Hypoxia-Induced Ethanol Production in Arabidopsis Root Apex Cells Affects Endocytic Vesicle Recycling and F-Actin Organisation

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Abstract

Ethanol (EtOH) is a short-chain alcohol that is abundant in nature. EtOH is endogenously produced by plants under hypoxic conditions, and exogenously applied EtOH improves plant stress tolerance at low concentrations (<1%). However, no direct observations have shown how EtOH affects cellular events in plants. In intact *Arabidopsis* roots, 0.1% EtOH promoted reactive oxygen species production in root apex cells. EtOH also accelerated exocytic vesicle recycling and altered F-actin organisation, both of which are closely related to cell membrane properties. In addition to exogenous EtOH application, hypoxic treatment resulted in EtOH production in roots and degradation of the cross-wall actin cytoskeleton in root epidermal cells. We conclude that hypoxia-induced EtOH production affects endocytic vesicle recycling and associated signalling pathways.

25 Introduction

26 Ethanol (EtOH) is a small volatile molecule that is abundant in nature. EtOH is
 27 produced through multiple chemical and biochemical reactions and in particular during
 28 yeast-mediated fermentation. Extensive consumption of EtOH produces a drunken state
 29 in animals and humans due to the molecular interactions with certain components of the
 30 nervous system. Plants are also susceptible to the effects of EtOH. High concentrations
 31 of EtOH can damage plants; however, low concentrations have been shown to moderate
 32 plant stress responses. For example, incubating cucumber seedlings in EtOH vapour
 33 (<3%) induced chilling tolerance [1]. Similarly, the application of 0.3% ethanol
 34 enhanced the salinity tolerance of Arabidopsis and rice roots [2,3]. Although EtOH
 35 treatments also promoted the expression of heat shock proteins in soybean, no
 36 improvements in heat tolerance were observed [4].

37 Some plants can also produce EtOH under hypoxic and anoxic conditions.
 38 During this process, pyruvate is converted into acetaldehyde by pyruvate decarboxylase,
 39 then acetaldehyde is converted into EtOH by alcohol dehydrogenase (ADH) [5-10].
 40 This reversible enzymatic reaction has been observed in yeast, bacteria,
 41 *Chlamydomonas* [11] and plants [12] under low oxygen conditions. Arabidopsis roots
 42 constitutively express the *ADH* gene, and Chung *et al.* [13] demonstrated that ADH is
 43 strongly upregulated when roots are exposed to low oxygen conditions upon penetrating
 44 solid-agar media. Hypoxic and anoxic conditions are sensed by roots and subsequently
 45 induce ADH expression in shoots, which suggests that EtOH plays a role in
 46 communication between shoots and roots [13]. Furthermore, low temperatures induce
 47 ADH expression and EtOH biogenesis in shoots and roots of rice seedlings, and EtOH
 48 treatments improve the cold tolerance of rice seedlings [14]. Together these
 49 observations suggest that in plants, endogenous EtOH is a stress-induced molecule that
 50 facilitates physiological adaptations to changes in environmental conditions.

51 Although numerous studies have investigated the role of EtOH in plants, the
 52 initial events and cellular components that mediate plant responses to EtOH remain
 53 poorly understood. In previous studies, EtOH was reported to fluidise *Escherichia coli*
 54 plasma membranes [15]; molecular dynamics simulations showed that EtOH interacts
 55 with membrane lipids [16]. The plasma membrane is a highly active and dynamic

structure in all organisms, and the elaborate control of membrane homeostasis is crucial to many cellular events. In human astrocytes, 0.15% EtOH treatments disrupted the actin cytoskeleton structures [17]. Thus, it is accepted that even small membrane disturbances can have substantial impacts on membrane proteins, ion channels and endo/exocytic vesicle recycling [18]. To our knowledge, the present study is the first to have monitored the membrane-associated effects of EtOH in intact Arabidopsis root cells. Under hypoxic conditions, endogenous EtOH production was observed in the root tissue. We used confocal microscopy to demonstrate that EtOH inhibits Arabidopsis root growth and interrupts vesicle recycling. In addition, we observed reactive oxygen species (ROS) accumulation in the root apex regions and perturbations of F-actin polarisation under both EtOH and hypoxic treatments.

Results and Discussion

Ethanol production under hypoxic conditions in Arabidopsis roots

Many plants produce considerable quantities of ethanol under anoxic and hypoxic growth conditions [5-7,9,10]. For example, Kato-Noguchi (2000) demonstrated that EtOH levels in lettuce roots increased following hypoxic treatments [8]. Here we first used spectrophotometry to determine whether Arabidopsis roots produce EtOH under low oxygen conditions. As shown in Fig. 1, the endogenous EtOH concentrations were significantly increased after incubation in anoxic conditions (nitrogen gas) for 24 h. The range of EtOH concentrations detected here ($\mu\text{mol g}^{-1}$ FW order) was consistent with a previous report [10]. EtOH production was also observed in our untreated controls, presumably due to the penetration of horizontally-incubated roots into the watery solid medium, as described previously [13]. In a recent study, Eysholdt-Derzsó and Sauter revealed that hypoxic conditions activate the group VII ethylene response factor (ERFVII), resulting in slanted root growth, possibly as a mechanism to avoid low oxygen conditions [19]. Changes in auxin distribution and signalling in root tips have also been reported under hypoxic conditions [19]. Next, we monitored changes in the root growth and gravitropism response following exogenous application of EtOH.

Inhibition of root growth by EtOH

We monitored *Arabidopsis* root growth in $0.5 \times$ Murashige-Skoog (MS) medium containing 0, 0.01%, 0.1% and 1% EtOH. The experimental EtOH concentrations did not affect seed germination (data not shown). However, as shown in Fig. 2A, 0.01%, 0.1% and 1% EtOH significantly inhibited root growth; the inhibition effect was strongest in the 1% EtOH treatment. Arcara and Ronchi [20] reported that treating onion (*Allium cepa* L.) with 0.4% EtOH delayed the mitotic cycle, but did not completely stop mitosis. The root growth inhibition observed here is likely the result of a similar response to EtOH in *Arabidopsis*. In contrast, root gravitropism was not affected by EtOH, even after 24 h in 1% EtOH (Fig. 2B). The DR5 auxin reporter expression was not affected, and auxin redistribution after 6 h gravistimulation in the presence of 0.1% or 1% EtOH was not significantly different from the control (Fig. 2C). Thus, EtOH at the concentrations tested did not affect the events required for graviresponse such as auxin redistribution.

EtOH induces ROS accumulation in root apex

ROS in root apex cells play a crucial role in root development and growth [21]. The accumulation of ROS has been observed in EtOH-treated yeast [22]. In hepatocytes, ROS production was induced by EtOH concentrations as low as 1 mM (~0.006%) [23]. To determine whether ROS homeostasis is altered in plant roots in the presence of EtOH, we visualised superoxide localisation in *Arabidopsis* root apices using nitroblue tetrazolium salt (NBT) staining (Fig. 3). Superoxide production was promoted by 0.1% and 1% EtOH, as demonstrated by the accumulation of superoxide in root apex regions. According to a previous report [21], the balance of superoxide and hydrogen peroxide is strictly regulated in these regions. Moreover, hydrogen peroxide promotes cell elongation, whereas superoxide promotes cell proliferation. Hence, superoxide accumulation under EtOH treatments is consistent with the inhibition of root growth but not with root responses to gravity. Nguyen *et al.* [2] recently showed that plant salt tolerance is improved by treatments with exogenous EtOH, reflecting the induction of ROS detoxifying enzymes such as peroxidases. Here we analysed the gene expression of transcription factor ZAT10, which provides an ROS signalling-dependent stress response [24]. After treatment with 1% EtOH, the ZAT10 expression level was about four-fold higher in the control. Hence, EtOH-mediated ROS production may regulate the transcription of genes, resulting in the regulation of antioxidant enzymes. However,

119 further studies are required to determine the mechanisms and sources of ROS
120 production.

121

122 **EtOH unbalances between endocytic and exocytic vesicle recycling pathways**

123 Because EtOH directly interacts with membrane structures, we assessed endocytic
124 vesicle recycling in the presence of EtOH in root epidermal cells. We used brefeldin A
125 (BFA) to monitor membrane recycling in Arabidopsis. BFA prevents the formation of
126 exocytic vesicles but not endocytic vesicles [25]; in particular, the area of BFA
127 compartments is indicative of the rate of membrane recycling through the endocytic
128 pathway. As shown in Figs 4A and B, treatments with 0.1% EtOH did not affect the
129 BFA-induced compartment area. However, 30 min of BFA treatment after 1% EtOH
130 pre-treatment inhibited the endocytic vesicular pathways that reflect small BFA-induced
131 compartments in Arabidopsis seedlings (Figs 4A and B). BFA does not modify
132 dopamine receptor localisation, indicating that endosomal recycling is accomplished
133 through BFA-independent pathways [26]. By washing the BFA from the EtOH-treated
134 roots, the retarded exocytic pathway traffic is released again and the speed of exocytosis
135 can be monitored. Our BFA washing-out experiments revealed that 0.1% or 1% EtOH
136 treatments sped up the outward exocytic pathway 30 min after BFA removal (Figs 4C
137 and D). Taken together, these data suggest that EtOH modifies BFA-sensitive signalling
138 pathways in plants. Inward endocytic pathway was inhibited and outward exocytic one
139 was accelerated. Hypoxia-induced root slanting occurs via the alteration of auxin
140 distribution [19]; however, our data show that exogenous EtOH does not affect DR5
141 expression and root gravitropic responses (Figs 2B and C). These findings imply that the
142 acceleration of exocytic vesicle recycling does not affect gravitropic responses and
143 auxin distribution for up to 24 h of EtOH treatment.

144

145 **Dispersion of F-actin bundles in root apex region cross-walls**

146 F-actin acts as a scaffold system for moving vesicles in the root transition zone [25].
147 The influence of EtOH on F-actin localisation in root epidermal cells was observed in
148 GFP-FABD2 transgenic *Arabidopsis* plants [27]. As shown in Fig. 5A, F-actin
149 alignment at the cross-wall (white arrow) disappeared in the presence of 0.1% and 1%
150 EtOH. The effects of EtOH on F-actin have been reported in other organisms. For

151 example, F-actin in budding yeast was dispersed following induction by 6% EtOH [28].
 152 EtOH treatments at 0.15% concentration clearly disrupted actin cytoskeleton structures
 153 in astrocytes [17]. In mouse cells, the actin cytoskeleton acts as both the target and
 154 effector of the EtOH effects on synapses, as indicated by disturbances in N-methyl-D-
 155 aspartate receptor signalling [29-31]. Furthermore, abundant F-actin and active
 156 endocytic vesicle recycling were previously observed in root apex transition regions
 157 [32-34]. Thus, the depletion of cross-wall localised F-actin in EtOH-treated root apex
 158 cells may disturb vesicle recycling, root growth and polarity, as well as influencing the
 159 general cell physiology.

160

161 **Depletion and reorganisation of F-actin under hypoxia**

162 To clarify whether hypoxic conditions change F-actin organisation without exogenous
 163 EtOH application, we monitored GFP-FADB2 root transition zone cells under hypoxia
 164 for 1, 3, 24 h. F-actin depletion was observed after 1-h hypoxic treatment and the signal
 165 disappeared from the cross-walls in the epidermal cells (Fig. 6A). The timing of GFP
 166 signal loss from the cross-walls was the same under the 1-h hypoxic and 0.1% EtOH
 167 treatments (Fig. 5A). This finding suggests that endogenous EtOH production under
 168 hypoxia may be initiated as soon as roots are exposed to low oxygen conditions. The
 169 accumulated F-actin bundle moved to the tonoplast surface under hypoxic conditions
 170 (Fig. 6B). Importantly, F-actin recovery following the 24-h hypoxia treatment was
 171 observed after just 1 h (Fig. 6C), suggesting that hypoxia-induced F-actin disruption
 172 may be mediated by endogenous EtOH production. Interestingly, the root apex cells in
 173 the transition zone show the most rapid and extensive F-actin redistribution [35]. This
 174 zone also contains cells with the highest demands for oxygen uptake [36,37], rates of
 175 endocytic vesicle recycling and auxin fluxes [39-40].

176

177 **Conclusions**

178 We conclude that EtOH produced under hypoxic conditions directly affects the physical
 179 properties of plasma membranes, contributing to ROS homeostasis, vesicle recycling
 180 and F-actin organisation. These EtOH-responsive processes help roots to rapidly and
 181 effectively adapt to hypoxic environments. The molecular details and spatio-temporal
 182 relationships between EtOH-modified membranes, endocytic vesicle recycling and F-actin

183 organisation will be investigated in future studies.

184

185 **Materials and Methods**

186 **Plant growth conditions and exogenous EtOH treatments**

187 *Arabidopsis thaliana* seeds were sterilised in 2% NaClO containing 0.1% Triton X-100
 188 for five minutes. Seeds were then washed with distilled water four times and planted in
 189 Petri dishes on 0.5 × MS basal salt medium containing 1% (w/v) sucrose (pH 5.8 with
 190 KOH) solidified with 0.4% (w/v) phytagel containing 0, 0.01, 0.1 or 1% EtOH (VWR
 191 chemicals, France). Approximately 20 seedling were placed on EtOH-containing plates
 192 of each concentration and three times biological replicates were conducted. Ecotype
 193 Col-0 was used for the wild type, the 35S: GFP-FABD2 transgenic line was used for F-
 194 actin visualisation [27] and DR5::GUS was used for visualising the localisation of auxin
 195 during gravitropism. The MS medium was autoclaved and sufficiently cooled before
 196 adding the EtOH to prevent evaporation. The Petri dishes were incubated at 4°C for one
 197 day, then incubated vertically at 23°C–25°C under a 16-h light/8-h dark cycle. Light
 198 was provided at an intensity of 120 μmol/m²/s. Three days after germination, images of
 199 roots were taken using an EOS Kiss X7 camera (Canon, Tokyo) and root lengths were
 200 measured using ImageJ software (ver. 1.43u for Mac OSX, <http://imagej.nih.gov/ij/>).
 201 The three-day-old seedlings were transferred to EtOH containing 0.5 × MS solid media
 202 (0%, 0.1% or 1%) and rotated 90 degrees to observe root gravitropism. The seedlings
 203 were then incubated in the dark for 24 h. Images to assess root curvature were captured
 204 and measured as described above.

205

206 **Hypoxic treatments and endogenous EtOH production measurement**

207 For the hypoxia treatments, seedlings growing on 0.5 × MS solid medium in Petri
 208 dishes (without lids) were placed in a gas-tight chamber five days after germination. To
 209 expel oxygen, pure nitrogen gas was infused into the closed chamber through small
 210 holes for 5 min. The holes were then tightly sealed and the plates in the chamber were
 211 incubated horizontally at 23°C–25°C for 24 h in the dark to prevent photosynthesis.
 212 Control seedlings were prepared without nitrogen gas treatments. After the hypoxic
 213 treatments, roots were cut from the seedlings and weighed. The root samples were then

214 ground in liquid nitrogen using a pestle and mortar. Subsequently, 300- μ L aliquots of
215 distilled water were added and mixed with the ground powder. The mixture was
216 centrifuged in 1.5 mL tubes at 15,000 rpm ($21,386 \times g$) for 1 min at 1°C and the
217 supernatant was collected. The EtOH content was then determined
218 spectrophotometrically using a commercial kit (Ethanol, R-Biopharm AG, Germany)
219 according to the manufacturer's instructions. Briefly, the kit contained nicotinamide-
220 adenine dinucleotide (NAD), alcohol dehydrogenase and aldehyde dehydrogenase. In
221 spectrophotometric reactions, alcohol dehydrogenase oxidises EtOH to acetaldehyde in
222 the presence of NAD, generating NADH. Aldehyde dehydrogenase also oxidises
223 acetaldehyde to acetic acid and generates NADH in the presence of NAD. Hence,
224 NADH absorbance at 334, 340 and 365 nm was determined using a spectrophotometer
225 (U-5100, Hitachi, Japan) and stoichiometry was used to calculate the root extract EtOH
226 concentrations using the extinction coefficient of NADH.

227

228 **Histochemical root staining**

229 For the GUS staining, Petri dishes with five-day-old DR5::GUS seedlings were rotated
230 90 degrees and placed in the dark for 6 h. After gravistimulation, images of the seedlings
231 were captured using an EOS Kiss X7 camera (Canon, Tokyo) and fixed with 90% cold
232 acetone for 15 min. The acetone was discarded and the seedlings were rinsed with GUS
233 buffer three times. The GUS buffer contained 50-mM potassium phosphate buffer (pH =
234 7.2), 0.5-mM potassium ferricyanide and 0.5-mM potassium ferrocyanide. Next, the
235 seedlings were stained with 1-mM X-Gluc/DMF dissolved in GUS buffer in a vacuum
236 chamber. The stained samples were incubated overnight at 37°C and rinsed with 70%
237 EtOH before observation.

238 To detect superoxide in root apices grown in various concentrations of EtOH,
239 five-day-old seedlings were incubated in EtOH solution for 1 h before staining in a solution
240 containing 300- μ M NBT (Fluka, Germany) dissolved in 0.1-M Tris-HCl, 0.1-M NaCl
241 and 0.05-M $MgCl_2$ (pH = 9.5) for 5 min. Seedlings were then observed and imaged
242 under a light stereomicroscope. The NBT-stained areas in the binarised images with
243 identical threshold were measured using ImageJ.

244

245 **Quantification of gene expression in EtOH-treated roots**

246 After the EtOH treatments, roots were cut from the seedlings and weighed. The root
247 samples were frozen with liquid nitrogen and ground to a powder using a pestle and
248 mortar. Total RNA was purified using an ISOSPIN Plant RNA kit (NIPPON GENE,
249 Japan) following the manufacturer's manual. Single-strand cDNA was also obtained
250 using SuperScript IV Reverse Transcriptase (Thermo-Fischer, Japan). DNA
251 amplification was conducted using a real-time PCR device, LightCycler Nano (Roche,
252 Switzerland) with the following primers: ZAT10-forward,
253 AGGCTCTTACATCACCAAGATTAG; ZAT10-reverse,
254 TACACTTGTAGCTCAACTTCTCCA; GAPDH3-forward,
255 TTGGTGACAACAGGTCAAGCA; GAPDH3-reverse,
256 AAAGTTGTCGCTCAATGCAATC. The relative gene expression was calculated using
257 the $\Delta\Delta C_t$ method with GAPDH3 (glyceraldehyde-3-phosphate dehydrogenase) as the
258 reference gene.

259

260 **Confocal observations of endo/exocytic vesicle recycling**

261 Five-day-old seedlings grown under the conditions described above were stained with
262 the membrane-staining fluorescence probe FM4-64 at 4 μ M (Sigma, Germany) for 10
263 min. The seedlings were then treated with 0, 0.1 or 1% (v/v) EtOH in 0.5 $\times$ MS for 1 h,
264 followed by incubation in 35- μ M BFA (Sigma, Germany) for 30 min. Confocal images of
265 the seedlings were taken using a confocal laser microscope (Fluoview FV1000, Olympus,
266 Tokyo, Japan) and the effects of EtOH on the endocytosis rates were compared. To
267 measure exocytosis, we monitored seedling recovery from BFA-inhibited recycling. The
268 seedlings were stained with 4- μ M FM4-64 and then soaked in 35- μ M BFA for 30 min.
269 After washing out the BFA, the seedlings were incubated in EtOH at the stated
270 concentrations for 1 h before confocal imaging. The FM4-64 dye was excited at 515 nm
271 using an Argon laser and fluorescence emissions of FM4-64 were measured at 630–700
272 nm. To quantify the BFA compartment size in the root transition zone, confocal images
273 of the epidermal cells were trimmed to a 50 μ m square (10–15 cells). The plasma
274 membrane red fluorescence was removed and the BFA compartments were extracted by
275 adjusting the threshold in ImageJ. The total BFA compartment area (~30–50 particles)
276 was calculated for each image and averaged.

277

278 **F-actin bundle visualisation**

279 Transgenic *Arabidopsis* GFP-FABD2 (actin-binding domain 2) seedlings [27] were
280 grown under the conditions described above. After five days, the seedlings were
281 prepared for confocal microscopy analysis by incubation in 0.5 × MS containing EtOH
282 for 2 h. GFP-FABD2 was excited at 488 nm using an Argon laser and the emissions
283 were measured at 500–550 nm.

284

285 **Statistical analysis**

286 All numerical data were analysed using Student's t-test and Tukey's honestly significant
287 difference (HSD) using Prism 9 for macOS (ver. 9.3.1 (350), December 7, 2021).
288 Differences were considered significant when $p < 0.05$.

289

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403 Acknowledgements

404 We thank to Dr. Yutaka Kodama (University of Utsunomiya) for his critical reading and
 405 suggestion. We also thank to Dr. Yoshihito Kohari (Kitami Institute of Technology) for his
 406 technical advice for EtOH quantification. This work was supported by JSPS KAKENHI,
 407 Grant-in-Aid for No. 18K14726 (T.K.) and in part by Leading Initiative for Excellent
 408 Young Researchers of MEXT, Japan (K.Y.). The authors would like to thank Enago
 409 (www.enago.jp) for the English language review.

410

411 Author Contribution Statement

412 K.Y. and F.B coordinated the project. T.K. and K.Y. designed the experiments. T.K.
 413 performed the microscopy and biochemical analysis. K.Y. analysed the data and
 414 performed all calculations. All authors wrote the manuscript and agreed with the final
 415 manuscript.

416

417 Competing Interests

418 The authors declare no competing interests.

419

420 **Figure Legends**

421 **Figure 1. Hypoxia-induced EtOH production in Arabidopsis root tissues.**

422 Ethanol contents were biochemically determined using a spectrophotometer based on the
423 amount of NADH metabolised by alcohol dehydrogenase and acetaldehyde
424 dehydrogenase. Seedlings were treated with pure nitrogen gas for 24 h to induce
425 hypoxia. Error bars indicate standard deviations of the mean from five independent
426 experiments. Each sample contained approximately 40 mg of root tissue (~100 roots).
427 Significant differences were identified using Student's t-test; * $P < 0.05$.

428

429 **Figure 2. Root lengths are affected by EtOH treatments but not gravitropism.**

430 (A) Lengths of roots grown in the presence of 0%, 0.01%, 0.1% or 1% EtOH for 3 days
431 ($n = 52-56$ with three biological replicates). Different letters indicate significant
432 differences (Tukey's HSD test, $P < 0.05$). (B) Angles of root gravitropic curvature in the
433 presence of EtOH. The x-axis shows the treatment time after gravistimulation started.
434 There were no significant differences in each EtOH treatment time. Error bars indicate
435 standard deviations of the mean ($n = 33-36$ with three biological replicates). (C)
436 Gravitropic curvature in the presence of EtOH. The left column shows root gravitropism
437 under three different EtOH concentrations. Scale bar = 5 mm. The right column shows
438 the expression of auxin reporter mutant DR5::GUS. Scale bar = 100 μm .

439

440 **Figure 3. Ethanol induces the production of superoxide in Arabidopsis root apices.**

441 (A) Seedlings were treated with EtOH for 1 h and then stained with nitroblue
442 tetrazolium salt (NBT) solution to visualise superoxide distribution. (B) NBT-stained
443 areas were quantified. Error bars indicate standard deviations from the mean ($n = 5-6$
444 with three biological replicates). Different letters indicate significant differences
445 (Tukey's HSD test, $P < 0.05$). (C) Real-time PCR analysis for ROS signalling-
446 dependent transcription factor, ZAT10; expression was analysed using the $\Delta\Delta\text{Ct}$ method
447 with GAPDH3 as the reference gene.

448

449 **Figure 4. BFA-induced compartments in root apex transition zone cells exposed to** 450 **EtOH.**

451 (A) Confocal images of BFA-induced compartments in the root epidermis of the
452 transition zone after EtOH treatments. Five-day-old seedlings were stained with 4- μ M
453 FM4-64 for 10 min, incubated in 0% or 1% EtOH in $0.5 \times$ MS for 1 h and then soaked
454 in 35- μ M BFA in $0.5 \times$ MS for 30 min. Scale bar = 50 μ m. (B) The total area of BFA-
455 induced compartments in root transition zone epidermis cells was measured using
456 ImageJ (n = 18-21 with three biological replicates). Error bars indicate standard
457 deviations from the mean. (C) Recovery from BFA-mediated inhibition of vesicle
458 recycling following washing. After FM4-64 staining, seedlings were soaked in 35- μ M
459 BFA in $0.5 \times$ MS for 30 min and then incubated in 0% or 1% EtOH in $0.5 \times$ MS for 1 h.
460 Scale bar = 50 μ m. (D) Reductions in BFA compartment sizes after recovery. The total
461 area of BFA-induced compartments in root transition zone epidermis cells was
462 measured using ImageJ (n = 10-14 with three biological replicates). Error bars indicate
463 standard deviations from the mean; ns, not significant; different letters indicate significant
464 differences according to Tukey's HSD test, $P < 0.05$.

465

466 **Figure 5. Visualisation of F-actin in epidermal cells in the root elongation zone.**

467 (A) Five-day-old light-grown GFP-FABD2 seedlings were incubated in 0%, 0.1% or
468 1% EtOH in $0.5 \times$ MS solid media for 1 h. The cross-wall position under each condition
469 is indicated with an arrow. Five replicates produced similar results and representative
470 examples are presented for each condition. (B) Enlarged confocal image corresponding
471 to Figure 5A. The cross-wall position under each condition is indicated with an arrow.

472

473 **Figure 6. Hypoxia-induced depletion of F-actin in Arabidopsis GFP-FABD2 root** 474 **apex.**

475 (A) Hypoxia-induced the depolymerisation of F-actin after just 1 hour. Both cytosolic
476 and cross-wall F-actin were depleted. The position of the cross-wall is indicated with an
477 arrow. Scale bar = 25 μ m. (B) The enlarged images from (A). An asterisk and an arrow
478 indicate the position of the nucleus and F-actin accumulation on the tonoplast
479 membrane in the treated root cells, respectively. Scale bar = 10 μ m. (C) F-actin recovery
480 from hypoxic conditions. Scale bar = 25 μ m. The position of the cross-wall is indicated
481 with an arrow. Representative images from six roots are shown.

482

Endogenous EtOH levels [$\mu\text{mol} / \text{g FW}$]

15
10
5
0

Control

Hypoxia

*

A **B** **C**

A

Control

0.1% EtOH

1% EtOH

C

Control

0.1% EtOH

1% EtOH

B **D**

A

Control

0.1% EtOH

1% EtOH

B

Control

1% EtOH

A **B** **C**