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2 Quantitative imaging reveals distinct contributions of SnRK2 and ABI3 in plasmodesmatal

3 permeability in *Physcomitrella patens*

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15 Six Figures

16 One Supplementary Table

17 Six Supplementary Figures

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19 **Short title**

20 ABA signaling for plasmodesmatal permeability

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24 permeability in *Physcomitrella patens*

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One-sentence summary

A protein kinase and a transcription factor are an essential and a promotive factor in the regulation of cell-to-cell connectivity in response to plant stress hormone abscisic acid.

Author contributions

T.T. and T.F. conceived the project. T.T. and K.K. designed the study. H.T. and T.F. directed the study. T.T. performed all experiments and analyzed the data. M.K. and Y.S. provided materials. T.T., K.K., and T.F. wrote the manuscript with input from co-authors.

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Abstract

Cell-to-cell communication is tightly regulated in response to environmental stimuli in plants. We previously used photoconvertible fluorescent protein Dendra2 as a model reporter to study this process. This experiment revealed that macromolecular trafficking between protonemal cells in *Physcomitrella patens* is suppressed in response to abscisic acid (ABA). However, it remains unknown what and how ABA signaling components contribute to this suppression.

Here, we showed that ABA signaling components SUCROSE NON-FERMENTING 1-RELATED PROTEIN KINASE 2 (PpSnRK2) and ABA INSENSITIVE 3 (PpABI3) play roles as an essential and a promotive factor, respectively in regulating <u>A</u>BA-induced <u>s</u>uppression of Dendra2 <u>d</u>iffusion between cells (ASD). Our quantitative imaging analysis revealed that disruption of *PpSnRK2* resulted in defective ASD onset itself, whereas disruption of *PpABI3* caused an 81-min delay in initiation of ASD. Live-cell imaging of callose deposition using aniline blue staining showed that, despite this onset delay, callose deposition on cross walls remained constant in the *PpABI3* disruptant, suggesting that PpABI3 facilitates ASD in a callose-independent manner. Given that ABA is an important phytohormone to cope with abiotic stresses, we further explored cellular physiological responses. We found that acquisition of salt stress tolerance is promoted by PpABI3 in a quantitative manner similar to ASD. Our results suggest that PpABI3-mediated ABA signaling may effectively coordinate cell-to-cell communication during acquisition of salt stress tolerance. This study will accelerate quantitative study for ABA signaling mechanism and function in response to various abiotic stresses.

KEYWORDS

abscisic acid signaling, cell-to-cell communication, *Physcomitrella patens*, quantitative imaging analysis, salt stress tolerance

Introduction

The phytohormone ABA plays a pivotal role in overcoming water-deficit stress conditions. Extensive molecular analyses have identified core components of ABA signaling in angiosperms which include an ABA receptor pyrabactin resistance 1-like (PYL) (also known as PYR or RCAR) (Ma et al., 2009; Park et al., 2009), a positive regulator of ABA signaling SnRK2 (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002), and its negative regulator of group A protein phosphatase type 2C (PP2C) (Umezawa et al., 2009; Vlad et al., 2009).

106   Under non-stressed conditions, PP2C interacts and suppress SnRK2 kinase activity (Yoshida

107   et al., 2019). Once plants are exposed to water-deficit stress conditions, ABA binds to PYL

108   receptor, which then inactivates PP2C to release SnRK2. This mechanism induces a

109   SnRK2-mediated phosphorylation relay to activate the ABA signaling (Yoshida et al., 2019).

110   Although details of molecular mechanisms are partially different, these ABA signaling

111   components are highly conserved in basal land plants bryophytes such as moss *Physcomitrella*

112   *patens* and liverwort *Marchantia polymorpha* (Khandelwal et al., 2010; Komatsu et al., 2013;

113   Saruhashi et al., 2015; Stevenson et al., 2016; Eklund et al., 2018; Shinozawa et al., 2019).

114   Because plasmodesmata (PD) are cytoplasmic channels which transverse the cell walls

115   to allow cell-to-cell communication, PD permeability is one of the critical elements to

116   determine the extent of cell-to-cell communication. Interestingly, in addition to various

117   phytohormones, downstream output of the ABA signaling is involved in the regulation of

118   cell-to-cell communication by modulating callose deposition at PD in angiosperms (Han and

119   Kim, 2016; Amsbury et al., 2018; Tylewicz et al., 2018; Wu et al., 2018). For example, in

120   response to shorter photoperiods, PD permeability in shoot apices of *Populus* is reduced

121   through the ABA signaling by formation of callosic sphincters to maintain bud dormancy

122   (Tylewicz et al., 2018). The effect of ABA is compromised by expression of a dominant

123   mutant allele *PP2C* of *Arabidopsis thaliana ABA INSENSITIVE 1* (*abi1-1*) (Tylewicz et al.,

124   2018). This *abi1-1* phenotype was rescued by overexpression of *PD-LOCATED PROTEIN 1*

125   (*PDLP1*), a type I membrane-receptor protein family (Tylewicz et al., 2018). Given that the

126   PDLP-mediated callose deposition is likely to occur through direct interactions with

127   *CALLOSE SYNTHASE* (*CALS*) *10/GLUCAN SYNTHASE LIKE* (*GSL*) *8* (Saatian et al., 2018),

128   the PDLPs are important regulators for PD permeability through callose deposition in

129   angiosperms. We recently reported that intercellular Dendra2 movement through PD in *P.*

130   *patens* protonemal cells is suppressed by ABA treatment (Kitagawa et al., 2019).

131   Transmission electron microscopy (TEM) revealed that ABA treatment decreased PD aperture

132   size from 38 nm to 29 nm but had little effect on PD density (Kitagawa et al., 2019). Aniline

133   blue staining indicated, however, that there is negligible change in callose deposition on cross

134   walls after ABA treatment during decrease in PD permeability (Kitagawa et al., 2019). These

135   data suggest that the ABA-induced suppression of intercellular Dendra2 movement is likely to

136   occur in a callose-independent fashion. Furthermore, genome database search identified no

137   *PDLP* orthologous genes in *P. patens* (Lee, 2014; Brunkard and Zambryski, 2017). Therefore,

138   it is of great interest to unravel how, in the absence of functional PDLPs the ABA

139   signaling-mediated PD permeability is regulated in *P. patens.*

140   Photoconvertible fluorescent proteins such as Dendra2 and DRONPA have been used to

141   analyze PD permeability. This quantitative approach revealed that, for example, in *A. thaliana*

142   roots different cell types have different PD permeability, and their sensitivities in response to

143   flagellin, low temperature and salicylic acid also differ (Wu et al., 2011; Gerlitz et al., 2018).

144   In addition, we previously established an experimental setup to visualize and analyze

145   intercellular Dendra2 movement in protonemal cells of *P. patens* (Kitagawa and Fujita, 2013;

146   Kitagawa and Fujita, 2015), and found a phenomenon of ABA-induced suppression of

147   Dendra2 movement between cells (ASD) (Kitagawa et al., 2019). However, due to a lack of

148   quantitative characterization of the Dendra2 movement, its biophysical property such as

149   diffusivity is still unclear. This prevents us from quantitatively dissecting the contributions of

150   ABA signaling components on the regulation of PD permeability. In this study, we examined

151   how the ABA signaling components *PpSnRK2* and *PpABI3* are involved in ASD by

152   quantitatively characterizing the Dendra2 movement between protonemal cells through PD.

153   Our analysis revealed different contributions of these two components on the regulation of PD

154   permeability in response to ABA. Furthermore, we detected a delay in the ASD initiation in a

155   *PpABI3* disruptant and that this delay is quite similar to the timescale required for salt stress

156   tolerance acquisition in response to ABA. Based on these results, we discuss a possible

157   function of *PpABI3* in coordinating the rapid initiation of ASD and salt stress tolerance

158   acquisition in *P. patens*.

159

160

161   **Results**

162   **Overexpression of *PpABI1A^{G333D}*, *PpSnRK2A*, and *PpABI3A* impacted on cellular growth**

163   **and morphology**

164 In response to the ABA treatment, protonemal cells of moss including *P. patens* exhibit drastic

165 morphological changes and growth suppression and eventually differentiate into brood cells

166 (or brachycytes) (Schnepf and Reinhard, 1997; Rowntree et al., 2007; Komatsu et al., 2009;

167 Amagai et al., 2018). We observed a similar morphological changes in our transgenic line

168 *ProEF1α:D2,* which constitutively expresses *Dendra2* (*D2*) under the control of an *EF1α*

169 promoter. ABA treatment on the *ProEF1α:D2* inhibited cell growth but cell division was

170 unaffected, and this resulted in the formation of round-shape cells like brood cells in their

171 apices and slightly swollen cells in their basal regions (Fig. 1A). To examine whether these

172 ABA-induced phenotypes requires the ABA signaling components, we constitutively

173 overexpressed $PpABI1A^{G333D}$ in the *ProEF1α:D2* background

174 (*PpABI1A^{G333D}OX/ProEF1α:D2*). The *PpABI1A^{G333D}* allele harbors a hypermorphic mutation

175 which phenocopies the *A. thaliana abi1-1* allele that acts as a negative regulator in ABA

176 signaling (Koornneef et al., 1984; Eklund et al., 2018). We observed that cellular phenotypes

177 in *PpABI1A^{G333D}OX/ProEF1α:D2* did not show significant difference between dimethyl

178 sulfoxide (DMSO) control and ABA-treated samples (Fig. 1B). This indicates that the

179 PpABI1A^{G333D} negatively impacted on the ABA signaling in *P. patens*, similar to that in *A.*

180 *thaliana.*

181 　　We next examined whether the ABA-induced cellular phenotypes are reproducible by

182 inducible overexpressions of *PpSnRK2A* and *PpABI3A* in the *ProEF1α:D2* background

183 (*PpSnRK2AiOX/ProEF1α:D2* and *PpABI3AiOX/ProEF1α:D2*). Transcripts of *PpSnRK2A* and

184 *PpABI3A* were readily detectable 24 hours after β-estradiol treatment (Supplemental Fig. S1,

185 A-D). Expression levels of the transgenic *PpSnRK2A* and *PpABI3A* were much higher than

186 the endogenous expression levels of *PpSnRK2A-D* and *PpABI3A-C* after ABA treatment

187 (Supplemental Fig. S1, E-H). We confirmed that its cellular growth and morphology in the

188 *ProEF1α:D2* were normal either in the absence or presence of β-estradiol (Fig. 1C). We

189 observed that the growth of protonemal cells in the *PpSnRK2AiOX/ProEF1α:D2* ceased and

190 exhibited swollen phenotypes upon β-estradiol treatment (Fig. 1D). On the other hand, the

191 *PpABI3AiOX/ProEF1α:D2* primarily showed growth suppression with little morphological

192 changes (Fig. 1E). We thus demonstrated that inducible expressions of either *PpSnRK2A* or

193 *PpABI3A* in the *PpSnRK2AiOX/ProEF1α:D2* and *PpABI3AiOX/ProEF1α:D2* lines can

194 partially phenocopies the ABA-induced morphological changes (Fig. 1, C-E).

195

Overexpression of *PpABI1A^{G333D}*, *PpSnRK2A*, and *PpABI3A* influences intercellular

197 **Dendra2 movement in protonemal cells**

198 Since overexpression of *PpABI1A^{G333D}*, *PpSnRK2A*, and *PpABI3A* impacted on the cellular

199 growth and morphology, we analyzed Dendra2 movement between protonemal cells in

200 *PpABI1A^{G333D}OX/ProEF1α:D2*, *PpSnRK2AiOX/ProEF1α:D2*, and

201 *PpABI3AiOX/ProEF1α:D2* to clarify contribution of these components on suppression of the

202 Dendra2 movement. In this system, intensity of the photoconverted Dendra2 red signal in

203 *ProEF1α:D2* decreased as the Dendra2 move away from the original cell over time in a

204 PD-mediated manner (Fig. 2A) (Kitagawa and Fujita, 2013; Kitagawa et al., 2019). Upon

205 ABA treatment, the Dendra2 intensity remained constant in the original cell, suggesting that

206 Dendra2 movement to neighboring cells was inhibited (Fig. 2A).

207 In the *PpABI1A^{G333D}OX/ProEF1α:D2*, although the Dendra2 movement was seemingly

208 suppressed upon ABA treatment as compared to the DMSO control, intercellular Dendra2

209 movement was still observed (Fig. 2B), suggesting a partial contribution of *PpABI1* on the

210 suppression of Dendra2 movement between cells. On the other hand, the Dendra2 movement

211 was fully suppressed in *PpSnRK2AiOX/ProEF1α:D2* and *PpABI3AiOX/ProEF1α:D2* upon

212 inducible overexpression of *PpSnRK2A* and *PpABI3A*, as compared to the corresponding

213 DMSO controls (Fig. 2, C-E). These observations indicate that inducible overexpression of

214 either *PpSnRK2A* or *PpABI3A* is sufficient to suppress the intercellular Dendra2 movement

215 even in the absence of ABA treatment.

216

Quantification workflow to analyze Dendra2 movement between protonemal cells in

218 **response to ABA**

219 We next established a method to quantitatively analyze the kinetics of Dendra2 intensity over

220 time based on a time-lapse Dendra2 imaging system reported previously (Kitagawa and Fujita,

221 2013). To rule out the possibility that disappearance of the Dendra2 intensity was due to

222   proteolytic protein turnover, we tracked the Dendra2 intensity in the presence of MG-132, a

223   proteasome inhibitor. We found that the Dendra2 intensity in protoplasts was unchanged over

224   the course of 720 min, both in MG-132-treated and DMSO control (Supplemental Fig. S2).

225   This indicated that proteolytic degradation of Dendra2 is negligible for at least 720 min. In

226   addition, we analyzed the Dendra2 intensity in the neighboring cells of the photoconverted

227   cells. As the Dendra2 intensity was decreased in the original 12th cell, the increase in the

228   Dendra2 intensity in the neighboring 11th and 13th cells was detected (Supplemental Fig. S3).

229   Thus our data suggest that the decrease in the Dendra2 intensity in the original cell was due to

230   the intercellular movement between the protonemal cells. In our previous report (Kitagawa

231   and Fujita, 2013), directional movement of the Dendra2 towards apical side of protonemata

232   was observed when protonemal cells were grown under the light condition but not under the

233   dark condition. Consistently, in the time-lapse imaging under the dark condition in this study,

234   directional bias of the Dendra2 movement was not found (Supplemental Fig. S3). It is also

235   noted that the decrease in the Dendra2 intensity in the 11th and 13th neighboring cells was

236   detectable over time, probably because the Dendra2 was diffused further.

237   We furthermore determined that this intercellular Dendra2 movement can be defined as a

238   simple diffusion process because the kinetics of the Dendra2 intensity in the photoconverted

239   cell was well fitted with a single exponential function (Fig. 3, A and B). This indicates that the

240   intercellular Dendra2 mobility is characterized by two parameters, a time constant (τ) and an

241   immobile fraction (B). The τ represents intercellular diffusivity of the Dendra2 through PD,

242   and the B refers to the ratio of immobilized Dendra2 in the original cell (Fig. 3C).

243   In addition, we found that this quantification method can be employed to evaluate the

244   effects of ABA on the intercellular Dendra2 diffusivity. Within 1 hour after ABA treatment,

245   intensity of the photoconverted Dendra2 became restricted in the original cell (Fig. 3, A and

246   B). Notably, Dendra2 in ABA-treated protoplasts was also stable to proteolytic degradation

247   (Supplemental Fig. S2). Consistently, by fitting the Dendra2 kinetics with the single

248   exponential function, the τ and B were smaller and larger in the ABA-treated sample,

249   respectively as compared to the DMSO control (Fig. 3B). As the τ represents how fast the

250   Dendra2 intensity reaches the B, the τ indicates how fast intercellular Dendra2 diffusivity is

251 suppressed in response to ABA and the *B* means the ratio of Dendra2 immobilized in the

252 original cell as a result of ASD. Thus, our quantitative imaging analysis succeeded in

253 characterizing the processes of intercellular diffusion of Dendra2 and its change in response to

254 ABA.

255

256 **Quantitative dissection revealed PpSnRK2 and PpABI3 contributions to ASD**

257 Our phenotypic analyses indicated that *PpSnRK2A* and *PpABI3A* play more predominant

258 roles than *PpABI1A* on ASD. We therefore next asked the question whether there is a

259 differential contribution between *PpSnRK2A* and *PpABI3A* in regulating ASD. To this end, we

260 measured the changes in the Dendra2 intensity over time in response to ABA in

261 *ppsnrk2a/b/c/d* quadruple knockout (*ppsnrk2qko*) and *ppabi3a/b/c* triple knockout (*ppabi3tko*)

262 mutants (*ProEF1α:D2/ppsnrk2qko* and *ProEF1α:D2/ppabi3tko*, respectively). We found that

263 intercellular Dendra2 diffusion in the *ProEF1α:D2/ppsnrk2qko* was observable irrespective of

264 the presence and absence of ABA treatment (Fig. 4, A and B). The τ and *B* obtained from the

265 fittings were comparable between *ProEF1α:D2* (τ = 180, *B* = 0.16 in the median) and

266 *ProEF1α:D2/ppsnrk2qko* (τ = 149, *B* = 0.23 in the median) in the absence of ABA or

267 *ProEF1α:D2/ppsnrk2qko* in the presence of ABA (τ = 187, *B* = 0.22 in the median; *P* = 7.9 $\times$

268 10^{-1} and *P* = 4.2 $\times$ 10^{-1}), respectively (Fig. 4, E and F). This indicates that PpSnRK2 is an

269 indispensable factor for ASD.

270 On the other hand, although intercellular Dendra2 diffusion was observed in

271 *ProEF1α:D2/ppabi3tko* in the DMSO control (Fig. 4, C and D), the τ in

272 *ProEF1α:D2/ppabi3tko* was calculated to be smaller than that in *ProEF1α:D2* (τ = 204 and

273 266 in the median), whereas the *B* was similar (*B* = 0.25 and 0.24 in the median) (Fig. 4, G

274 and H). This suggests that the intercellular Dendra2 diffusivity is higher in

275 *ProEF1α:D2/ppabi3tko* than in *ProEF1α:D2* under the normal culture condition, possibly due

276 to responsiveness to endogenous ABA. Upon ABA treatment, the observed ASD in

277 *ProEF1α:D2/ppabi3tko* was similar to that in *ProEF1α:D2* (Fig. 3A and Fig. 4C). However,

278 the τ and *B* in *ProEF1α:D2/ppabi3tko* was significantly higher and lower (τ = 46, *B* = 0.72 in

279 the median), respectively compared to that in the *ProEF1α:D2* control (τ = 10, *B* = 0.82 in the

280   median) (Fig. 4, G and H). Since the τ indicates responsiveness to ABA to initiate ASD, the

281   higher τ in *ProEF1α:D2/ppabi3tko* suggests that the disruption of *PpABI3* causes a temporal

282   delay to initiate ASD. The lower *B* in *ProEF1α:D2/ppabi3tko* can be also explained by this

283   temporal delay before initiation of ASD. During the delay of ASD initiation, more Dendra2

284   can diffuse from the original cells to neighboring cells in *ProEF1α:D2/ppabi3tko* than

285   *ProEF1α:D2*. Thus, the Dendra2 intensity in the original cell decreased to a greater extent in

286   *ProEF1α:D2/ppabi3tko* than *ProEF1α:*D2 at the onset of ASD, which can result in the lower

287   *B* in *ProEF1α:D2/ppabi3tko*.

288   Our results suggest that PpABI3 is responsible for the normal initiation of ASD. To gain

289   better quantitative resolution on the temporal control of how PpABI3 facilitates this process,

290   we introduced a time to the initiation of ASD (*A*) in this study (Fig. 4I). Because complete

291   suppression of the Denra2 diffusion between cells requires infinite time due to the exponential

292   indeterminate form (see Materials and Methods), we here considered the duration required for

293   the Dendra2 intensity to the 90% of the steady state level (A_{90}). The A_{90} allows us to compare

294   the temporal difference to initiate ASD in the absence and presence of *PpABI3*. We

295   determined that in *ProEF1α:D2* the A_{90} is 24 min, whereas in *ProEF1α:D2/ppabi3tko* the A_{90}

296   is increased to 105 min. This indicates an 81-min delay to arrest the Dendra2 diffusion in

297   response to ABA in *ProEF1α:D2/ppabi3tko*. We obtained a similar temporal delay in the

298   *ProEF1α:D2/ppabi3tko* when A_{85} and A_{95} were used, further confirming the robustness of our

299   interpretation.

300

Suppression of intercellular Dendra2 diffusion by PpABI3 is callose-independent

302   Using TEM approach, we previously reported that the PD aperture size was reduced by 9-nm

303   after ABA treatment. Aniline blue staining also showed that there are little changes in the

304   callose deposition on cross walls (Kitagawa et al., 2019). However, it is still unclear whether

305   conventional aniline blue staining in fixed cells is sufficient to support the

306   callose-independent regulation of ASD. We therefore improved the aniline blue staining

307   method and established its live-imaging method to observe changes in callose deposition on

308   cross walls between targeted cells after ABA treatment. Callose accumulation at the cross wall

309   was detectable during cell divisions in living protonemal cells even in the presence of aniline

310   blue solution (Supplemental Fig. S4A). Moreover, we were able to observe intercellular

311   Dendra2 diffusion and ASD in this method (Supplemental Fig. S4B), indicating that link

312   between intercellular Dendra2 diffusivity and the extent of callose deposition on cross walls is

313   evaluable. We analyzed changes of callose deposition in the *ProEF1α:D2* on the same cross

314   wall between the 11th and 12th cell for 180 min after ABA treatment, and found that callose

315   signal intensity was constant throughout the observation, irrespective of ABA treatment. (Fig.

316   5, A and D). Intriguingly, callose intensity was also unchanged in the *ProEF1α:D2/ppabi3tko*

317   as well as the *ProEF1α:D2/ppsnrk2qko* (Fig. 5, B-D). To investigate the callose deposition on

318   cross walls between the 11th and 12th cell in greater details, we acquired images with better

319   resolution from control and ABA-treated samples, at 20-, 100- and 180-min time points.

320   When observed at 0.3-μm optical slice, we detected punctate signals on cross walls (Fig. 5,

321   E-G; Supplemental Fig. S5, A and B), which resembles the PD-associated callose

322   accumulation as detected previously by callose immunostaining (Kitagawa et al., 2019).

323   There was no significant increase in the density of punctate signals between the control and

324   ABA-treated samples in *ProEF1α:D2*, *ProEF1α:D2/ppsnrk2qko*, and *ProEF1α:D2/ppabi3tko*

325   (Fig. 5H), and also *PpABI3AiOX/ProEF1α:D2* (Supplemental Fig. S5C). Our results suggest

326   that PpABI3 does not increase the density of PD-associated callose in response to endogenous

327   and exogenous ABA. Collectively, our data further supported that PpSnRK2 and PpABI3

328   facilitates ASD in a callose-independent manner.

329

330   **PpABI3 is required in salt stress tolerance acquisition of protonemal cells in response to**

331   **ABA**

332   Given that PpABI3 facilitates the initiation of ASD in protonemal cells, we wonder whether

333   this observation is related to any physiological response(s). One possible response is

334   acquisition of salt stress tolerance since ABA plays crucial roles on this process (Takezawa et

335   al., 2015). To test this hypothesis, we set out to determine the protonemal viability rate after

336   salt stress treatment by counting propidium iodide (PI)-stained dead cells (Fig. 6A). In the

337   control, all cells in *ProEF1α:D2*, *ppsnrk2qko/ProEF1α:D2*, and *ppabi3tko/ProEF1α:D2* were

338   alive (viability: 100 ± 0 %) (Fig. 6B). In *ProEF1α:D2*, the cell viability under salt stress

339   without and after 5-, 20-, and 100-min ABA pretreatment was $51 \pm 33\%$, $47 \pm 41\%$, $83 \pm 9\%$,

340   and $87 \pm 12\%$, respectively (Fig. 6B), indicating that salt stress tolerance was significantly

341   induced after 20-min ABA pretreatment. In *ppsnrk2qko/ProEF1α:D2*, cell viability under salt

342   stress without and after 5-, 20-, and 100-min ABA pretreatment was $13 \pm 26\%$, $9 \pm 19\%$, $5 \pm$

343   9%, and $14 \pm 26\%$ (Fig. 6B). This result showed that *ppsnrk2qko/ProEF1α:D2* is

344   ABA-insensitive during acquisition of salt stress tolerance and is consistent with the essential

345   role of PpSnRK2 in ASD (Fig. 4, A, B, E and F). On the contrary, cell viability in

346   *ProEF1α:D2/ppabi3tko* under salt stress without and after 5-, 20-, and 100-min ABA

347   pretreatment was $43 \pm 26\%$, $35 \pm 37\%$, $54 \pm 27\%$ and 76 ± 18 % (Fig. 6B), indicating that salt

348   stress tolerance was significantly induced after 100-min ABA treatment. There was about

349   80-min delay in acquisition of salt stress tolerance in *ProEF1α:D2/ppabi3tko* when compared

350   to *ProEF1α:D2*. These results indicate that the PpABI3-mediated ABA signaling contributes

351   to the acquisition of salt stress tolerance in around 80 min, which is quite similar to the value

352   observed in PpABI3-mediated ASD response (Fig. 4I). This suggests a plausible mechanism

353   connecting the regulation of PD permeability and viability against salt stress tolerance by

354   PpABI3.

355

356

357   **Discussion**

358   This study provides quantitative dissection on the contributions of ABA signaling components

359   in regulating protonemal PD permeability. Our analysis revealed that, in contrast to the

360   essential roles of PpSnRK2 in ASD, PpABI3 plays a specific role in promoting the initiation

361   of ASD within 80 min. We further unraveled that PpABI3 is required for the rapid acquisition

362   of salt stress tolerance in protonemal cells, thereby contributing to the survival strategy during

363   abiotic stress conditions. Interestingly, these two processes that mediated by PpABI3 occur in

364   a similar timescale of tens of minutes, suggesting the promotive role of PpABI3 in

365   coordinating PD permeability and stress tolerance through the ABA signaling.

366

Regulatory mechanism of ASD in protonemal cells of *P. patens*

Although our previous study reported the phenomenon of ASD and the contribution of endogenous ABA in ASD (Kitagawa et al., 2019), it remains elusive which of the ABA signaling components and how they are involved in this process. Here, we provide evidence to show that PpSnRK2 and PpABI3 are essential and promotive factors in the ABA signaling to drive ASD in protonemal cells. Our quantitative analysis based on the newly introduced A_{90} further revealed that ASD is almost initiated within this time frame after ABA treatment. Since PpSnRK2 is essential for ASD and is involved in the phosphorylation of ABA signaling, PpSnRK2 seems to phosphorylate ASD-related factors within 24 min based on A_{90} in *ProEF1α:D2*. Previously a phosphoproteomic analysis of the ABA signaling pathway identified 74 phosphopeptides (51 phosphorylated proteins except for PpSnRK2s) within 15 min after ABA treatment (Amagai et al., 2018). We speculate that the downstream targets of PpSnRK2 to trigger ASD are present in this phosphoprotein list. In consistent with this, seven of these phosphoproteins are orthologues found in the proteome of *A. thaliana* PD (Fernandez-Calvino et al., 2011; Amagai et al., 2018). However, their functions in regulating PD permeability and localizations around PD have not yet been demonstrated. It would be worth examining the contribution of their phosphomimic forms on ASD to uncover molecular mechanism of the ABA signaling in ASD.

In contrast to these downstream targets of the PpSnRK2, PpABI3 is not phosphorylated in the ABA signaling (Nakashima et al., 2009; Umezawa et al., 2013; Wang et al., 2013; Amagai et al., 2018). ABA INSENSITIVE 5 (ABI5) of *P. patens* is instead phosphorylated within 15 min after ABA treatment (Amagai et al., 2018). PpABI3A is known to interact with barley ABI5, which enhances transactivation of an ABA-responsive wheat Em promoter without ABA treatment (Marella et al., 2006). Therefore, one possible regulatory mechanism in ASD may be the co-regulation by a PpABI3 and PpABI5 heterodimer which accelerates the ASD downstream of PpSnRK2. If this is the case, the PpABI3-mediated ASD should involve transcriptional regulation since PpABI3 is a known transcriptional factor. Because basal expression of *PpABI3* is required for the protonemal growth in *P. patens* (Zhao et al., 2018), this weak but constitutive expression of *PpABI3* may also contribute to promote the rapid

396 initiation of ASD. This hypothesis is consistent with our observation that intercellular

397 Dendra2 diffusion in the *ProEF1α:D2/ppabi3tko* is slightly higher than *ProEF1α:D2* under

398 normal culture conditions. In addition, *de novo* synthesized PpABI3 in response to ABA may

399 further augment the ABA signaling to boost ASD.

400   A key question relevant to the PpABI3-mediated regulation of ASD would be, what is

401 the downstream mechanism? A previously reported proteome analysis study identified

402 SYNAPTOTAGMIN (SYT) as a candidate target of PpABI3-mediated ABA signaling (Yotsui

403 et al., 2016). SYT is annotated as a C2 domain containing protein. In *A. thaliana*, AtSYTA

404 was shown to be a PD-localized protein (Schapire et al., 2008; Fernandez-Calvino et al., 2011;

405 Levy et al., 2015; Perez-Sancho et al., 2015). It is tempting to hypothesize that PpSYT may

406 tether plasma membrane and endoplasmic reticulum to restrict PD permeability as shown in

407 other multiple C2 domains and transmembrane region proteins (Brault et al., 2019).

408 Importantly, this regulation by the SYT may occur in a callose-independent manner, which is

409 consistent with observation that the PpABI3-mediated ASD did not involve any callose

410 deposition around PD.

411   Among the ABA signaling components, contribution of PpABI1 on ASD was less

412 prominent compared to that of PpSnRK2 and PpABI3. In contrast to our finding,

413 ABI1-mediated ABA signaling in *Populus* is involved in the regulation of PD permeability in

414 bud dormancy (Tylewicz et al., 2018). This discrepancy can be explained by an interaction

415 between ABI1 and SnRK2 and/or emergence of PDLP which can modify the ABA sensitivity

416 in *Populus* (Komatsu et al., 2013; Lee, 2014; Brunkard and Zambryski, 2017). Our data

417 warrant further investigations to unravel the molecular mechanisms of PpSnRK2-, PpABI3-,

418 and PpABI1-mediated ABA signaling in ASD. This would be helpful to extend our

419 understanding on how PD permeability is regulated in response to abiotic stresses.

420

421 **Cellular physiological responses associated with ABA signaling in ASD**

422 We previously established a simple simulation model for PD permeability based on a

423 relationship between PD aperture and molecule size (Kawade and Tanimoto, 2015; Kawade et

424 al., 2017). Although this model is well recapitulated in *in vivo* experiments using GFP

425 diffusivity through PD under the normal condition, it failed to explain the connection between
426 ASD occurrence and the reduction in PD aperture size. Although upon ABA treatment the PD
427 aperture was reduced from 38 nm to 29 nm (Kitagawa et al., 2019), the Dendra2 molecules
428 with a Stokes radius of Dendra2 (2.4 nm) should still be able to diffuse through the tightened
429 PD aperture without much difficulty (Sadovsky et al., 2017). However this is not the case as
430 ABA treatment inhibits the intercellular ABA diffusion. These observations suggest that
431 additional elements independent of PD structural modification such as cytoplasmic viscosity
432 can be involved in ASD. Indeed, in response to ABA and some environmental stresses, sugar
433 and sugar alcohol has been reported to accumulate in *P. patens* protonemal cells as compatible
434 solutes (Nagao et al., 2005; Nagao et al., 2006; Oldenhof et al., 2006; Komatsu et al., 2013;
435 Arif et al., 2018). This increment in the compatible solutes can enhance cytoplasmic viscosity
436 (Wolkers et al., 1998; Golovina et al., 2001) which can affect macromolecular diffusivity
437 (Verkman, 2002).

438   Lastly we pursued cellular physiological responses regulated by PpABI3 in protonemal
439 tissues and found that salt stress tolerance is confered by the PpABI3 in a time frame similar
440 to that of ASD. A series of studies had been carried out to investigate mechanisms involved to
441 acquire various stress tolerance against desiccation, hyperosmolality, low temperature, and
442 freezing in an order of 24 hours after ABA treatment (Minami et al., 2005; Komatsu et al.,
443 2009; Khandelwal et al., 2010; Komatsu et al., 2013; Saruhashi et al., 2015; Tan et al., 2017).
444 Involvement of PpABI3-mediated transcriptional regulation in these processes has also been
445 reported (Khandelwal et al., 2010; Tan et al., 2017). However, our results showed that the
446 acquisition of salt stress tolerance mediated by PpABI3 was much more rapid and occurred
447 within 20 min of ABA pretreatment. Disruption of *PpABI3* compromised this rapid induction
448 of salt stress tolerance and resulted in an aprroximately 80-min delay for the tolerance
449 acquisition. Our studies, together with these results, indicate that PpABI3 plays roles in
450 acquiring stress tolerance both in short and long time frame. We speculate that basal
451 expression of the PpABI3 contributes to short term environmental stresses tolerance, and this
452 "pre-existing" PpABI3 in combination with *de novo* synthesized PpABI3 are involved in the
453 regulation of a transcriptional network to induce more robust and persisting stress tolerances.

454   Autoregulation of the ABI3 transcription may accelerate the downstream reaction until

455   endogenous ABA is gradually synthesized (Minami et al., 2005; Bedi and Chaudhuri, 2018).

456   Thus at the moment, it is tempting to speculate that whether and how PpABI3-mediated ASD

457   and acquisition of salt stress tolerance are mechanistically and functionally coordinated in a

458   relatively short duration. Nevertheless, it is also possible that the decreased viability in

459   *ProEF1α:D2/ppsnrk2ako* or *ProEF1α:D2/ppabi3tko* is attributed to the repression of

460   PpSnRK2- and/or PpABI3-mediated regulation of salt stress tolerance, which is independent

461   of the regulation of cell-to-cell communication. Future experiments to address this point

462   should provide further understanding of the relationship between cell-to-cell communication

463   and physiological response at a cellular level.

464

465

466   **Materials and Methods**

467   **Plant materials and growth conditions**

468   The *Physcomitrella patens* WT strain used in this study was Gransden 2004 (Rensing et al.,

469   2008). The *ProEF1α:D2*, *ppsnrk2qko*, and *ppabi3tko* lines were described previously

470   (Khandelwal et al., 2010; Kitagawa and Fujita, 2013; Shinozawa et al., 2019). The plants

471   were cultured on BCDAT medium with 0.8% (w/v) agar under continuous white light at 25°C

472   for three to five days (Nishiyama et al., 2000).

473

474   **Vector constructions**

475   We obtained the full-length cDNA clone of *pphn8m2* including *PpABI1A* from the RIKEN

476   BioResource Center (Resource number: pdp31463). A synonymous mutation in the open

477   reading frame (ORF) of *PpABI1A* (*PpABI1A^{G1610A}*) in pdp31463 was corrected with the

478   primer set of   5'-ATGAGGCGGTCTGCGATATTG-3'   (Forward)   and

479   5'-CTATCTATCCCTGGGAACTTTTAAGTC-3'(Reverse). The resulting fragment was used

480   to   amplify   the   ORF   of   *PpABI1A*   with   the   primer   set   of

481   5'-CACCATGGCCACAGCTAAAACTTGTAGAAG-3'   (Forward)   and

482   5'-CTATCTATCCCTGGGAACTTTTAAGTC-3' (Reverse). The ORF fragment of *PpABI1A*

483 was cloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific) and confirmed by

484 sequencing. To introduce a mutation mimicking *abi1-1* of *A. thaliana* (Koornneef et al.,

485 1984) in the ORF fragment of *PpABI1A* (*PpABI1A*^{G333D}), PCR was performed with the primer

486 set of 5'-ATGGGCATGATGGATCGC-3' (Forward) and

487 5'-CGTATACTCCAAAATAATGTAAAGGAGC-3' (Reverse) using the pENTR/D-TOPO

488 vector containing *PpABI1A* as the template, and then the resultant fragment was ligated with

489 DNA ligation kit (TaKaRa). The resultant plasmid was subjected to LR reaction with the

490 destination pPOG1 vector using LR clonase II plus enzyme mix (Thermo Fisher Scientific)

491 (pPOG1-PpABI1A^{G333D}).

492   The ORF fragment of *PpSnRK2A* from pGAD424 vector containing *PpSnRK2A* was

493 amplified with the primer set of 5'-CACCATGGATATTCCGAGCATGCATGACCAC-3'

494 (Forward) and 5'-CATTGCGCACACAAACTCCCCAC-3' (Reverse). The ORF fragment of

495 *PpSnRK2A* was cloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific) and

496 confirmed by sequencing. The resultant plasmid was subjected to LR reaction with the

497 destination pPGX8 (Kubo et al., 2013) vector using LR clonase II plus enzyme mix (Thermo

498 Fisher Scientific) (pPGX8-PpSnRK2A).

499   We obtained the full-length cDNA clones of *pphn37a15* including *PpABI3A* from the

500 RIKEN BioResource Center (Resource number: pdp41729). The ORF fragment of *PpABI3A*

501 in pdp41729 was amplified with the primer set of

502 5'-CACCATGGTGCTCCTATCGAGTGTG-3' (Forward) and

503 5'-TCCTGCGGGCTCGGTC-3' (Reverse). The ORF fragment of *PpABI3A* was cloned into

504 the pENTR/D-TOPO vector (Thermo Fisher Scientific) and confirmed by sequencing. The

505 resultant plasmid was subjected to LR reaction with the destination pPGX8 (Kubo et al.,

506 2013) vector using LR clonase II plus enzyme mix (Thermo Fisher Scientific)

507 (pPGX8-PpABI3A).

508   The pPOG1-PpABI1A^{G333D}, pPGX8-PpSnRK2A, and pPGX8-PpABI3A were digested

509 with the restriction enzyme *Sse*8387I (TaKaRa) before transformation.

510

511 **Transgenic lines**

512 Transformation was performed based on the polyethylene glycol-mediated method

513 (Nishiyama et al., 2000).

514 The *PpABI1A^{G333D}OX/ProEF1a:D2* lines were generated by introducing the

515 *Sse*8387I-digested pPOG1-PpABI1A^{G333D} fragments into the *ProEF1α:D2* genetic

516 background. Stable transformants were selected on BCDAT agar medium containing 30 mg/L

517 hygromycin B (Wako) twice. The expression of *PpABI1A^{G333D}* in

518 *PpABI1A^{G333D}OX/ProEF1α:D2* lines was confirmed through RT-PCR with the primer set of

519 5'-TGTGGTGCCTAGTGACGCTG-3' (Forward) and 5'-ATTCGCTGCCTGCGATCCAT-3'

520 (Reverse) for *PpABI1A^{G333D}* and the primer set of 5'-TGCCATTAAGACGGCTATCA-3'

521 (Forward) and 5'-CGAGATTATTTCCAACAGATGGTCTA-3' (Reverse) for *PpGAPDH* (as

522 an internal control) (Supplemental Fig. S6).

523 *PpSnRK2AiOX/ProEF1α:D2* lines were generated by introducing the *Sse*8387I-digested

524 pPGX8-PpSnRK2A fragments into the *ProEF1α:D2* genetic background. Stable

525 transformants were selected on BCDAT agar medium containing 30 mg/L hygromycin B

526 (Wako) twice. The gene targeting was verified by genomic PCR with the primer set of

527 5'-ACAAGTTTGTACAAAAAAGCAGGCT-3' (Forward) and

528 5'-CATTGCGCACACAAACTCCC-3' (Reverse) which bind the outside from the 5'-end of

529 ORF and the 3'-end of ORF of *PpSnRK2A*, and the primer set of

530 5'-CGCTTAGGCAGGAGGCCGTT-3' (Forward) and

531 5'-CACCCTCTGGCCCGTGACAA-3' (Reverse) which bind the inside of transgene and the

532 outside of the 3'-end of the *P. patens* inter-genic 1 (PIG1) site.

533 *PpABI3AiOX/ProEF1α:D2* lines were generated by introducing *Sse*8387I-digested

534 pPGX8-PpABI3A fragments into the *ProEF1α:D2* genetic background. Stable transformants

535 were selected on BCDAT agar medium containing 30 mg/L hygromycin B (Wako) twice. The

536 gene targeting was verified by genomic PCR with the primer set

537 5'-ACAAGTTTGTACAAAAAAGCAGGCT-3' (Forward) and

538 5'-TCCTGCGGGCTCGGTCTTCA-3' (Reverse) which bind the outside from the 5'-end of

539 ORF and the 3'-end of ORF of *PpABI3A*, and with the primer set

540 5'-CGCTTAGGCAGGAGGCCGTT-3' (Forward) and

541 5'-CACCCTCTGGCCCGTGACAA-3' (Reverse) which bind the PIG1 site.

542 The *ProEF1α:D2/ppsnrk2qko* and *ProEF1α:D2/ppabi3tko* lines were established by

543 introducing the *ProEF1α:D2* construct (pT1OG-Dendra2) (Kitagawa and Fujita, 2013) into

544 the *ppsnrk2qko* and *ppabi3tko* genetic backgrounds, respectively (Khandelwal et al., 2010;

545 Shinozawa et al., 2019). Stable transformants were selected on BCDAT agar medium

546 containing 100 mg/L zeocin (Invitrogen) twice.

547 The independently generated transgenic lines underwent the following quantitative

548 RT-PCR, microscopy and photoconversion assay, callose staining with aniline blue, and

549 viability test against salt stress, in which we confirmed reproducibility.

550

551 **Quantitative RT-PCR**

552 Protonemal tissues of *ProEF1α:D2,* *PpSnRK2AiOX/ProEF1α:D2*, and

553 *PpABI3AiOX/ProEF1α:D2* lines were cultured for 10 days under red light on BCDAT agar

554 medium overlaid with cellophane sheets. The cellophane with the 10-day-old culture of

555 *ProEF1α:D2* were then transferred onto BCDAT agar medium with 50 μM ABA, and

556 subjected to additional culture for 1, 6, or 12 hours to investigate the expression levels of

557 *PpSnRK2A-D* (Pp3c5_21160V3, Pp3c6_16600V3, Pp3c6_11090V3 and Pp3c5_17150V3,

558 respectively) and *PpABI3A-C* (Pp3c2_3370V3, Pp3c17_16470V3 and Pp3c4_7320V3,

559 respectively) in response to ABA. In the negative control, the cellophanes with the 10-day-old

560 culture of *ProEF1α:D2* were directly used to investigate the expression level of *PpSnRK2A-D*

561 and *PpABI3A-C* without additional culture on BCDAT agar medium with 50 μM ABA. For

562 inducible experiments, the cellophanes with the 10-day-old cultures of *ProEF1α:D2,*

563 *PpSnRK2AiOX/ProEF1α:D2*, and *PpABI3AiOX/ProEF1α:D2* lines on BCDAT agar medium

564 were transferred onto BCDAT agar medium with 1 μM β-estradiol (or 0.1% (v/v) DMSO as

565 the negative control). The plant tissues were used for RT-PCR analysis 24 hours after transfer

566 to confirm the inducible overexpression of *PpSnRK2A* or *PpABI3A*.

567 Total RNA was purified from the protonemal tissues with RNeasy Plant Mini Kit

568 (QIAGEN). First-strand cDNA was synthesized with ReverTra Ace qPCR RT Master Mix

569 with gDNA Remover (TOYOBO) according to the manufacture's instruction. qRT-PCR was

570 performed using an ABI 7500 RealTimePCR System (Applied Biosystems) with the

571 THUNDERBIRD qPCR mix (TOYOBO). The data were analyzed by the ΔΔCt method for

572 relative quantification of the transcript levels. The transcript levels were normalized against

573 riboflavin kinase gene (*RFK*) (Pp3c12_7730V3) or 3-hydroxyisobutyryl-CoA hydrolase gene

574 (*HIBCH*) (Pp3c4_17830V3), whose expression levels were reported to be robust against ABA

575 treatment (Khraiwesh et al., 2015). The data were collected from three independent biological

576 replicates with technical triplicates, and subjected to the statistical analysis of Welch's *t*-test.

577 The used primers in the qRT-PCR is listed in Supplemental Table S1.

578

579 **Microscopy and photoconversion assay**

580 Three- to five-day-old protonemal tissues were inoculated into 500 μL of BCDAT medium

581 containing 0.5% glucose (BCDATG) solidified with 0.5% gellan gum in 4-well chamber

582 slides (no. 5222-004; Iwaki), and then covered with the additional 250 μL of the medium. To

583 suppress branching of the protonemata, the samples were cultured under continuous red light

584 for 10 days as previously reported (Kitagawa and Fujita, 2013) except for

585 *ProEF1α:D2/ppsnrk2qko* (for 15 days) due to its slower growth under red light. *ProEF1α:D2*

586 was also cultured for 15 days when compared with *ProEF1α:D2/ppsnrk2qko*. In the test of

587 ABA effects, 50 mM ABA or DMSO was added into wells of the 4-well chamber slides just

588 after photoconversion to final concentrations of 50 μM and 0.1% (v/v), respectively. In the

589 test of overexpression effect of *PpSnRK2A* or *PpABI3A*, 1 mM β-estradiol or DMSO was

590 added into wells of the 4-well chamber slides 24 hours before photoconversion to final

591 concentrations of 1 μM and 0.1% (v/v), respectively. For assessment of protonemal growth

592 and morphology, the samples were observed under a microscope (DM 2500, Leica).

593 Photoconversion of Dendra2 was carried out using a confocal laser-scanning microscope

594 (Nikon A1, Nikon) in the 12th cells from the apices in protonemata with 405-nm diode laser

595 (10.0% laser output, 36 mW) with 3 iterations (8 sec/frame, zoom 4×) using the PlanApo

596 10x/0.45 NA objective. Time-lapse imaging was subsequently performed with the objective

597 lens PlanApo 10x /0.45 NA and 561-nm solid laser (2.0% laser output, 10 mW) at 20-min

598 intervals for 720 min. Five z-stack images of size 1024 × 1024 pixels were acquired at 6-μm

599   steps in each interval. The pinhole size was set to 16.60 μm in diameter, which yields a 7.29

600   μm optical slice in this condition.

601   We manually measured the mean fluorescence intensity of photoconverted Dendra2 in

602   the whole regions of the laser-irradiated cells or the neighboring cells, and subtracted the

603   mean fluorescence intensity of the surrounding medium region at each time point with Fiji

604   software (ImageJ; https://imagej.net/Fiji). Based on the previous analysis (Kawade et al.,

605   2017), the kinetics of Dendra2 intensity from 0 to 720 min after photoconversion was fitted

606   by an exponential function as

$$I(t) = (I_0 - B)e^{(-t/\tau)} + B, \tag{1}$$

608   where $I(t)$ and I_0 are fluorescence intensity of Dendra2 at a time t and just after

609   photoconversion, respectively, normalized with the mean fluorescence intensity per pixel just

610   after photoconversion ($I_0 = 1$); τ is a time constant of Dendra2 diffusivity or responsiveness to

611   ABA treatment for ASD; B is an immobile fraction of Dendra2 in the original cell. τ and B are

612   free parameters determined by minimizing the residual sum of squares (RSS) between the

613   measured data and the fitting function (1) by using custom MATLAB scripts.

614   Dendra2 intensity decreased over time and reached the level at the steady state in our

615   experiment. To evaluate how this profile is affected in response to ABA during the

616   progression to ASD, we here calculated a decrease ratio of Dendra2 intensity ($x\%$) at an

617   arbitrary point of time t against the Dendra2 intensity at the steady state. The decrease ratio of

618   Dendra2 intensity at a time t is described as follows:

$$(I(t) - B)/(I_0 - B) = 1 - x/100. \tag{2}$$

620   When solving for $I(t)$, we obtained the following equation:

$$I(t) = (1 - x/100) \times (I_0 - B) + B. \tag{3}$$

622   By solving for x after introduction of (3) into (1), the decreased ratio of Dendra2

623   intensity x was summarized by using t and τ, given as:

$$x = 100 \times (1 - e^{(-t/\tau)}). \tag{4}$$

625   Because, in this case, t represents time required for reaching the Dendra2 intensity to the

626    $x\%$ of the steady state level, we introduced a new parameter Ax for this specific time instead

627   of t to avoid confusion as follows:

628
$$x = 100 \times (1 - e^{(-Ax/\tau)}). \tag{5}$$

629   By using this equation, we were able to evaluate how rapidly ASD is observed in

630   response to ABA. A_{90} nearly equals to 2.3-fold value of τ.

631

632   **Assessment of Dendra2 degradation in protoplasts**

633   Protoplasts were obtained from three- to five-day-old protonemal tissues of *ProEF1α:D2*

634   through 20-min treatment of 2% (w/v) Driserase dissolved in 8% (w/v) mannitol (Kyowa

635   Hakko Kogyo Co., Ltd.) solution with gentle mix at 5-min intervals. The protoplasts were

636   filtered through a mesh (CellTrics ®, 50 μm, Partec) into a 12 mL tube, collected by

637   centrifugation at 1,000 rpm, and suspended in 8% (w/v) mannitol solution. After

638   centrifugation again, the protoplasts were suspended in 250 μL of BCDATG medium

639   containing 0.5% (w/v) gellan gum and 6% (w/v) mannitol, and put into each well of 8-well

640   chamber slides (no. 5232-008; Iwaki), and then covered with additional 500 μL of BCDATG

641   medium containing 0.5% (w/v) gellan gum and 6% (w/v) mannitol .

642   Images of protoplasts were obtained just after and 12 hours after photoconversion. Mean

643   fluorescence intensity per pixel of photoconverted Dendra2 in the protoplasts was analyzed

644   with Fiji software (ImageJ; https://imagej.net/Fiji). Dendra2 degradation was assessed with

645   the ratio of mean fluorescence intensity just after photoconversion and at 12 hours after

646   photoconversion. MG-132 (final concentration 50 μM) was added into the wells of 8-well

647   chamber slides 1.5 hours before photoconversion of Dendra2 for the negative control of

648   proteolytic degradation.

649

650   **Callose staining with aniline blue**

651   Aniline blue (Wako) was dissolved in 100 mM phosphate buffer (pH 8.7) to a concentration

652   of 5.0% (w/v), and sterilized by filtration (pore size = 0.22 µm). The 5.0% (w/v) aniline blue

653   solution was further dissolved in the buffer to a concentration of 1.6% (w/v). The color of

654   aniline blue solution was changed from blue to brownish yellow when incubated more than 2

655   days at room temperature and exposure to air at several times. Staining was carried out after

656   the color change. We found that the color aniline blue solution changed into yellow-brown

657 when dissolving in the pH 8.7 phosphate buffer. This is similar to the case of dissolving

658 aniline blue in K_3PO_4 (pH 12) solution as the previous reported (Zavaliev and Epel, 2015).

659 The aniline blue solution was added to samples to a concentration of 0.1% (w/v) one day

660 before observation under Nikon A1 confocal microscope (Nikon). To detect change in callose

661 signal with aniline blue, time-lapse imaging was performed with the PlanApo-VC 20x/0.75

662 NA objective (zoom 4×) and 405-nm diode laser (2.0% laser output, 36 mW) at 20-min

663 intervals for 180 min. Five z-stack images of size 1024 × 1024 pixels were acquired at 3

664 μm-steps in each interval. The pinhole size was set to 14.05 μm in diameter, which yields a

665 1.77 μm optical slice in this condition. To calculate the density of punctate signals from

666 callose, images were acquired with the PlanApo-VC 100xH/1.4 NA objective (zoom 4×) and

667 405-nm diode laser (100% laser output, 36 mW). The pinhole size was set to 40.87 μm in

668 diameter, which yields a 0.31 μm optical slice in this condition. We calculate the density of

669 punctate signals from the obtained images with manual adjustments of brightness and contrast.

670 For counting the punctate signals, we manually adjust contrast and/or brightness so that we

671 can distinguish each punctate signal in the same image possessing different signal intensity of

672 the cross wall.

673

674 **Viability test against salt stress**

675 Three- to five-day-old protonemal tissues were inoculated into 200 μL of BCDATG with

676 0.5% gellan gum, in glass-based dishes with 27-mm-diameter chambers (no. 3960-035;

677 Iwaki) and then covered with additional 300 μl of the medium. In addition, 8.5 mL of liquid

678 BCDATG medium was poured on the culture medium to avoid drying. The samples were

679 cultured for 10 days under continuous light with the glass side up.

680 The liquid medium was exchanged with 8.5 mL of liquid BCDATG medium containing

681 ABA to final concentration 50 μM. The samples were incubated for 20 and 100 min. These

682 steps were skipped in the case without ABA treatment. To give salt stress to protonemal

683 tissues, the liquid medium was exchanged with 8.5 mL of liquid BCDATG medium

684 containing NaCl to a final concentration of 600 mM for 60 min. To assess cell viability, the

685 liquid medium containing NaCl in the samples were exchanged with 3 mL of 50 μg/mL

propidium iodide (PI) and incubated for 5 min, and then washed out with 8.5 mL of liquid BCDATG medium for 5 min. In the control, the samples were directly subjected to the PI staining without salt stress treatment.

Under a stereomicroscope (M165 FC, Leica), we counted the number of dead cells among 14 cells from the apices in each protonema as judged with the PI signal in nuclei.

Supplementary data

Supplemental Figure S1. Expression level of *PpSnRK2* and *PpABI3* in protonemal tissues.

Supplemental Figure S2. Measurement of the Dendra2 degradation rate.

Supplemental Figure S3. Change in fluorescence intensity of Dendra2 in the neighboring cells of the photoconverted cells.

Supplemental Figure S4. Callose signal on newly formed cross walls and intercellular Dendra2 diffusivity under the condition of callose staining with aniline blue.

Supplemental Figure S5. Live-cell imaging of callose staining in *PpABI3AiOX/ProEF1α:D2*.

Supplemental Figure S6. Expression of *PpABI1A^{G333D}* in protonemal tissue of *PpABI1A^{G333D}OX/ProEF1α:D2*.

Supplemental Table S1. Primer sequences in qPCR.

715

Figure legends

Figure 1. Morphology and growth of protonemal cells in transgenic lines overexpressing
$PpABI1A^{G333D}$**,** $PpSnRK2A$**, or** $PpABI3A$**.**

A and B, Representative images of protonemal cells at apical and basal positions after DMSO
or ABA treatment in $ProEF1\alpha:D2$ (A) and $PpABI1A^{G333D}OX/ProEF1\alpha:D2$ (B). C to E,
Representative images of protonemal cells at apical and basal positions after DMSO or
β-estradiol treatment in $ProEF1\alpha:D2$ (C), $PpSnRK2AiOX/ProEF1\alpha:D2$ (D), and
$PpABI3AiOX/ProEF1\alpha:D2$ (E). Times after treatment are indicated. The 12th cells were
observed as the basal cells. Scale bars = 50 μm. Arrowheads indicate cross walls of the
original branches.

Figure 2. Dendra2 movement between protonemal cells in transgenic lines
overexpressing $PpABI1A^{G333D}$**,** $PpSnRK2A$**, or** $PpABI3A$**.**

A and B, Representative differential interference contrast (DIC) and photoconverted Dendra2
fluorescence (gray) images in protonemal cells of $ProEF1\alpha:D2$ (A) and
$PpABI1A^{G333D}OX/ProEF1\alpha:D2$ (B) treated with DMSO or ABA. C to E, Representative DIC
and photoconverted Dendra2 fluorescence (gray) images in protonemal cells of $ProEF1\alpha:D2$
(C), $PpSnRK2AiOX/ProEF1\alpha:D2$ (D), and $PpABI3AiOX/ProEF1\alpha:D2$ (E) treated with
DMSO or β-estradiol. Times after photoconversion are indicated. Scale bars = 100 μm.

Figure 3. Quantitative characterization of Dendra2 movement between protonemal cells
and its suppression by ABA.

A, Representative DIC, non-photoconverted (green), and photoconverted (gray) Dendra2
fluorescence images in protonemal cells of $ProEF1\alpha:D2$ treated with DMSO or ABA. Times
after photoconversion are indicated. Scale bars = 100 μm. B, Mean fluorescence intensity of
Dendra2 at 20-min intervals after photoconversion in photoconverted protonemal cells of
$ProEF1\alpha:D2$ treated with DMSO ($n = 15$) or ABA ($n = 27$). Standard deviation (SD) is
indicated by shaded area. The time constant (τ) and immobile fraction (B) were determined by

744   fitting the exponential function to the kinetics of mean fluorescence intensity (black solid

745   lines). RSS, residual sum of squares. C, Schematic diagram of kinetics obtained by using

746   different combinations of fitting parameters. Intercellular diffusivity of macromolecules is

747   determined by τ and B. Highly permeable PD allow Dendra2 to move more rapidly from the

748   original cell (black) than lower permeable PD (blue). More Dendra2 is trapped in the original

749   cell when immobile fraction is increased (red).

750

751   **Figure 4. Intercellular Dendra2 diffusivity in protonemal cells of**

752   ***ProEF1α:D2/ppsnrk2qko* and *ProEF1α:D2/ppabi3tko*.**

753   A and C, Representative DIC and photoconverted Dendra2 fluorescence (gray) images in

754   protonemal cells of *ProEF1α:D2/ppsnrk2qko* (A) and *ProEF1α:D2/ppabi3tko* (C) treated

755   with DMSO or ABA. Times after photoconversion are indicated. Scale bars = 100 μm. B and

756   D, Mean fluorescence intensity of Dendra2 at 20-min intervals after photoconversion in

757   protonemal cells of *ProEF1α:D2/ppsnrk2qko* (B) and *ProEF1α:D2/ppabi3tko* (D) treated

758   with DMSO or ABA with exponential fits to the mean data. SD is indicated by the shaded

759   area. The τ and B were determined by fitting the exponential function to the kinetics of mean

760   fluorescence intensity (black solid lines). E to H, Values of the time constant τ and B obtained

761   through the fittings. The τ and B in *ProEF1α:D2* and *ProEF1α:D2/ppsnrk2qko* (E and F), and

762   in *ProEF1α:D2* and *ProEF1α:D2/ppabi3tko* (G and H). Each violin plot shows the density

763   distribution of the data by the box plot (median as a yellow horizontal line, interquartile range

764   as a box, and data range as whiskers). The P value was determined by the Mann-Whitney

765   *U*-test. *ProEF1α:D2* with DMSO ($n = 17$) and ABA ($n = 24$) (E and F), and

766   *ProEF1α:D2/ppsnrk2qko* with DMSO ($n = 13$) and ABA ($n = 22$) (B, E and F). *ProEF1α:D2*

767   with DMSO ($n = 15$) and ABA ($n = 27$) (G and H), and *ProEF1α:D2/ppabi3tko* with DMSO

768   ($n = 14$) and ABA ($n = 17$) (D, G and H). I, Time to initiation of ASD calculated from the

769   median of fitting parameters in *ProEF1α:D2* and *ProEF1α:D2/ppabi3tko*. The grey line

770   indicates A_{90}.

771

772   **Figure 5. Live-cell imaging of aniline blue staining for callose after ABA treatment.**

773   A to C, Representative images of DIC and aniline blue fluorescence for callose staining in

774   protonemal cells of *ProEF1α:D2* (A), *ProEF1α:D2/ppsnrk2qko* (B), and

775   *ProEF1α:D2/ppabi3tko* (C) after DMSO or ABA treatment. Times after treatment are

776   indicated. The callose signal is shown in Fire look-up table of ImageJ. Scale bars = 20 μm. D,

777   Change in aniline blue fluorescence intensity on cross walls in protonemal cells of

778   *ProEF1α:D2*, *ProEF1α:D2/ppsnrk2qko*, and *ProEF1α:D2/ppabi3tko* after DMSO or ABA

779   treatment. *ProEF1α:D2* with DMSO ($n = 12$) and ABA ($n = 12$), *ProEF1α:D2/ppsnrk2qko*

780   with DMSO ($n = 12$) and ABA ($n = 8$), and *ProEF1α:D2/ppabi3tko* with DMSO ($n = 10$) and

781   ABA ($n = 12$). The *P* value was determined by the Welch's *t*-test, n.s., non-significance ($P \geq$

782   0.05). E to G, Representative images of DIC and punctate callose signal of aniline blue

783   fluorescence at cross walls between the 11th and 12th cell of *ProEF1α:D2* (E),

784   *ProEF1α:D2/ppsnrk2qko* (F), and *ProEF1α:D2/ppabi3tko* (G) protonemal cells after ABA

785   treatment. Times after treatment are indicated. Scale bars = 5 μm. Arrowheads indicate

786   punctate signals included in the number as an example (E). H, The density of punctate signals

787   on cross walls between the 11th and 12th cell in *ProEF1α:D2*, *ProEF1α:D2/ppsnrk2qko*, and

788   *ProEF1α:D2/ppabi3tko* after ABA treatment. Each violin plot shows the density distribution

789   of the data by the box plot (median as a cyan horizontal line, interquartile range as a box, and

790   data range as whiskers). *ProEF1α:D2* without and after 20-, 100- and 180-min ABA treatment

791   ($n = 19, 17, 18$ and 30, respectively), *ProEF1α:D2/ppsnrk2qko* without and after 20-, 100-

792   and 180-min ABA treatment ($n = 18, 8, 9$ and 9, respectively), and *ProEF1α:D2/ppabi3tko*

793   without and after 20-, 100- and 180-min ABA treatment ($n = 10, 18, 19$ and 18, respectively).

794   The *P* value was determined by the Mann-Whitney *U*-test, n.s., non-significance $P \geq 0.05$).

795

796   **Figure 6. Viability under salt stress after different pretreatment time of ABA.**

797   A, Representative bright field, PI and non-photoconverted Dendra2 fluorescence images after

798   the salt stress treatment. Cells with PI signal indicate dead cells. B, Survival rate after the salt

799   stress with ABA pretreatment. "w/o NaCl" indicates the condition without salt stress and ABA

800   treatment. The bar graph shows mean ± SD of the survival rate ($n = 75$). The *P* value was

801   determined by the Mann-Whitney *U*-test, n.s., non-significance ($P \geq 0.05$), *$P < 0.01$

802   compared to the non-ABA pretreated corresponding line.

803

804

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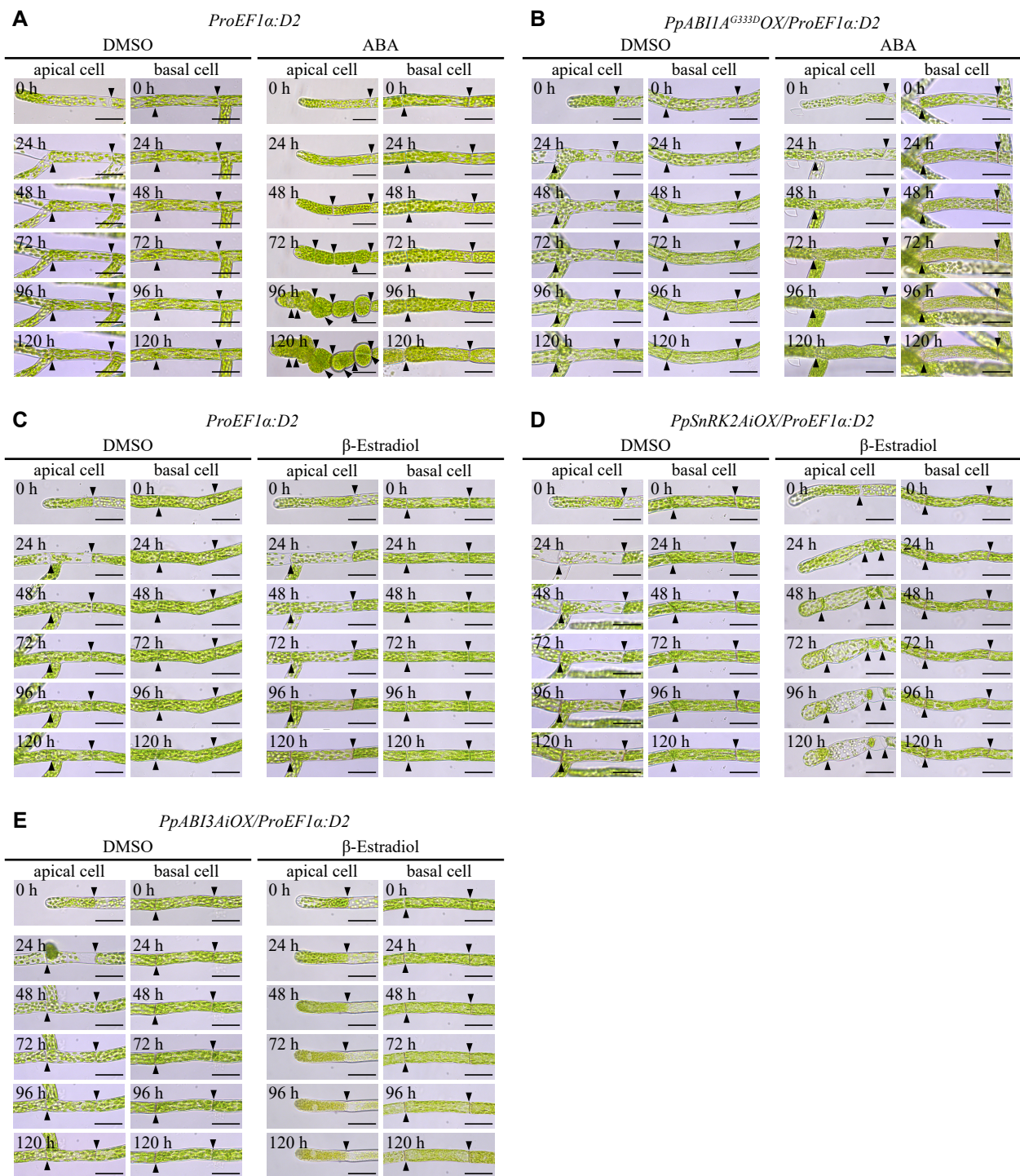
Figure 1. Morphology and growth of protonemal cells in transgenic lines overexpressing *PpABI1A^{G333D}*, *PpSnRK2A*, or *PpABI3A*.

A and B, Representative images of protonemal cells at apical and basal positions after DMSO or ABA treatment in *ProEF1α:D2* (A) and *PpABI1A^{G333D}OX/ProEF1α:D2* (B). C to E, Representative images of protonemal cells at apical and basal positions after DMSO or β-estradiol treatment in *ProEF1α:D2* (C), *PpSnRK2AiOX/ProEF1α:D2* (D), and *PpABI3AiOX/ProEF1α:D2* (E). Times after treatment are indicated. The 12th cells were observed as the basal cells. Scale bars = 50 μm. Arrowheads indicate cross walls of the original branches.
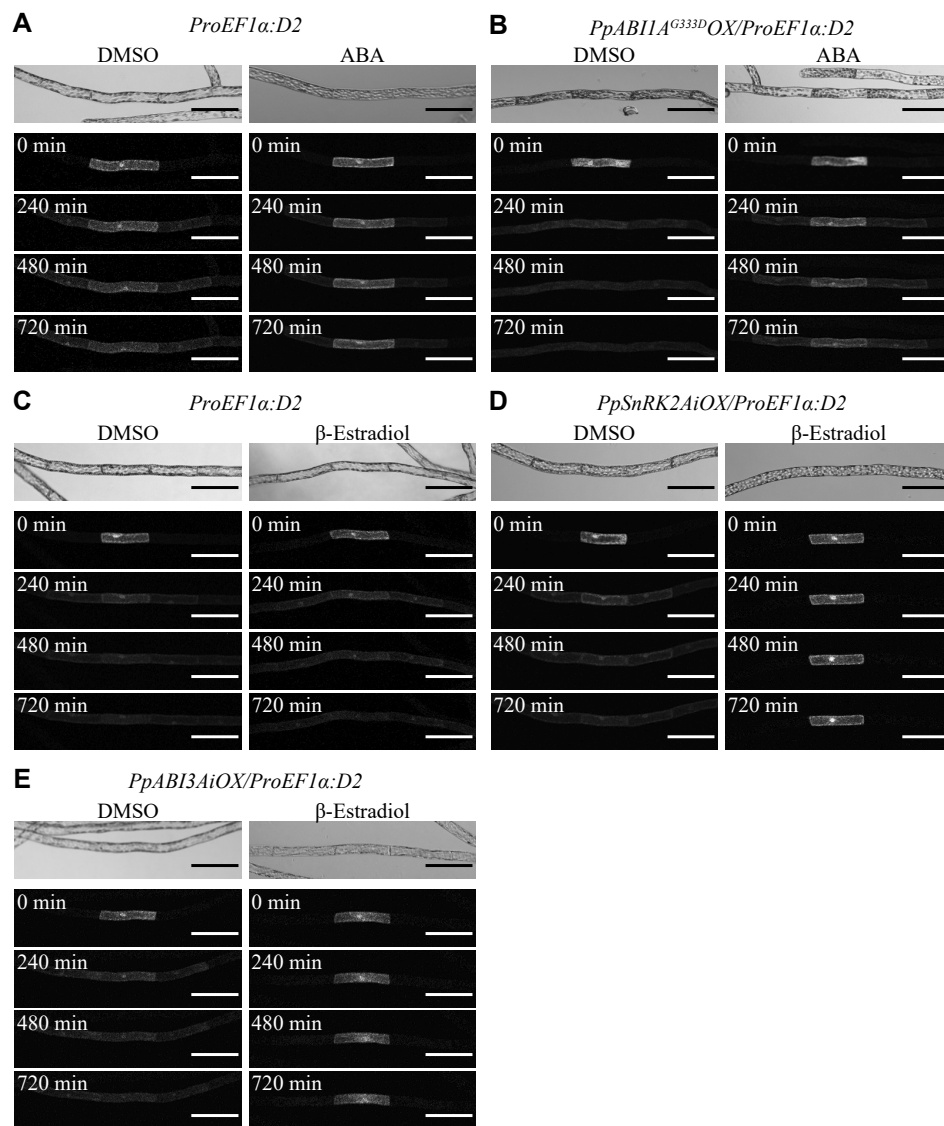
Figure 2. Dendra2 movement between protonemal cells in transgenic lines overexpressing *PpABI1A^{G333D}*, *PpSnRK2A*, or *PpABI3A*.

A and B, Representative differential interference contrast (DIC) and photoconverted Dendra2 fluorescence (gray) images in protonemal cells of *ProEF1α:D2* (A) and *PpABI1A^{G333D}OX/ProEF1α:D2* (B) treated with DMSO o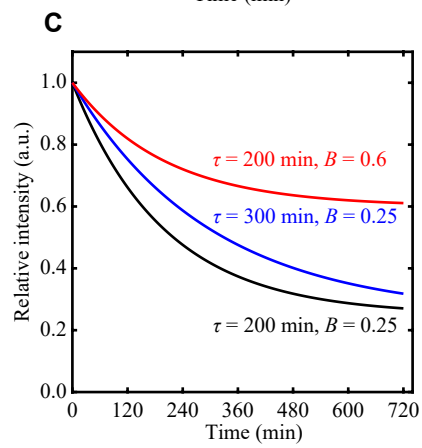r ABA. C to E, Representative DIC and photoconverted Dendra2 fluorescence (gray) images in protonemal cells of *ProEF1α:D2* (C), *PpSnRK2AiOX/ProEF1α:D2* (D), and *PpABI3AiOX/ProEF1α:D2* (E) treated with DMSO or β-estradiol. Times after photoconversion are indicated. Scale bars = 100 μm.

A

ProEF1α:D2

DMSO   ABA

before   before

0 min   0 min

before   before

0 min   0 min

240 min   240 min

480 min   480 min

720 min   720 min

B

ProEF1α:D2

τ = 17 min, B = 0.83
RSS = 0.0018

τ = 250 min, B = 0.26
RSS = 0.0010

- DMSO
- ABA

Normalized intensity (a.u.)

Time (min)

C

τ = 200 min, B = 0.6

τ = 300 min, B = 0.25

τ = 200 min, B = 0.25

Relative intensity (a.u.)

Time (min)

Figure 3. Quantitative characterization of Dendra2 movement between protonemal cells and its suppression by ABA.

A, Representative DIC, non-photoconverted (green), and photoconverted (gray) Dendra2 fluorescence images in protonemal cells of *ProEF1α:D2* treated with DMSO or ABA. Times after photoconversion are indicated. Scale bars = 100 μm. B, Mean fluorescence intensity of Dendra2 at 20-min intervals after photoconversion in photoconverted protonemal cells of *ProEF1α:D2* treated with DMSO (n = 15) or ABA (n = 27). Standard deviation (SD) is indicated by shaded area. The time constant (τ) and immobile fraction (B) were determined by fitting the exponential function to the kinetics of mean fluorescence intensity (black solid lines). RSS, residual sum of squares. C, Schematic diagram of kinetics obtained by using different combinations of fitting parameters. Intercellular diffusivity of macromolecules is determined by τ and B. Highly permeable PD allow Dendra2 to move more rapidly from the original cell (black) than lower permeable PD (blue). More Dendra2 is trapped in the original cell when immobile fraction is increased (red).

A *ProEF1α:D2/ppsnrk2qko*

DMSO | ABA

0 min | 0 min
240 min | 240 min
480 min | 480 min
720 min | 720 min

C *ProEF1α:D2/ppabi3tko*

DMSO | ABA

0 min | 0 min
240 min | 240 min
480 min | 480 min
720 min | 720 min

B *ProEF1α:D2/ppsnrk2qko*

τ = 185 min, B = 0.23
RSS (ABA) = 0.0034

τ = 179 min, B = 0.24
RSS (DMSO) = 0.0068

D *ProEF1α:D2/ppabi3tko*

τ = 42 min, B = 0.70
RSS = 0.0027

τ = 201 min, B = 0.25
RSS = 0.0008

E

DMSO | ABA

ProEF1α:D2
ProEF1α:D2/ppsnrk2qko

P = 6.8 ×10⁻¹ | P = 5.4 ×10⁻⁸

G

DMSO | ABA

ProEF1α:D2
ProEF1α:D2/ppabi3tko

P = 5.8 ×10⁻² | P = 1.9 ×10⁻²

F

DMSO | ABA

ProEF1α:D2
ProEF1α:D2/ppsnrk2qko

P = 4.2 ×10⁻¹ | P = 4.7 ×10⁻⁸

H

DMSO | ABA

ProEF1α:D2
ProEF1α:D2/ppabi3tko

P = 9.8 ×10⁻¹ | P = 2.6 ×10⁻³

I

ProEF1α:D2
ProEF1α:D2/ppabi3tko

105 min

24 min

**Figure 4. Intercellular Dendra2 diffusivity in protonemal cells of *ProEF1α:D2/ppsnrk2qko* and *ProEF1α:D2/ppabi3tko*.**

A and C, Representative DIC and photoconverted Dendra2 fluorescence (gray) images in protonemal cells of *ProEF1α:D2/ppsnrk2qko* (A) and *ProEF1α:D2/ppabi3tko* (C) treated with DMSO or ABA. Times after photoconversion are indicated. Scale bars = 100 μm. B and D, Mean fluorescence intensity of Dendra2 at 20-min intervals after photoconversion in protonemal cells of *ProEF1α:D2/ppsnrk2qko* (B) and *ProEF1α:D2/ppabi3tko* (D) treated with DMSO or ABA with exponential fits to the mean data. SD is indicated by the shaded area. The τ and B were determined by fitting the exponential function to the kinetics of mean fluorescence intensity (black solid lines). E to H, Values of the time constant τ and B obtained through the fittings. The τ and B in *ProEF1α:D2* and *ProEF1α:D2/ppsnrk2qko* (E and F), and in *ProEF1α:D2* and *ProEF1α:D2/ppabi3tko* (G and H). Each violin plot shows the density distribution of the data by the box plot (median as a yellow horizontal line, interquartile range as a box, and data range as whiskers). The P value was determined by the Mann-Whitney U-test. *ProEF1α:D2* with DMSO ($n = 17$) and ABA ($n = 24$) (E and F), and *ProEF1α:D2/ppsnrk2qko* with DMSO ($n = 13$) and ABA ($n = 22$) (B, E and F). *ProEF1α:D2* with DMSO ($n = 15$) and ABA ($n = 27$) (G and H), and *ProEF1α:D2/ppabi3tko* with DMSO ($n = 14$) and ABA ($n = 17$) (D, G and H). I, Time to initiation of ASD calculated from the median of fitting parameters in *ProEF1α:D2* and *ProEF1α:D2/ppabi3tko*. The grey line indicates A_{90}.

A *ProEF1α:D2*

DMSO / ABA
0 min, 20 min, 100 min, 180 min

B *ProEF1α:D2/ppsnrk2qko*

DMSO / ABA
0 min, 20 min, 100 min, 180 min

C *ProEF1α:D2/ppabi3tko*

DMSO / ABA
0 min, 20 min, 100 min, 180 min

D

n.s. n.s. n.s.

Normalized intensity (a.u.)

- ○ *ProEF1α:D2* (DMSO)
- ● *ProEF1α:D2* (ABA)
- △ *ProEF1α:D2/ppsnrk2qko* (DMSO)
- ▲ *ProEF1α:D2/ppsnrk2qko* (ABA)
- □ *ProEF1α:D2/ppabi3tko* (DMSO)
- ■ *ProEF1α:D2/ppabi3tko* (ABA)

Time after treatment (min)

E *ProEF1α:D2*   **F** *ProEF1α:D2/ppsnrk2qko*   **G** *ProEF1α:D2/ppabi3tko*

w/o
20 min
100 min
180 min

H

Density of punctate signals on septa (/μm)

n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s.

n.s.
n.s.
n.s.

w/o   20 min   100 min   180 min

ProEF1α:D2
ProEF1α:D2/ppsnrk2qko
ProEF1α:D2/ppabi3tko

Figure 5. Live-cell imaging of aniline blue staining for callose after ABA treatment.

A ~~and~~to ~~B~~C, Representative images of DIC and aniline blue fluorescence for callose staining in protonemal cells of *ProEF1α:D2* (A), *ProEF1α:D2/ppsnrk2qko* (B), and *ProEF1α:D2/ppabi3tko* (~~B~~C) after DMSO or ABA treatment. Times after treatment are indicated. The callose signal is shown in Fire look-up table of ImageJ. Scale bars = 20 μm. ~~C~~D, Change in aniline blue fluorescence intensity on cross walls in protonemal cells of *ProEF1α:D2*, *ProEF1α:D2/ppsnrk2qko*, and *ProEF1α:D2/ppabi3tko* after DMSO or ABA treatment. *ProEF1α:D2* with DMSO (n = 12) and ABA (n = 12), *ProEF1α:D2/ppsnrk2qko* with DMSO (n = 12) and ABA (n = 8), and *ProEF1α:D2/ppabi3tko* with DMSO (n = 10) and ABA (n = 12). The P value was determined by the Welch's t-test, n.s., non-significance (P ~~>~~$\geq$ 0.05). ~~D and E~~E to G, Representative images of DIC and punctate callose signal of aniline blue fluorescence at cross walls between the 11th and 12th cell of *ProEF1α:D2* (~~D~~E), *ProEF1α:D2/ppsnrk2qko* (F), and *ProEF1α:D2/ppabi3tko* (G) protonemal cells (~~E~~) after ABA treatment. Times after treatment are indicated. Scale bars = 5 μm. Arrowheads indicate punctate signals included in the number as an example (~~D~~E). ~~F~~H, The ~~number~~density of punctate signals on cross walls between the 11th and 12th cell in *ProEF1α:D2*, *ProEF1α:D2/ppsnrk2qko*, and *ProEF1α:D2/ppabi3tko* after ABA treatment. Each violin plot shows the density distribution of the data by the box plot (median as a ~~yellow~~cyan horizontal line, interquartile range as a box, and data range as whiskers). *ProEF1α:D2* without and after 20-, 100- and 180-min ABA treatment (n = 19, 17, 18 and 30, respectively), *ProEF1α:D2/ppsnrk2qko* without and after 20-, 100- and 180-min ABA treatment (n = 18, 8, 9 and 9, respectively), and *ProEF1α:D2/ppabi3tko* without and after 20-, 100- and 180-min ABA treatment (n = 10, 18, 19 and 18, respectively). The P value was determined by the Mann-Whitney U-test, n.s., non-significance P ~~>~~$\geq$ 0.05).
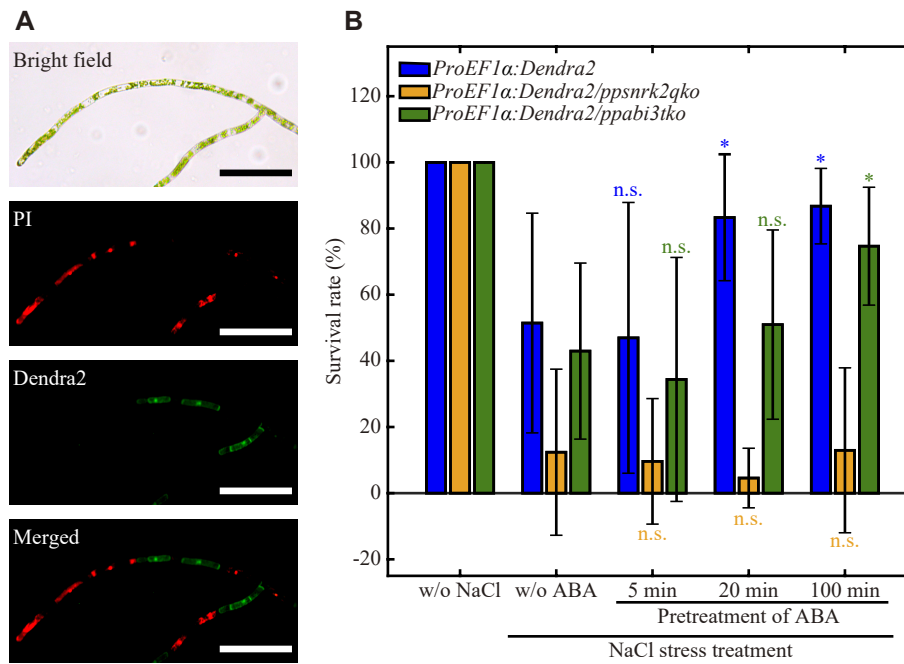
Figure 6. Viability under salt stress after different pretreatment time of ABA.

A, Representative bright field, PI and non-photoconverted Dendra2 fluorescence images after the salt stress treatment. Cells with PI signal indicate dead cells. B, Survival rate after the salt stress with ABA pretreatment. "w/o NaCl" indicates the condition without salt stress and ABA treatment. The bar graph shows mean ± SD of the survival rate (n = 75). The P value was determined by the Mann-Whitney U-test, n.s., non-significance ($P \geq 0.05$), *$P < 0.01$ compared to the non-ABA pretreated corresponding line.