1 **Stomatal density affects gas diffusion and CO₂ assimilation dynamics in**
 2 **Arabidopsis under fluctuating light**

3
 4 **Running title**

5 Stomatal density affects photosynthetic induction

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42

43 **Abbreviations**

44 *A*, CO₂ assimilation rate; *CAR*, cumulative CO₂ assimilation rate; *CER*, cumulative

45 transpiration rate; *C_i*, intercellular CO₂ concentration; *E*, transpiration rate; *gs*, stomatal

46 conductance; *L_g*, guard cell length; PPFD, photosynthetic photon flux density; *SD*,

47 stomatal density; *WUE*, water use efficiency; *WUE_i*, integrated water use efficiency

48 **Abstract**

49 Stomatal density (*SD*) is closely associated with photosynthetic and growth
50 characteristics in plants. In the field, light intensity can fluctuate drastically throughout
51 a day. The objective of the present study is to examine how the change in *SD* affects
52 stomatal conductance (*gs*) and CO₂ assimilation rate (*A*) dynamics, biomass production,
53 and water use under fluctuating light. Here, we compared the photosynthetic and
54 growth characteristics under constant and fluctuating light among four lines of
55 *Arabidopsis thaliana* (L.): a wild-type (WT), a *STOMAGEN/EPFL9*-overexpressing
56 line, *STOMAGEN/EPFL9*-silencing line, and an *EPIDERMAL PATTERNING FACTOR*
57 *1* knockout line (*epf1*). Lower *SD* resulted in faster response of *A* owing to the faster
58 response of *gs* to fluctuating light and higher water use efficiency without decreasing *A*.
59 Higher *SD* resulted in a faster response of *A* because of the higher initial *gs*. *epf1*, with
60 a moderate increase in *SD*, showed the larger carbon gain, attributable to the high
61 capacity and fast response of *A*, yielding higher biomass production than WT under
62 fluctuating light. The present study suggests that higher *SD* can be beneficial to
63 improve biomass production in the plant under field conditions.

64 65 **Key words**

- 66 leaf photosynthesis, fluctuating light, photosynthetic induction, stomata, stomatal
- 67 density, water use efficiency

68 ***Introduction***

69 Stomata, pores on the epidermis of plant leaves, function to maintain the balance
70 between CO₂ uptake for photosynthesis and water loss for transpiration (Mcadam and
71 Brodribb, 2012). The regulation of stomatal development has an important role in plant
72 adaption to long-term environmental changes, and stomatal differentiation and
73 patterning are tightly regulated by various signals influenced by plant hormones and
74 environmental stimuli (Qi and Torii, 2018). The peptide signals in a family of
75 EPIDERMAL PATTERNING FACTOR (EPF) were identified to function in the
76 stomatal development of Arabidopsis (*Arabidopsis thaliana* (L.) Heynh) (Hara et al.,
77 2007). It is demonstrated that EPF1 and EPF2 combine with the receptor-like protein,
78 TOO MANY MOUTHS (TMM) and ERECTA family leucine-rich repeat-receptor-like
79 kinases and, consequently, restrain a specific process in stomatal development.
80 Contrastingly, STOMAGEN/EPFL9 combines with TMM competitively to EPF1 and
81 EPF2, and promote stomatal development (Lee et al., 2015; Sugano et al., 2010).
82 Elucidation of the genetic factors involved in stomatal development has enabled us to
83 modify the stomatal size, number, and patterning in plant leaves by transgenic
84 approach.

85 Enhancing leaf photosynthesis has been attempted to drive further increase in

86 biomass production in crop plants (von Caemmerer and Evans, 2010; Sakoda *et al.*,
87 2016; Yamori *et al.*, 2016b). Gas diffusional resistance from the atmosphere to the
88 chloroplast is one of the limiting factors for leaf photosynthetic capacity (Farquhar and
89 Sharkey, 1982). Furthermore, it has been highlighted that the conductance to gas
90 diffusion via stomata (g_s) can be a major determinant of CO₂ assimilation rate (A)
91 (Wong *et al.*, 1979). The potential of g_s is mainly determined by the size, depth, and
92 opening of single stoma, and their density (Franks and Beerling, 2009). It has been
93 controversial how the change in the stomatal density (SD), defined as the stomata
94 number per unit leaf area, affects photosynthetic and growth characteristics in plants
95 (Lawson and Blatt, 2014). Doheny-Adams *et al.*, (2012) reported that lower SD yielded
96 higher growth rate and biomass production in Arabidopsis under constant light owing
97 to the favorable water condition and temperature for metabolism and low metabolic
98 cost for stomatal development (Doheny-Adams *et al.*, 2012). Contrastingly, lower SD
99 resulted in the depression of g_s and/or A in Arabidopsis and poplar plants (Bussis *et al.*,
100 2006; Yoo *et al.*, 2010; Wang *et al.*, 2016). An *SDD1* knockout line of Arabidopsis with
101 higher SD showed higher g_s and A than a wild-type line, depending on light condition
102 (Schlüter *et al.*, 2003). Previously, we reported that higher SD by overexpressing
103 *STOMAGEN/EPFL9* resulted in the enhancement of g_s and A in Arabidopsis under

104 constant and saturated light conditions (Tanaka et al., 2013). Therefore, *SD*
 105 manipulation could have the potential to enhance photosynthetic and growth
 106 characteristics in plants, even though that effect can depend on the species or
 107 environmental conditions.

108 In the field, light intensity can fluctuate at different scales, from a few seconds to
 109 minutes, over the course of a day owing to changes in the solar radiation, cloud cover,
 110 or self-shading in the plant canopy. The gradual increase in *A* can be shown after the
 111 transition from low to high light intensity, and this phenomenon is called
 112 "photosynthetic induction." A simulation analysis demonstrated that the potential loss
 113 of the cumulative amount of CO₂ assimilation caused by photosynthetic induction can
 114 reach at least 21% in wheat (*Triticum aestivum* L.) and soybean (*Glycine max* (L.)
 115 Merr.) (Tanaka et al., 2019; Taylor and Long, 2017). In rice (*Oryza sativa* L.) and
 116 soybean, there is genotypic variation in the speed of photosynthetic induction, which
 117 causes significant differences in the cumulative carbon gain under fluctuating light
 118 (Adachi et al., 2019; Soleh et al., 2017, 2016). Consequently, speeding up
 119 photosynthetic induction can yield more efficient carbon gain, which will open a new
 120 pathway to improve biomass production in plants under field conditions.

121 Photosynthetic induction is typically limited by three phases of the biochemical and

diffusional processes: (1) activation of electron transport, (2) activation of the enzymes in the Calvin-Benson cycle, and (3) stomatal opening (Percy, 1990; Yamori, 2016a). Especially, the activation of Rubisco (5–10 min for full induction) and stomatal opening (20–30 min for full induction) constitute a major limitation to photosynthetic induction (Carmo-Silva and Salvucci, 2013; Yamori et al., 2012). The overexpression of *PATROL1*, controlling the translocation of a major H⁺-ATPase (AHA1) to the plasma membrane, resulted in faster *gs* response to fluctuating light in Arabidopsis without the change in *SD* (Hashimoto-Sugimoto et al., 2013). Arabidopsis knockout mutants of ABA transporter, which plays pivotal roles in stomatal closure, improved stomatal response to fluctuating light and photosynthesis (Shimadzu et al., 2019). Furthermore, the rapid movement of stomata is important for plants to achieve high water use efficiency (*WUE*) (Qu et al., 2016). Notably, the overexpression of *BLINK1*, light-gated K⁺ channel in guard cells, accelerates the stomatal opening and closing, which improved biomass production without additional water loss in Arabidopsis under the fluctuating light (Papanatsiou et al., 2019). These facts evidence that rapid stomatal movement can be beneficial for the effective carbon gain and water use under fluctuating light conditions. However, how *SD* changes affect *gs* and *A* dynamics, biomass production, and water use under these conditions has been understudied

140 (Drake et al., 2013; Papanatsiou et al., 2016; Schuler et al., 2018).

141 The objective of this study was to examine how the change in *SD* affects the
142 photosynthetic and growth characteristics in plants under fluctuating light conditions.

143 Therefore, we investigated the dynamics of *g_s*, *A*, and transpiration rate (*E*) by gas
144 exchange measurements and biomass production under fluctuating light conditions in

145 four *Arabidopsis* lines differing in *SD*. The results shine a light on the manipulation of

146 *SD* to improve the biomass production and water use in plants under field.

147 **Results**

148 **Stomatal density, size, and clustering**

149 Compared with WT, ST-OX showed 268.1% higher SD ($p < 0.05$) while ST-RNAi
 150 showed 70.9% lower SD ($p < 0.05$) as reported in Sugano *et al.* (2010) and Tanaka *et*
 151 *al.* (2013) (Fig. 1A). L_g of ST-OX was 10.0% lower than that of WT ($p < 0.01$) (Fig.
 152 1B). SD in *epfl* was 46.5% higher than that of WT ($p < 0.1$) and L_g of *epfl* was slightly
 153 higher than that of WT as reported in Dow. *et al.* (2014) ($p < 0.1$). Stomatal clustering
 154 was not observed in WT and ST-RNAi, while two to five stomata were clustered in
 155 ST-OX and *epfl* (Fig. 1C). The ratio of stomata in each clustering category was higher
 156 in ST-OX than that in *epfl*.

157

158 **Photosynthetic characteristic under fluctuating light conditions**

159 ST-OX and *epfl* maintained higher g_s , A , and E than WT under fluctuating light
 160 condition (Fig. 2B, D, and E). ST-OX showed higher C_i and lower WUE than WT
 161 throughout the measurement period, while *epfl* only showed higher C_i and lower WUE
 162 than WT during the initial 50–60 min after the dark condition (Fig. 2C and F).
 163 ST-RNAi showed higher g_s , C_i , A , and E than WT in the initial induction phase and,
 164 subsequently, similar or lower values of these parameters than those of WT. WUE of

165 ST-RNAi was constantly higher than that of WT except for the initial induction phase.

166 After the light intensity change from dark to high ($500 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$), *gs*

167 response was faster in ST-RNAi and slower in ST-OX than that in WT (Fig. 3A). In

168 ST-OX, *gs* did not reach the steady state even after 120 min with illumination (Fig. 2B).

169 $t_{\text{dark_gs}0.6}$ in ST-RNAi was smaller ($p < 0.05$) and $t_{\text{dark_gs}0.9}$ in ST-OX was larger than

170 those in WT ($p < 0.05$) (Fig. 3B and C). *gs* response in *epf1* was similar to that in WT.

171 The response of *A* in all transgenic lines was faster than that in WT after light intensity

172 change from dark to high intensity (Fig. 3D). $t_{\text{dark_A}0.6}$ in ST-OX ($p < 0.05$), ST-RNAi

173 ($p < 0.1$), and *epf1* ($p < 0.05$) were shorter than that in WT (Fig. 3E and F). There was

174 no significant variation in $t_{\text{dark_A}0.9}$ between WT and each line, although $t_{\text{dark_A}0.9}$ in *epf1*

175 was 37.0% shorter than that of WT.

176 After light intensity changed from low ($50 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$) to high ($500 \mu\text{mol}$

177 $\text{photon m}^{-2} \text{ s}^{-1}$), a large variation in *gs* and *A* responses was not shown between WT and

178 each transgenic line (Fig. 4A and C). There was no significant variation in $t_{\text{low_gs}0.6}$

179 between WT and each transgenic line (Fig. 4B). Although *gs* response in ST-OX

180 seemed faster than that in WT (Fig. 4A), $gs_{\text{low_induction}}$ was overestimated in ST-OX

181 because *gs* did not reach the steady state in 120 min with illumination after the dark

182 period (Fig. 2B and 4A). $t_{\text{low_gs}0.9}$ was not calculated since *gs* did not reach 0.9 to gs_{max}

183 in any lines except for ST-OX. $t_{\text{low_A0.6}}$ was significantly shorter in three transgenic
184 lines than that in WT ($p < 0.05$), while the variation was quite small (Fig. 4D). $t_{\text{low_A0.9}}$
185 in ST-OX was 52.8% shorter than that in WT ($p < 0.1$) (Fig. 4E).

186 In the steady state under dark conditions, gs_{dark} in ST-OX and *epfl* were 264.5% ($p <$
187 0.01) and 160.6% ($p < 0.1$) higher, respectively, than that in WT (Fig. 5A). $t_{\text{dark_A0.6}}$
188 decreased with the increase in gs_{dark} when $gs_{\text{dark}} < 0.055$, and it was constantly
189 independent of gs_{dark} for $gs_{\text{dark}} > 0.055$ (Fig. 5B). $t_{\text{dark_A0.9}}$ showed no relation to gs_{dark}
190 (Fig. 5C). gs_{low} in ST-OX was 95.6% higher than that in WT ($p < 0.01$) (Fig. 5D).
191 $t_{\text{low_A0.6}}$ and $t_{\text{low_A0.9}}$ were independent of gs_{low} (Fig. 5E and F). Under the steady state at
192 $500 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, there was no significant difference in gs , A , and E between
193 WT and ST-OX or *epfl*, although the transgenic lines showed values of these
194 parameters higher than those in WT (Fig. S1). ST-RNAi showed lower C_i ($p < 0.05$),
195 higher WUE ($p < 0.05$), and similar A than WT. gs and E in ST-RNAi tended to be
196 lower than those in WT although there was no significant variation.

197 CAR in *epfl* was 20.6% higher than that in WT ($p < 0.1$) (Fig. 6A). The loss rate in
198 *epfl* was 33.4% lower than that in WT, although the variation was not significant (Fig.
199 6B). CER in ST-OX and *epfl* were 30.6% and 27.5% higher, respectively, than that of
200 WT with no significant variation (Fig. 6C). ST-RNAi showed similar CAR to that of

201 WT, while it showed 21.0% lower CER , which contributed to WUE_i being 32.1%
202 higher than that of WT ($p < 0.1$) (Fig. 6D).

203

204 **Biomass production under constant and fluctuating light conditions**

205 Compared with WT, dry weight of the above ground biomass under constant light
206 (DW_{constant}) of *epfl* was similar, while that under fluctuating light ($DW_{\text{fluctuating}}$) of *epfl*
207 was 25.6% higher than that of WT ($p < 0.05$) (Fig. 7C and D). DW_{constant} and
208 $DW_{\text{fluctuating}}$ of ST-OX were slightly lower or higher than those of WT, respectively,
209 with no significance variation. DW_{constant} and $DW_{\text{fluctuating}}$ of ST-RNAi were 44.9% and
210 36.5% lower than those of WT, respectively ($p < 0.01$).

211 **Discussion**

212 Stomata play a significant role in the regulation of gas exchange between the outside
 213 and inside of the leaf. However, how the *SD* change affects photosynthetic and growth
 214 characteristics in plants has been controversial, and the effect of *SD* change on
 215 photosynthesis and growth can vary depending on the plant species or environmental
 216 conditions. Previously, we reported that higher *SD* resulted in the enhancement of *g_s*
 217 and *A* in Arabidopsis under constant and saturated light conditions (Tanaka et al.,
 218 2013). Lawson and Blatt (2014) suggested that with higher *SD*, it would be instructive
 219 to determine biomass productivity under fluctuating light, although only a few studies
 220 investigated the relationship between *SD* and photosynthetic or growth characteristics
 221 under that condition (Drake et al., 2013; Papanatsiou et al., 2016; Schuler et al., 2018).
 222 Here, we attempted to examine how the change in *SD* affects *g_s* and *A* dynamics,
 223 biomass production, and water use in Arabidopsis under fluctuating light.

224 In this study, the four Arabidopsis lines with contrasting *SD*s showed significant
 225 differences in the light response of *g_s* in the steady and non-steady state. *SD*
 226 differences had no significant effect on the response of *g_s* to the light intensity change
 227 from low to high in the present (Fig. 4A and B) and previous studies (Papanatsiou et al.,
 228 2016; Schuler et al., 2018). Contrastingly, ST-OX and ST-RNAi showed *g_s* responses

229 slower or faster, respectively, than that of WT when pre-adapted to the dark condition
 230 (Fig. 3A–C). This suggests that the relationship between *SD* and *gs* dynamics can
 231 depend on the light condition history of the leaf. The different responses of *gs* could be
 232 attributable to the difference in the size, density, and patterning of stomata. Drake *et al.*
 233 (2013) reported that smaller stomata respond the fluctuating light faster than larger
 234 stomata among several species of the genus *Banksia*. On the contrary, smaller stomata
 235 resulted in the slower response of *gs* to fluctuating light in the genus *Oryza* (Zhang *et*
 236 *al.*, 2019). In this study, the variation in stomatal size evaluated as L_g did not
 237 correspond to that of *gs* response (Fig. 1 and 3), indicating that the stomatal size would
 238 have a minor effect on the dynamics of *gs* in *Arabidopsis* under fluctuating light.

239 The stomatal opening is regulated by at least three key components, blue-light
 240 receptor phototropin, plasma membrane H^+ -ATPase, and plasma membrane inward
 241 rectifying K^+ channels in the guard cell (Inoue and Kinoshita, 2017). The activation of
 242 H^+ -ATPase induced by blue light as the initial signal facilitates K^+ uptake through the
 243 inward rectifying K^+ channel to increase the turgor pressure of guard cells, resulting in
 244 the stomatal opening. In addition, stomatal opening dynamics depend on the water
 245 status in the plant (Lawson and Blatt, 2014). With more stomata, higher metabolic cost
 246 and water uptake would be required for the movement. The gas-exchange and

247 theoretical-modeling analysis indicated that the stomatal clustering decreased the
248 maximum value of g_s and A under the steady state because of the misplacement of
249 stomatal pores over mesophyll cells (Dow. *et al.*, 2014; Lehmann and Or, 2015). It was
250 also shown that clustering suppressed stomatal movement owing to the decreased
251 capacity of the K^+ flux and K^+ accumulation in the guard cells (Papanatsiou et al.,
252 2016). Additionally, g_s response to fluctuating light in *Begonia* species with clustered
253 stomata was slower than that in those without clustered stomata (Papanatsiou et al.,
254 2017). In this study, ST-OX, with the highest SD and clustering rate, showed the
255 slowest g_s response among Arabidopsis lines, while ST-RNAi, with the lowest SD and
256 no clustered stomata, showed the fastest response (Fig. 1 and 3). *epf1*, with a moderate
257 increase in SD and clustering rate, showed g_s response similar to that of WT. These
258 results suggest that the drastic change in stomatal density and patterning rather than
259 size can largely affect g_s response to fluctuating light owing to the change in metabolic
260 cost and water uptake for stomatal opening and the opening speed of single stoma.

261 Stomatal patchiness, the spatially or temporally-heterogeneous distribution of
262 stomatal apertures on the leaf, can occur in response to light fluctuation and affects the
263 kinetics of the stomatal response (Mott and Buckley, 2000). Patchiness has been
264 observed in many studies under water-limited conditions such as low humidity or soil

265 water potential (Terashima, 1992). ST-OX showed higher E under the steady and
 266 non-steady state than WT (Fig. 2), leading to increased water loss during growth. This
 267 might induce stomatal patchiness more severely in ST-OX, which would require longer
 268 time to reach the full induction of g_s than WT. This consideration can be partly
 269 supported by the fact that ST-RNAi, with the lowest E and highest WUE , showed the
 270 fastest g_s response to fluctuating light of all lines (Fig. 1–3).

271 In ST-OX, ST-RNAi, and *epfl*, A response to light intensity change from dark to
 272 high intensity was faster than that of WT (Fig. 3D–F). Photosynthetic induction is
 273 typically limited by three phases of the biochemical or diffusional processes; (1)
 274 activation of electron transport, (2) activation of the enzymes of the Calvin-Benson
 275 cycle, and (3) stomatal opening (Percy, 1990; Yamori, 2016). The significance of
 276 stomatal limitation to photosynthetic induction depends on the initial value of g_s
 277 (Kirschbaum and Percy, 1988). Activation of the electron transport and enzymes of
 278 the Calvin-Benson cycle can be largely affected by CO_2 concentration when the small
 279 portion of Rubisco is activated (Jackson et al., 1991; Kaiser et al., 2017; Urban et al.,
 280 2008). The variation of g_s under dark or low light conditions corresponded to that in
 281 the speed of photosynthetic induction in several plant species (Kaiser et al., 2016;
 282 Soleh et al., 2017). Kaiser *et al.*, (2016) reported that $t_{\text{dark}_A0.9}$ correlated with initial g_s

when g_s was less than $0.13 \text{ mol m}^{-2} \text{ s}^{-1}$. In this study, $t_{\text{dark_A0.6}}$ correlated with $g_{s\text{dark}}$ if $g_{s\text{dark}} < 0.055 \text{ mol m}^{-2} \text{ s}^{-1}$, and it was constant regardless of $g_{s\text{dark}}$ for $g_{s\text{dark}} > 0.055 \text{ mol m}^{-2} \text{ s}^{-1}$ (Fig. 5B). $g_{s\text{dark}}$ of WT, ST-OX, and *epfl* were 0.032, 0.118, and $0.085 \text{ mol m}^{-2} \text{ s}^{-1}$, respectively, suggesting that the variation in $g_{s\text{dark}}$ would cause the response difference of A (Fig. 5A and B). Contrastingly, ST-RNAi showed a slight difference in $g_{s\text{dark}}$ from WT, but the faster response of g_s , which contributed to higher g_s and C_i than those of WT in the initial induction phase (Fig. 2, 3, and 5). Therefore, higher SD resulted in higher initial value of g_s while lower SD resulted in faster g_s response, which would contribute to faster photosynthetic induction owing to the rapid activation of RuBP regeneration and carboxylation in the Calvin-Benson cycle.

After the change in light intensity from low to high, the response of A had no relation to initial g_s because $g_{s\text{low}}$ was over $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$ in all lines (Fig. 5D–F). Thus, g_s would not be a major limitation factor to photosynthetic induction. $t_{\text{low_A0.6}}$ in the three transgenic lines was shorter than that in WT ($p < 0.05$), and $t_{\text{low_A0.9}}$ in ST-OX was 52.8% shorter than that of WT ($p < 0.1$), suggesting that the activation of RuBP regeneration and carboxylation in the Calvin-Benson cycle in the transgenic lines might be faster than that in WT. Metabolic limitation to photosynthetic induction will be examined among Arabidopsis lines with contrasting SD s in a future study.

301 $DW_{\text{fluctuating}}$ was much lower than DW_{constant} in all Arabidopsis lines, although the
302 total amount of light intensity exposed to the plants was equal between both light
303 conditions. This difference would be caused by the loss of carbon gain owing to
304 photosynthetic induction under fluctuating light condition (Fig. 7). While there was no
305 variation in A between WT and ST-RNAi in the steady state (Fig. S1), DW_{constant} in
306 ST-RNAi was significantly lower than that in WT as reported in Tanaka *et al.* (2013)
307 (Fig. 7C). In Arabidopsis, a near linear relationship was reported between leaf area and
308 plant biomass during the vegetative stage (Weraduwaage et al., 2015). Furthermore,
309 Tanaka *et al.* (2013) showed a significant decrease in leaf area of ST-RNAi, suggesting
310 that the silencing of *STOMAGEN/EPLF9* would decrease biomass production owing to
311 the significant decrease in leaf area. Contrastingly, transgenic lines with lower *SD*
312 exhibited improved performance in growth under drought stress owing to high *WUE* in
313 several plant species (Caine et al., 2018; Wang et al., 2016; Yoo et al., 2010).
314 Additionally, the rapid movement of stomata can be important for plants to achieve
315 high *WUE* (Qu et al., 2016). In this study, compared with WT, ST-RNAi, with lower
316 *SD*, showed faster g_s response, similar amount of carbon gain, lower water loss, and
317 lower biomass production loss caused by photosynthetic induction under fluctuating
318 light condition (Fig. 3, 6, and 7). It is indicated that lower *SD* would be advantageous

319 for plant growth under water-limited and fluctuating light condition.

320 DW_{constant} in ST-OX was slightly lower than that in WT, although A_{max} was

321 significantly or slightly higher in Tanaka *et al.* (2013) and this study, respectively (Figs.

322 7 and S1). The increase in water loss and metabolic cost of stomatal movements would

323 have a negative effect on biomass production in ST-OX under constant light (Tanaka et

324 al., 2013). Although these penalties resulting from the drastic increase in SD might

325 have negative effects on carbon gain, $DW_{\text{fluctuating}}$ in ST-OX was 10.5% higher than that

326 in WT with no significant variation. Moreover, biomass production in *epf1*, with

327 moderate increase in SD , was significantly higher than that in WT under fluctuating

328 light, but there was no difference between these two lines under constant light (Fig. 7).

329 It is possible that a moderate increase in SD could achieve more efficient carbon gain

330 attributable to the high capacity and fast response of A in Arabidopsis under fluctuating

331 light (Fig. 1–6), while it would cause small penalties on water loss and metabolic cost

332 for stomatal movement. Overall, higher SD can be beneficial to improve biomass

333 production in plants under fluctuating light conditions.

334

335 **Conclusion**

336 Under fluctuating light, there was a significant variation in the photosynthetic and

337 growth characteristics among Arabidopsis lines contrasting in the stomatal density, size,
 338 and patterning. Lower *SD* resulted in faster *A* response owing to the faster *gs* response
 339 and higher *WUE* without the decrease in *A*. This suggests that lower *SD* would be
 340 advantageous for plant growth under water-limited and fluctuating-light conditions.
 341 Higher *SD* also resulted in faster *A* response to fluctuating light owing to the higher
 342 initial value of *gs*. *epf1*, with a moderate increase in *SD*, achieved more efficient
 343 carbon gain under fluctuating light attributable to the high capacity and fast response
 344 of *A*, which contributed to higher biomass production than that in WT. This study
 345 suggests that higher *SD* can be beneficial to improve biomass production in plants
 346 under field conditions.

347 ***Materials and Methods***

348 **Plant materials and cultivation for the gas exchange and biomass analyses**

349 Columbia-0 (CS60000) of *Arabidopsis thaliana* (L.) Heynh, was used as a wild-type
350 line (WT). In addition, we used three transgenic lines, *STOMAGEN/EPFL9*
351 overexpressing (ST-OX) and artificial microRNA (amiRNA)-mediated silencing
352 (ST-RNAi) lines, and an *EPF1* knockout line (*epf1*), which were developed by Sugano
353 *et al.* (2010). The six plants per line were sown and grown on the soil at an air
354 temperature of 22°C and a photosynthetic photon flux density (PPFD) of 100 μmol
355 $\text{photon m}^{-2} \text{s}^{-1}$ for the gas exchange analysis. The day/night length was set to 8/16 h.

356 For the biomass analysis, plants were sown and grown on the soil at an air
357 temperature of 22°C and a PPFD of 120 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 24 days after sowing
358 with the day/night length of 8/16 h. Subsequently, four plants per line were subjected
359 to constant and fluctuating light conditions, for 20 days with a day/night cycle of 12/12
360 h. During daytime, the light intensity in the constant light condition was changed from
361 a PPFD of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 4 h to 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 4h, followed by 60 $\mu\text{mol m}^{-2}$
362 s^{-1} for 4h, while a PPFD of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 10 min after 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 5 min
363 was repeated for 12 h in the fluctuating light condition (Fig. 7A and B). Plants were
364 exposed to the same total amount of light intensity per day under both light conditions.

365 Dry weight of above ground biomass grown under each light condition was evaluated
366 at 44 days after sowing.

367

368 **Evaluation of stomatal density, size, and clustering**

369 *SD*, guard cell length (L_g), and stomatal clustering were evaluated in the leaves of the
370 six plants per line at the same growth stage as the gas exchange measurements were
371 conducted. A section of the leaf (5×5 mm) was excised and immediately fixed in the
372 solution (Ethanol : acetic acid = 9:1, v/v) overnight. The fixed tissues were cleared in
373 chloral hydrate solution (chloral hydrate : glycerol : water = 8:1:2, w/v/v) overnight.
374 The cleared tissues were stained with safranin-O solution ($200 \mu\text{g ml}^{-1}$) for 30 min to 1
375 h. The abaxial side of the leaves was observed at a $200\times$ magnification using an optical
376 microscope and digital images were obtained (CX31 and DP21, Olympus, Tokyo,
377 Japan). We used imaging analysis software, ImageJ (NIH, Bethesda, MD, USA) to
378 assess the stomatal number and guard cell length from the images. The percentage of
379 clustered stomata to total number was measured for each clustering category.

380

381 **Gas exchange measurements**

382 Gas exchange measurements were conducted using a portable gas-exchange system

LI-6400 (LI-COR, Lincoln, NE, USA). All plants were kept in the dark overnight before and during the measurements. In the leaf chamber, we set flow rate at 300 $\mu\text{mol s}^{-1}$, CO_2 concentration at 400 $\mu\text{mol mol}^{-1}$, and air temperature at 25°C. After the leaf was clamped in the chamber, light intensity was kept at dark condition for the initial 10 min and, subsequently, under a series of 500, 50, 500, 50, 500, 50, and 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 120, 1, 20, 5, 20, 30, and 30 min as described in Fig. 2A. A , g_s , intercellular CO_2 concentration (C_i), and E were recorded every 10 s during the measurements. WUE was calculated as the ratio of A to E . Gas exchange measurements were conducted with three plants per line during 68 to 73 days after sowing.

Data processing and statistical analysis

The response of A and g_s was evaluated at 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 120 min after the dark period and at 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 30 min after the illumination with 50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 30 min, based on $A_{\text{induction}}$ and $g_{s\text{induction}}$ defined as the following equations:

$$\text{(eqn. 1) } g_{s\text{dark_induction}} = \frac{g_{s_t} - g_{s\text{dark}}}{g_{s_{\text{max}}} - g_{s\text{dark}}}$$

400

$$401 \quad (\text{eqn. 2}) \quad A_{\text{dark_induction}} = \frac{A_t - A_{\text{dark}}}{A_{\text{max}} - A_{\text{dark}}}$$

402

$$403 \quad (\text{eqn. 3}) \quad g_{\text{low_induction}}^s = \frac{g_t^s - g_{\text{low}}^s}{g_{\text{max}}^s - g_{\text{low}}^s}$$

404

$$405 \quad (\text{eqn. 4}) \quad A_{\text{low_induction}} = \frac{A_t - A_{\text{low}}}{A_{\text{max}} - A_{\text{low}}},$$

406

407 where A_{dark} and g_{dark} represent steady-state values under the dark condition, A_{low} and

408 g_{low} are steady-state values under a PPFD of 50 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, A_{max} and g_{max}

409 are maximum values under a PPFD of 500 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, and A_t and g_t

410 represent A and g_s at a given time under illumination. We evaluated the differences in

411 the time when $A_{\text{induction}}$ and $g_{\text{induction}}$ reached 0.6 and 0.9 after the light intensity change

412 from dark to high ($t_{\text{dark_gs0.6}}$, $t_{\text{dark_gs0.9}}$, $t_{\text{dark_A0.6}}$, and $t_{\text{dark_A0.9}}$) or from low to high

413 ($t_{\text{low_gs0.6}}$, $t_{\text{low_gs0.9}}$, $t_{\text{low_A0.6}}$, and $t_{\text{low_A0.9}}$) between WT and each transgenic line. To

414 compare the photosynthetic characteristics under the steady state, we evaluated A , g_s ,

415 C_i , and E in the four lines when A reached a maximum value throughout the

416 measurement.

417 The cumulative CO₂ assimilation rate (*CAR*) and transpiration rate (*CER*) under
 418 fluctuating light were calculated by summing *A* and *E* after the initial dark period. An
 419 integrated *WUE* (*WUE_i*) was calculated as the ratio of *CAR* to *CER*. Assuming the
 420 absence of induction response of *A* to fluctuating light, a theoretical maximum *CAR*
 421 (*CAR_t*) was defined by the following equation:

422

423 (eqn. 5)
$$CAR_t = A_{\max} \cdot T_{500} + A_{\text{low}} \cdot T_{50},$$

424

425 where *T*₅₀₀ and *T*₅₀ are the seconds for which the light intensity was maintained at 500
 426 and 50 μmol photon m⁻² s⁻¹, respectively. The potential loss rate of carbon gain caused
 427 by photosynthetic induction was defined by the following equation:

428

429 (eqn. 6)
$$\text{Loss rate} = \left(1 - \frac{CAR}{CAR_t}\right) \cdot 100$$

430

431 The variation in stomatal size and all the parameters of photosynthetic and growth
 432 characteristics were compared between WT and each transgenic line by a Dunnett's
 433 test. Steel test was applied to evaluate *SD* variation between WT and each transgenic

434 line because the distribution of values was extremely different among the lines.

435 Statistical analysis was conducted using R software version 3. 6. 1 (R Foundation for

436 Statistical Computing, Vienna, Austria).

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579

580 ***Legends to figures***

581 Fig. 1. Stomatal density, size, and clustering

582 (A) The stomatal density (SD), (B) guard cell length (L_g) and (C) the rate of stomata in
583 2–5 clustering category were evaluated on the fully expanded leaf in a wild-type line
584 (WT), a *STOMAGEN/EPFL9* overexpressing (ST-OX) or amiRNA-mediated silencing
585 line (ST-RNAi), and an *EPFL1* knockout line (*epfl1*) of *Arabidopsis thaliana*. The
586 vertical bars indicate the standard error ($n = 6$). †, * and ** indicate the significant
587 variation in each parameter between WT and each transgenic line at $p < 0.1$, 0.05, and
588 0.01, respectively, according to the Steel test in (A) or Dunnett's test in (B) and (C).
589 The values in each column represent the relative value of each line to WT.

590

591 Fig. 2. Photosynthetic characteristics under the non-steady state

592 (A) Light intensity set-up as for (B) stomatal conductance (g_s), (C) intercellular CO_2
593 concentration (C_i), (D) CO_2 assimilation rate (A), (E) transpiration rate (E), and (F)
594 water use efficiency (WUE) measurements on the fully expanded leaf in the four lines
595 of *Arabidopsis*. The gas exchange measurements were conducted at a CO_2
596 concentration of 400 ppm and air temperature of 25°C. Vertical bars indicate the
597 standard error ($n = 3$).

598

599 Fig. 3. Time lapsed until reaching 0.6 and 0.9, out of a maximum value of 1, for
600 stomatal conductance and CO₂ assimilation rate increase after changing light intensity
601 from dark to high

602 The induction state of (A) stomatal conductance (g_s) and (D) CO₂ assimilation rate (A)
603 was evaluated in the four lines of Arabidopsis based on $g_{s_{\text{dark_induction}}}$ and $A_{\text{dark_induction}}$
604 defined as eqn. 1 and 2, respectively, under a PPFD of 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 120
605 min after the dark period. The time when (B, C) $g_{s_{\text{dark_induction}}}$ and (E, F) $A_{\text{dark_induction}}$
606 reached 0.6 ($t_{\text{dark_}g_s0.6}$, $t_{\text{dark_}A0.6}$) and 0.9 ($t_{\text{dark_}g_s0.9}$, $t_{\text{dark_}A0.9}$), respectively, was compared
607 between WT and each transgenic line. Vertical bars indicate the standard error ($n = 3$).
608 † and * indicate significant differences in each parameter between WT and each
609 transgenic line at $p < 0.1$ and 0.05, respectively, according to Dunnett's test. The values
610 in each column represent the relative value of each line to WT.

611

612 Fig. 4. Time lapsed until reaching 0.6 and 0.9, out of a maximum value of 1, for
613 stomatal conductance and CO₂ assimilation rate increase after changing light intensity
614 from low to high

615 The induction state of (A) stomatal conductance (g_s) and (C) CO_2 assimilation rate (A)
616 was evaluated in the four lines of Arabidopsis based on $g_{s_{\text{low_induction}}}$ and $A_{\text{low_induction}}$
617 defined as eqn. 3 and 4, respectively, under a PPFD of $500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 30
618 min after a PPFD of $50 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 30 min. The time when (B) $g_{s_{\text{low_induction}}}$
619 and (D, E) $A_{\text{low_induction}}$ reached 0.6 ($t_{\text{low_}g_{s0.6}}$, $t_{\text{low_}A0.6}$) and 0.9 ($t_{\text{low_}A0.9}$) was compared
620 between WT and each transgenic line. Vertical bars indicate the standard error ($n = 3$).
621 †, *, and ** indicate significant differences in each parameter between WT and each
622 transgenic line at $p < 0.1$, 0.05, and 0.01, respectively, according to Dunnett's test. The
623 values in each column represent the relative value of each line to WT.

624

625 Fig. 5. Relationship between the initial stomatal conductance and time lapsed until
626 reaching 0.6 and 0.9, out of a maximum value of 1, for CO_2 assimilation rate increase
627 after changing light intensity

628 The steady-state value of stomatal conductance under (A) the dark condition ($g_{s_{\text{dark}}}$)
629 and a PPFD of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ($g_{s_{\text{low}}}$) were compared between WT and each transgenic
630 line. The relationship was investigated between $g_{s_{\text{dark}}}$ or $g_{s_{\text{low}}}$ and the time to reach (B,
631 E) 0.6 ($t_{\text{dark_}A0.6}$, $t_{\text{low_}A0.6}$) or (C, F) 0.9 ($t_{\text{dark_}A0.9}$, $t_{\text{low_}A0.9}$), out of a maximum value of 1,
632 for CO_2 assimilation rate increase after changing the light intensity from the dark or

low to high. Vertical bars indicate the standard error ($n = 3$). † and ** indicate significant differences in each parameter between WT and each transgenic line at $p < 0.1$ and 0.01, respectively, according to Dunnett's test. The values in each column represent the relative value of each line to WT.

637

Fig. 6. Cumulative carbon gain and water use under fluctuating light

(A) Cumulative rate of CO_2 assimilation (CAR) and (B) transpiration (CER) was measured in the four lines of Arabidopsis under the fluctuating light described in Fig. 2A. (C) The loss rate of CO_2 assimilation caused by the induction response was calculated based on eqn. 3. (D) Integrated water use efficiency (WUE_i) was calculated as the ratio of CAR to CER . Vertical bars indicate the standard error ($n = 3$). † indicates significant differences in each trait between WT and each transgenic line at $p < 0.1$ according to Dunnett's test. The values in each column represent the relative value of each transgenic line to WT.

647

Fig. 7. Biomass production under the constant and fluctuating light condition

Dry weight of the above ground biomass was evaluated in the four lines of Arabidopsis under (C) the constant (DW_{constant}) and (D) fluctuating ($DW_{\text{fluctuating}}$) light condition as

651 described in (A) and (B). Vertical bars indicate the standard error ($n = 4$). * and **
 652 indicate significant differences in each parameter between WT and each transgenic line
 653 at $p < 0.05$ and 0.01 , respectively, according to Dunnett's test. The values in each
 654 column represent the relative value of each line to WT.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7