsis thaliana*.  
789 Measurements were made on fully expanded sixth or seventh leaf of 35- to 45-d-old non-  
790 flowering rosettes for WT and mutants and 80-d-old non-flowering rosettes for *1a2b3b*. The  
791  $A/C_i$  curves show the response of net CO<sub>2</sub> assimilation ( $A$ ) to different sub-stomatal  
792 concentration of CO<sub>2</sub> ( $C_i$ ) under saturating light ( $1,800 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) for A) *1b* and *2b*  
793 mutants, B) *1a* and *3b* mutants, and C) *2b3b*, *1a2b*, *1a3b*, and *1a2b3b* mutants. Each value  
794 represents the means  $\pm$  SE of measurements made on individual leaves from three to four  
795 different rosettes.

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796 **Table 1.** Editing efficiency of paired gRNAs targeting each *rbcS* gene. T1 plants containing  
 797 Cas9 were screened by PCR for large deletions and Sanger sequencing for indels and point  
 798 mutations (PMs). T1 lines containing large deletions or indels/PMs were screened for  
 799 heritable mutations in Cas9-free plants in the T2 generation. Six of Cas9-free T2 plants were  
 800 sequenced for each T1 line (34 lines for 1AP2, 11 lines for 1BP2, 13 for 2BP2, 11 lines for  
 801 3BP1 and 8 lines for 1B3B).

802

gRNA pair	Target gene	Background	Number of T1 plants (with transgene)		Number of T2 plants (Transgene-free)	
			Large deletion	Indels/PM	Large deletion	Indels/PM
1AP2	1A		8/92 (9%)	26/106 (25%)	0/8 (0%)	24/204 (12%)
1BP2	1B	WT	0/112 (0%)	11/35 (31%)	N/A	42/66 (64%)
2BP2	2B		1/69 (1%)	12/63 (19%)	0/1 (0%)	30/78 (38%)
3BP1	3B		10/70 (14%)	1/32 (3%)	0/10 (0%)	24/66 (36%)
	1B					0/64 (0%)
1B3B	2B	WT	8/76 (11%)	N/A	0/8 (0%)	1/64 (2%)
	3B					8/64 (13%)
1BP2	1B	1a2b	0/33 (0%)	8/33 (24%)	N/A	N/A
3BP2	3B	1a2b	0/30 (0%)	3/30 (10%)	N/A	N/A

**Table 2.** Variables derived from photosynthetic CO<sub>2</sub> response curves, based on leaf gas exchange analysis. Values are means ± SE of measurements made on three or four leaves from different plants (35- to 45-d-old non-flowering rosettes for WT and mutants and 80-d-old non-flowering rosettes for *1a2b3b*). Values followed by the same letters in the same column are not significantly different ( $P < 0.05$ ) as determined by ANOVA followed by Tukey's HSD tests. Abbreviations:  $\Gamma$ , sub-stomatal CO<sub>2</sub> compensation point;  $g_s$ , stomatal conductance at 400 ppm CO<sub>2</sub>;  $R_d$ , mitochondrial respiration in the light;  $V_{\text{cmax}}$ , maximum rate of Rubisco carboxylation.

	$V_{\text{cmax}}$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	$R_d$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	$g_s$ ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )	$\Gamma$ ( $\mu\text{mol CO}_2 \text{ mol}^{-1}$ )
WT	55.5 ± 3.5 <sup>a</sup>	1.01 ± 0.27 <sup>a</sup>	0.25 ± 0.03 <sup>a</sup>	52.4 ± 4.7 <sup>a</sup>
<i>1a</i> T-DNA	38.0 ± 3.2 <sup>bc</sup>	1.18 ± 0.11 <sup>a</sup>	0.20 ± 0.03 <sup>a</sup>	65.7 ± 10.1 <sup>ab</sup>
<i>1a</i> CC1	38.1 ± 2.0 <sup>bc</sup>	0.93 ± 0.08 <sup>a</sup>	0.25 ± 0.01 <sup>a</sup>	61.3 ± 1.0 <sup>ab</sup>
<i>1b</i> T-DNA	50.3 ± 5.2 <sup>ab</sup>	1.20 ± 0.11 <sup>a</sup>	0.24 ± 0.01 <sup>a</sup>	63.2 ± 3.2 <sup>ab</sup>
<i>1b</i> CC1-3	49.4 ± 1.4 <sup>ab</sup>	0.83 ± 0.08 <sup>a</sup>	0.28 ± 0.01 <sup>a</sup>	53.8 ± 1.3 <sup>a</sup>
<i>2b</i> T-DNA	48.2 ± 4.7 <sup>ab</sup>	1.17 ± 0.08 <sup>a</sup>	0.24 ± 0.07 <sup>a</sup>	60.1 ± 2.3 <sup>ab</sup>
<i>2b</i> CC1-3	48.9 ± 1.5 <sup>ab</sup>	0.81 ± 0.06 <sup>a</sup>	0.28 ± 0.02 <sup>a</sup>	52.7 ± 1.3 <sup>a</sup>
<i>3b</i> T-DNA	39.8 ± 2.0 <sup>b</sup>	0.73 ± 0.05 <sup>a</sup>	0.26 ± 0.01 <sup>a</sup>	52.5 ± 0.8 <sup>a</sup>
<i>3b</i> CC1-3	38.5 ± 1.5 <sup>bc</sup>	0.77 ± 0.03 <sup>a</sup>	0.27 ± 0.01 <sup>a</sup>	54.7 ± 2.4 <sup>a</sup>
<i>2b3b</i>	36.9 ± 3.2 <sup>bc</sup>	0.85 ± 0.07 <sup>a</sup>	0.25 ± 0.02 <sup>a</sup>	65.3 ± 2.9 <sup>ab</sup>
<i>1a2b</i>	36.3 ± 2.9 <sup>bc</sup>	0.78 ± 0.08 <sup>a</sup>	0.27 ± 0.01 <sup>a</sup>	64.7 ± 2.3 <sup>ab</sup>
<i>1a3b</i>	24.6 ± 1.0 <sup>c</sup>	0.71 ± 0.04 <sup>a</sup>	0.26 ± 0.01 <sup>a</sup>	70.0 ± 1.5 <sup>b</sup>
<i>1a2b3b</i>	5.4 ± 0.3 <sup>d</sup>	0.84 ± 0.13 <sup>a</sup>	0.23 ± 0.04 <sup>a</sup>	200.1 ± 3.8 <sup>c</sup>

Figure 1

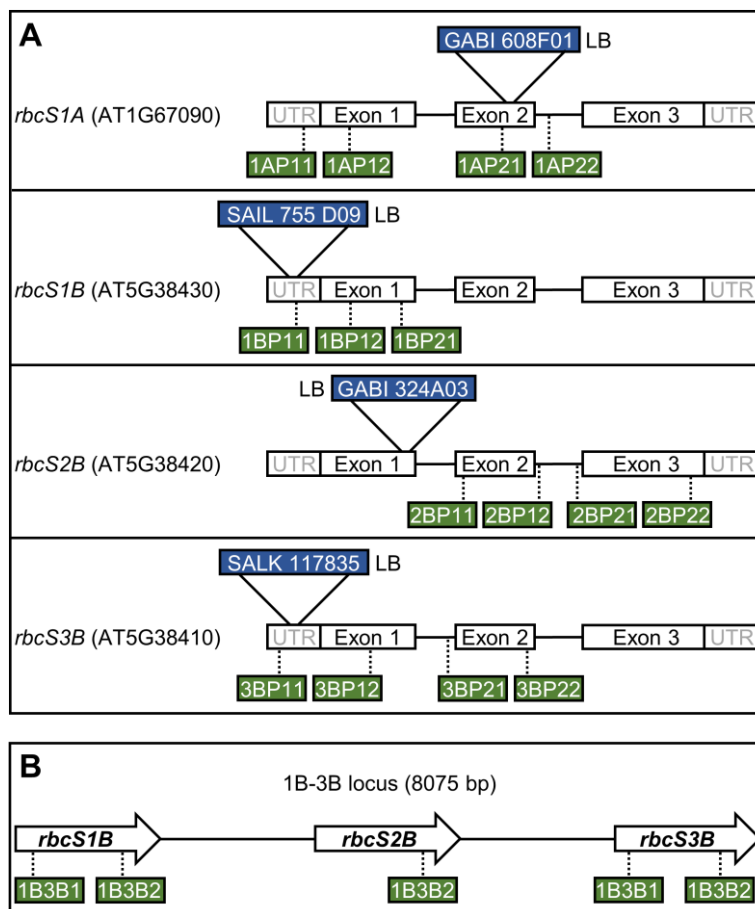
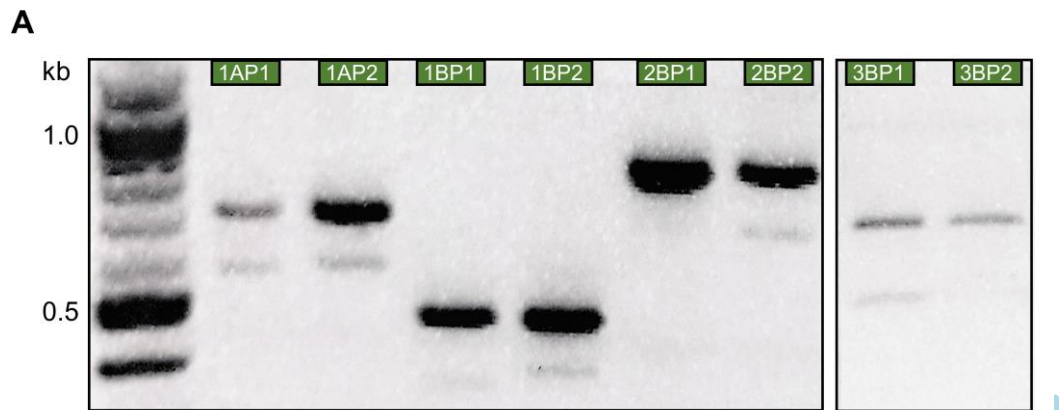


Figure 2



**B**

1AP1  
 TGTATCCTAGACCCTCCGATCACTCCA [130 bp] TATGCTCTCTTCCGCTACTATGGTTGCCT  
 TGTATCCTAGA-----CTATGGTTGCCT

1AP2  
 TCTTACCTTCCTGACCTTACCGATTCCG [126 bp] ATATAAACTAGCTAGATCTTAGGAAAATT  
 TCTTACCTTCC-----TCTTAGGAAAATT

1BP1  
 GCTCTCCTCTGCCGCTGTGGTTACCTCC [119 bp] TTACTTCCATCACAAGCAATGGGGGAAG  
 GCTCTCCTCTG-----AATGGGGGAAG

1BP2  
 TTTTGCCCTTACGGTTCTCACTATATA [96 bp] CCTCTGCCGCTGTGGTTACCTCCC GGCC  
 TTTTGCCCTCTTA-----CCGCTGTGGTTACCTCCC GGCC

2BP2  
 CAATATATATATCAATTGTATTGAATGG [168 bp] CCC TGGCGCCTTCATTAGGATCATCGGA  
 CAATATATATATCAATTGTATT-----CGCCTTCATTAGGATCATCGGA

3BP1  
 ATTATATAAAGATGACAACACCAGTAGG [180 bp] GGTCACCCGCAAGACCAACAAGGACATC  
 ATTATATAAAGATGACAACACC-----AACAAAGGACATC

ACU

Figure 3

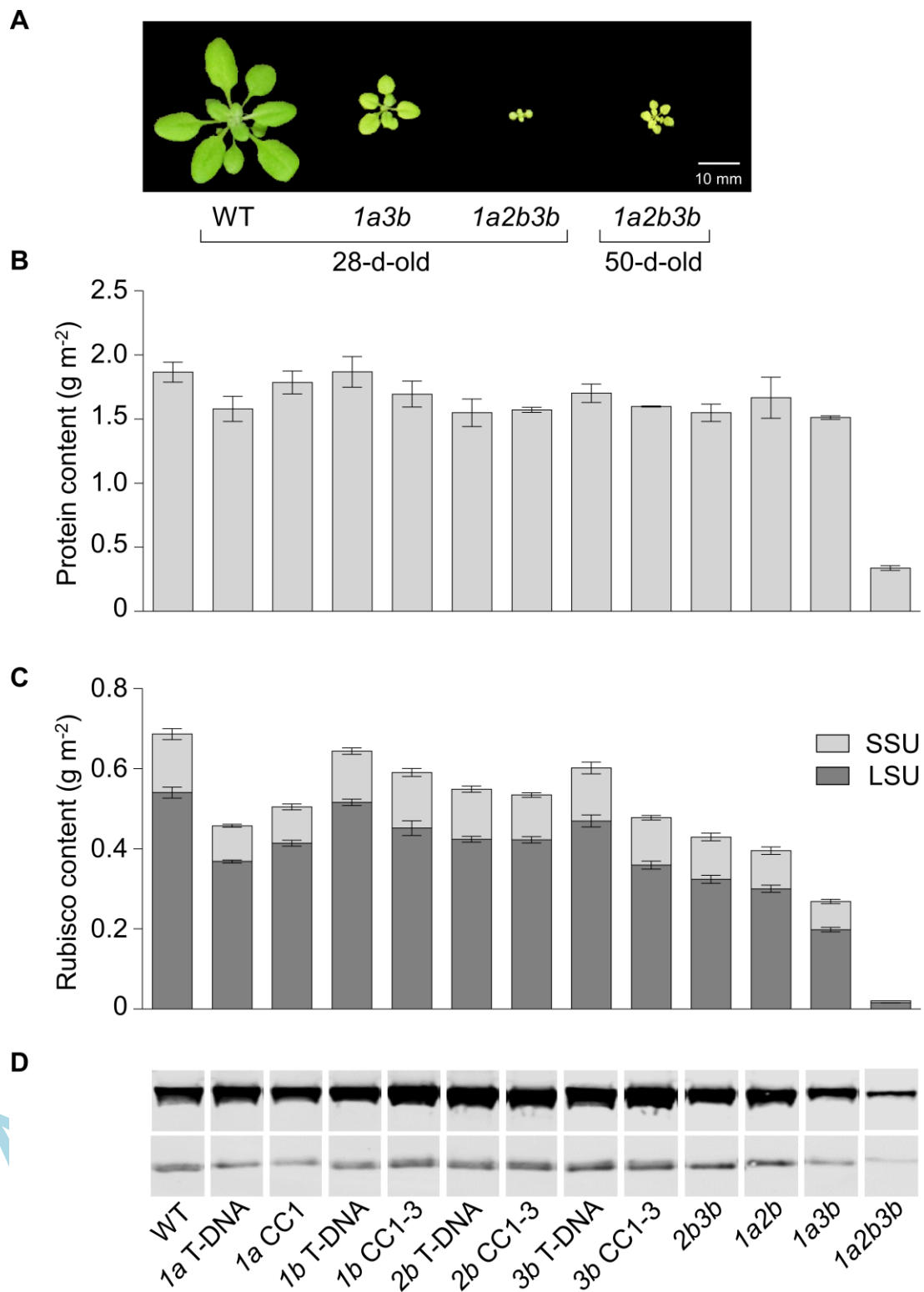




Figure 4

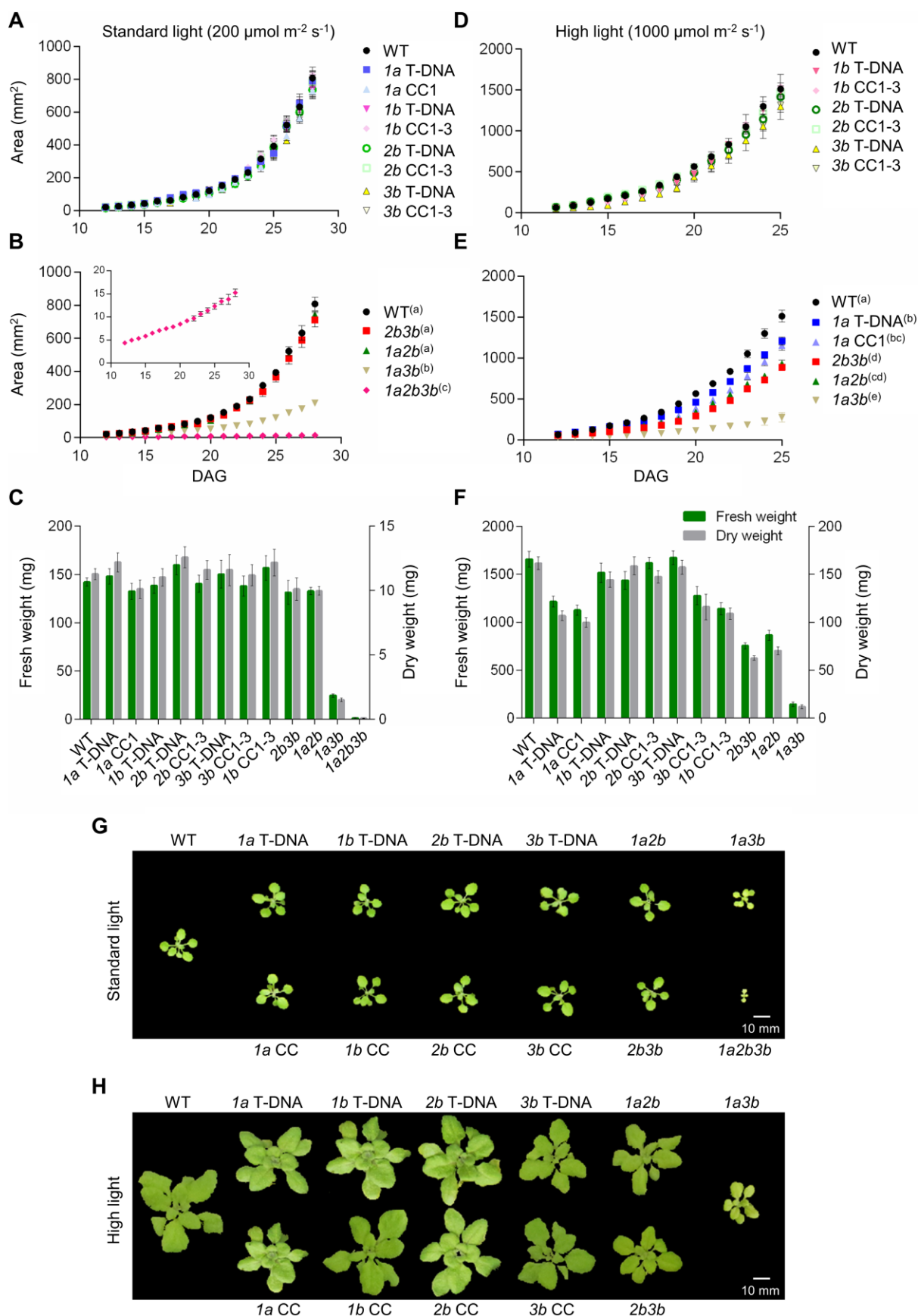


Figure 5

