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A plasma membrane nanodomain ensures signal specificity during osmotic signaling in plants

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29 **SUMMARY**

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In the course of their growth and development plants have to constantly perceive and react to their environment. This is achieved in cells, by the coordination of complex combinatorial signaling networks. However, how signal integration and specificity are achieved in this context is unknown. With a focus on the hyperosmotic stimulus, we use live super-resolution light imaging methods to demonstrate that a Rho GTPase, Rho-of-Plant 6 (ROP6), forms stimuli-dependent nanodomains within the plasma membrane (PM). These nanodomains are necessary and sufficient to transduce production of reactive oxygen species (ROS), that act as secondary messengers and trigger several plant adaptive responses to osmotic constraints. Furthermore, osmotic signal triggers interaction between ROP6 and two NADPH oxidases that subsequently generate ROS. ROP6 nanoclustering is also needed for cell surface auxin signaling, but short-time auxin treatment does not induce ROS accumulation. We show that auxin-induced ROP6 nanodomains, unlike osmotically-driven ROP6 clusters, do not recruit the NADPH oxidase, RBOHD. Together, our results suggest that Rho GTPase nano-partitioning at the PM ensures signal specificity downstream of independent stimuli.

INTRODUCTION

Biological membranes can be seen as a patchwork where lipids and proteins are grouped in a juxtaposition of domains of various shapes and sizes. Paradoxically, membranes are also a fluid-structure allowing lateral diffusion of its constituents by thermal agitation. This property of membranes is central as it participates in the dynamic partitioning of proteins and lipids between different plasma membrane (PM) domains and consequently regulates cell-surface signaling processes [1,2]. In plants, the vast majority of PM proteins observed with improved fluorescent microscopy technics was described to be organized in nanodomains of long dwell time (several minutes). It is especially the case of REMORIN3.1 (REM3.1), PLASMA MEMBRANE INTRINSIC PROTEIN2;1 (PIP2;1), PINFORMED2 (PIN2), AMMONIUM TRANSPORTER3.1 (AMT3.1), BRASSINOSTEROID INSENSITIVE1 (BRI1), RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D (RBOHD), FLAGELLIN SENSING2 (FLS2) and NITRATE TRANSPORTER1.1 (NRT1) [3-9]. Nevertheless, the functional relevance of this particular membrane organization remains poorly understood and its role in cell signaling just begins to be explored.

Among other signals, plant cells respond to changes in water availability generated by osmotic constraints. Despite tremendous effort in the last decades, the molecular mechanisms that allow plant cells to perceive and induce early signaling events in response to osmotic stress has just begun to be understood [10,11]. One of the first cellular responses is an accumulation of reactive oxygen species (ROS) [12] in cells, which act as secondary messengers, regulating cell endocytosis but also root water conductivity and intracellular accumulation of osmotica (e.g. proline) [13,14]. Two processes are under action to generate ROS during osmotic signalling. One is non-enzymatic and requires reduction of apoplastic iron. The second is mediated by the PM-localized NADPH oxidases, RBOHD and F [12]. RBOHs catalyze the production of superoxide free radicals by transferring one electron to oxygen from the cytoplasmic NADPH. Even if the mechanism that drives ROS production is now better understood, it is still unclear how it is triggered by a change in osmolarity.

The Rho of plant (ROP), belonging to the super clade of Ras/Rho GTPases, have a key role in cell surface signaling including response to hormones such as auxin or ABA, but also during biotic stimulation [15]. In some cases, they also appear to regulate ROS accumulation, like in tip growing cells or in response to chitin elicitation [16,17]. ROPs are functioning as molecular switches due to a change in conformation between an active GTP-bound form and an inactive GDP-bound form. However, ROP function is also tightly associated with its lipid environment. For instance, the rice type-II ROP OsRAC1 interacts with OsRBOHB in the presence of specific sphingolipids containing 2-hydroxy fatty acids [18]. Besides, the role of charged lipids was recently exemplified in a work on a type-I ROP from *Arabidopsis thaliana*. In this study, the anionic lipid phosphatidylserine (PS) was shown to interact directly with ROP6 C-terminal hypervariable domain, to determine ROP6 organization at the PM and to quantitatively control plant response to the phytohormone auxin [19]. Therefore, the ROP gene family may provide good candidates to regulate osmotic signaling.

Here, we show that ROP6 is a master regulator of osmotically-induced ROS accumulation and participates in a set of plant responses to this signal. Using super-resolution microscopy, we found that ROP6 co-exists in the same cell in different states and that osmotic stimulation induces ROP6 nanodomain formation. These nanodomains are needed for a correct ROS accumulation in cells and their composition differs when triggered by other stimuli, suggesting that ROP6 nanodomains may encode for signal specificity.

RESULTS

ROP6 is necessary for osmotically induced ROS accumulation and participates in plant responses to osmotic signal

To investigate the potential role of ROPs in osmotic signaling, we used medium or high sorbitol concentration ($\psi_{\text{medium}} = -0.26$ MPa and $\psi_{\text{high}} = -0.75$ MPa, respectively), and challenged *rop* loss-of-function mutant lines corresponding to the three isoforms that are highly expressed in roots (Figure S1 A). ROS accumulation in cells, as revealed by DHE dye, was used as a fast readout for activation of osmotic signalling (Figure 1B) [12]. Compared to WT, *rop6.2* seedlings, but not *rop2.1* nor *rop4.1*, show impaired ROS accumulation (Figure 1A-C, Figure S1 B). No additive effect was detected in *rop2.1xrop6.2* or *rop2.1xrop6.2xROP4RNAi* (Figure S1 B). The defect in ROS accumulation observed in *rop6.2* is independent of the type of osmoticum (Figure S1 C) and was fully complemented by a transgene containing mCitrine-tagged *ROP6* genomic DNA driven by its promoter (*rop6.2xmCit-ROP6*, Figure 1C). The regulation of ROS signalling was extensively studied in response to PAMPs and ABA. As roots also react to stimulation with Flg22 and ABA, we tested if some of the well-described ROS regulators are involved in osmotically induced ROS (Figure S1 D and E). Whereas the OST1/SNRK2.6 is probably not involved in osmotically-induced ROS production, we found that knock-out plants for *BIK1*, *BIK1/PLB1* and *CPK5/6/11* show no or attenuated ROS response, respectively. This suggests a potential interaction between osmotic and PAMP signalling, as it was previously postulated [20].

Because ROS accumulation in roots has been tightly associated with deposition of the secondary wall, especially lignin [21], we wondered if an osmotic constraint could enhance cell lignification. Roots exposed to -0.75 MPa for 24 hours have a strong autofluorescence signal compared to control situation, and when stained with phloroglucinol that reveals lignin specifically, a typical cherry-red staining was observed [22] (Figure S2 A and B). We tested if the osmotically-enhanced lignin deposition is indeed associated with ROS accumulation. Loss-of-function plants for the two highly expressed NADPH oxidases (*RBOHD* and *F*), showed a reduced autofluorescence after exposure to -0.75 MPa and control plants exposed to 1mM H₂O₂ for an hour revealed a strong fluorescent signal, showing a connection between osmotically-induced lignin deposition and ROS production (Figure S2 C). This response was partially regulated by *ROP6*, as *rop6-2* plants show dimmer root autofluorescence signal after -0.75 MPa treatments than control plants either Col0 or *rop6.2xmCit-ROP6* (Figure S2 C-D).

Interestingly, after 48 hours on -0.75 MPa plate, root tip cells displayed local isotropic cellular growth (Figure S2E). This change in cell polarity has been suggested to reflect an acclimation process of the root facing hyperosmotic condition, as was described for salt or drought responses [23,24]. Because, ROPs are known to regulate cell polar growth of pavement cells, pollen tube and root hairs [15], we wondered if *ROP6* may participate in the osmotically induced cell isotropic growth. *rop6.2* shows a significantly smaller circularity index than wild type or complemented lines on treated plate (-0.75 MPa), whereas no difference between genotypes was found in control conditions (Figure S2 F and G).

Because, *ROP6* seems to participate in multiple phenotypes associated to plant acclimation to osmotic constraint, we wondered if *ROP6* can also participate in the changes of root growth and development. Whereas indistinguishable when 5DAG plants were transplanted in control conditions, *rop6.2* plants grew slightly faster than *rop6.2xmCit-ROP6* in -0.75 MPa plate (rate constant^{*rop6.2xmCit-ROP6*} = $0.011 \pm 0.0005 \cdot \text{h}^{-1}$, rate constant^{*rop6.2*} = $0.009 \pm 0.0008 \cdot \text{h}^{-1}$, t-test p-value = 0.02, Figure S2H-K). Indeed, plants have longer primary and lateral roots in loss-of-function *rop6.2* mutant in this stress condition, while no significant effect was observed for lateral root density (Figure S2 L-N). Interestingly, *ROP6* expression pattern fits a potential role in root growth, as

158 mCit-ROP6 fluorescence is mostly present in the root meristem and elongation zone and
159 in lateral root primordia (Figure S2 O and P). As a whole, ROP6 appears to be necessary
160 for osmotically-induced ROS accumulation, but to some extent it also participates in plant
161 adaptations to hyperosmotic treatments (Figure S2 D, G and K).

162

163 **ROP6 activation, but not protein quantity, is rate-limiting to** 164 **trigger osmotic signaling.**

165 Next, we tested if *ROP6* is sufficient to trigger osmotic signaling. Although GFP-ROP6ox
166 overexpressing lines accumulate high amounts of ROP6 proteins, no enhancement of
167 osmotically-induced ROS was observed in control condition or after osmotic treatment,
168 suggesting that ROP activation rather than protein quantity might be a limiting factor
169 (Figure S3 A and Figure 1 C). To test this hypothesis, we used point mutated proteins
170 that are either constitutive active GTP-lock (ROP6-CA) or constitutive inactive GDP-lock
171 (ROP6-DN) ROP6. Transient expression in tobacco leaves of FRET based sensors (iROP)
172 shows that ROP6-CA but not ROP6-DN interacts with the CIRB domain of PAK1,
173 confirming their respective GTP or GDP-lock behavior (Figure S3 B, C and D). Stable
174 *rop6.2* plants expressing mCit-ROP6-CA, under its endogenous promoter, showed a
175 constitutively high ROS accumulation, independent of the stimulus (Figure 1 D).
176 Oppositely, in *rop6.2xmCit-ROP6-DN* plants, ROS induction was attenuated after
177 exposure to -0.75 MPa and totally suppressed after -0.26 MPa treatments, compared to a
178 control situation (Figure 1 D). This suggests that ROP6 itself might be sufficient for a part
179 of the osmotically-induced ROS production.

180

181 These results showed that ROP6 is necessary and its activation sufficient to trigger ROS
182 production. Then, we addressed if ROP6 activation could act upstream of ROS producing
183 enzymes. Therefore, *rop6.2xmCit-ROP6-CA* line, that has constitutively high ROS, was
184 treated alone or in combination with specific inhibitors for each of the two ROS pathways
185 activated by the osmotic stimulus [12]. Diphenyleneiodonium (DPI) was used to inhibit
186 NADPH oxidase activity and bathophenanthrolinedisulfonic acid (BPDS) to block ROS
187 mediated by ferric iron[12]. In co-treatment, ROS generated by mCit-ROP6-CA is
188 diminished drastically, suggesting that mCit-ROP6-CA is acting upstream of ROS
189 production machinery (Figure 1 E). Next, we determined if ROP6 activation is associated
190 with a change in its subcellular localization, as described for many small GTPases [25]. A
191 sharp fluorescent signal was observed delimiting root cells expressing mCit-ROP6, which
192 overlaid with the FM4-64 PM dye (Figure S2 Q). Only a minor difference in PM
193 fluorescence intensity or relative PM localisation was observed between wild type and
194 GTP or GDP lock ROP6 (Figure S3 E, F and G), suggesting that ROP6 is at most
195 marginally regulated by cytoplasmic/PM shuttling.

196

197 **Two populations of ROP6 molecules co-exist within the plasma** 198 **membrane and vary in frequency minutes after osmotic treatment**

199

200 We recently showed that ROP6 organization at the PM is critical for auxin signalling[19].
201 We thus addressed whether ROP6 lateral segregation at the PM could contribute to
202 osmotic signaling. Total internal reflexion fluorescent microscopy (TIRFM) in two
203 independent transgenic lines showed that GFP-ROP6 has a uniform localization within the
204 PM in control conditions, while in -0.26 MPa and even more in -0.75 MPa treated cells,
205 GFP-ROP6 appeared in diffraction-limited spots at the cell surface (Figure 2 A and B).
206 This suggests that ROP6 clustered in response to osmoticum treatment in a dose-
207 dependent manner (Figure 2 B). Kymograph analysis showed straight lines for up to 50
208 seconds, suggesting that GFP-ROP6 clusters are stable within the PM during this period
209 (Figure 2 C). We then wondered if ROP6 clustering could not go along with its
210 dissociation from the PM. Indeed, GFP-ROP6 shows a lower PM association index after
211 osmotic treatment (Figure S3 H and I). When ROP6 was locked in GTP bound form (RFP-
212 ROP6-CA), this effect was not observed although this form was able to cluster (Figure S3

213 H, I, J and K). Taken together, this suggests that ROP6 clustering and its membrane
214 dissociation are not strictly linked.

215
216 The average GFP-ROP6 spot size is close to the limit of diffraction (radius = 235 +/-
217 60.57 nm). Therefore, we next used sptPALM, a super-resolution imaging technic,
218 recently developed on plant samples [12,19,26]. Upon stochastic photoswitching on live
219 roots expressing mEOS2-ROP6, sub-diffraction spots are appearing with blinking
220 behaviour and small life span (< 0.5sec), as expected from single molecule behaviour
221 (Video S1 and Figure S4 A and B). By retrieving the displacement of each ROP6 single
222 molecule along with the videos, two behaviours can be observed in control condition
223 (Figure 2 D, highly diffusible molecules in orange and lowly diffusible molecules in blue,
224 Figure 2 E). Distribution of instantaneous diffusion coefficient of ROP6 single molecules,
225 extrapolated from mean square displacement plots, is bimodal (Figure 2 F, green curve
226 Figure 2 G). This result shows that diffusible ($D_{diff}=0.05\pm 0.007\mu\text{m}^2.\text{s}^{-1}$) and relatively
227 immobile ($D_{imm}=0.002\pm 0.0007\mu\text{m}^2.\text{s}^{-1}$) mEOS2-ROP6 molecules coexist within the PM
228 of a single cell. Minutes after -0.75 MPa treatments, the frequency of immobile mEOS2-
229 ROP6 doubles (Figure 2 G, H and I). Clustering analysis on live PALM images, using
230 Voronoi tessellation [27] (Figure 2 J), showed that the occurrence of molecules with high
231 local density increases after -0.75 MPa treatment (Figure 2 K and L, $\text{Log}_{local\ density}>3$).
232 Nevertheless, at this stage it was not possible to distinguish between three different
233 cases: (i) the sizes of nanodomains are increasing after treatment, (ii) cells have the
234 same number of nanodomains between control and treatment but with more ROP6
235 molecules in it or (iii) more nanodomains are formed in response to -0.75 MPa, with a
236 similar amount of ROP6 protein. To distinguish between these possibilities, segmented
237 images were generated based on detection local density, where only ROP6 molecules
238 with a local density higher than $\text{Log}_{local\ density}>3$ were investigated (Figure S3 C, D and E).
239 Whereas no effect on domain size, nor percentage of mEOS-ROP6 molecules per
240 nanodomains was found, the density of nanodomains per μm^2 of PM doubles after
241 osmotic treatment (Figure 2 M-O). Together our results suggest that in response to
242 osmotic stimulation, ROP6 molecules are clustering in nanometer-sized domains (i.e.
243 nanodomains), with a relatively fixed size and constant number of ROP6 molecules, and
244 in which ROP6 barely diffuses. This ROP6 diffusion behaviour differs substantially from
245 what we know for other PM proteins, such as the P-type ATPase, AHA2, or the aquaporin,
246 PIP1;2, which show an enhanced diffusion when cells are exposed to hyperosmotic
247 stimulation [12].

248 249 **ROP6 nanodomains are necessary to trigger osmotically-induced** 250 **ROS**

251 Next, we addressed whether ROP6-containing nanodomains are involved in osmotic
252 signaling. Because GTP-locked ROP6 (ROP6-CA) is constitutively producing ROS (Figure 1
253 D), we quantified diffusion and local density of mEOS2-ROP6-CA molecules by sptPALM.
254 In comparison to the wild-type protein, ROP6-CA has a higher proportion of immobile
255 molecules and a bigger fraction of molecules with high local density in control condition.
256 No difference was recorded between ROP6 and ROP6-CA after treatment, suggesting that
257 ROP6-CA is constitutively associated with nanodomains (Figure 3 A, B and C). In addition
258 to its C-terminal prenylation, ROP6 is transitorily S-acylated on cysteines 21 and 158
259 [17]. These modifications are required for localization in detergent-resistant membranes
260 and cause retarded lateral diffusion of the constitutive active GTP-lock ROP6 but have no
261 impact on ROP6 GTPase activity or PM targeting [17]. To test if ROP6 acylation is
262 required for nanoclustering, we generated mEOS2-ROP6^{C21S/C158S} expressing plants. Using
263 sptPALM and clustering analysis, we found that mEOS2-ROP6^{C21S/C158S} was insensitive to -
264 0.75 MPa treatments (Figure 3 D, E and F). Because mEOS2-ROP6^{C21S/C158S} is not
265 associated with nanodomains in response to osmotic treatment, we compared the ROS
266 response in *rop6.2xmCit-ROP6^{C21S/C158S}* and *rop6.2xmCit-ROP6* complemented lines.
267 Treatments with -0.26 MPa or -0.75 MPa did not trigger any ROS accumulation in
268 *rop6.2xmCit-ROP6^{C21S/C158S}* (Figure 3 G). Importantly, mCit-ROP6^{C21S/C158S} expressed
269 under the control of its own promoter localized at the PM in root cells (Figure 3 H and I),

270 as previously reported for 35S::GFP-ROP6-CA^{C21S/C158S} in leaves [17]. Together, our
271 results suggest that ROP6 nanodomain formation, rather than only ROP6 PM localization,
272 is necessary to activate osmotic signaling in cells.
273

274 **Activated ROP6 interacts with RBOHD and F in PM nanodomains to** 275 **generate ROS**

276 We checked first if *ROP6*, *RBOHD* and *RBOHF* are co-expressed in similar Arabidopsis root
277 cells. Transcriptional fusion for *RBOHD*, and translational fusion for *ROP6* and *RBOHF* all
278 showed an expression signal in root epidermis (Figure S5 A, B and C). Next, we tested if
279 the two NADPH oxidases isoforms that are activated by osmotic signal, *RBOHD* and
280 *RBOHF*, could interact with *ROP6*. FLIM experiments were performed in tobacco leaf cells
281 that transiently expressed the two putative interacting proteins tagged with GFP or
282 mRFP. We found a significant diminution of GFP life time when GFP-*RBOHD* was co-
283 expressed with RFP-*ROP6*-CA compared to cells expressing GFP-*RBOHD* and RFP-*ROP6*-
284 DN or when cells expressed only the donor GFP-*RBOHD* (Figure 4 A and B). Similar
285 results were observed with GFP-*RBOHF*, suggesting that both RBOHs interact *in planta*
286 with the GTP-, but not the GDP-locked form of *ROP6* (Figure S5 D). This is in line with
287 recent observations made in yeast two hybrid experiments, where *RBOHD* and *ROP6*-CA
288 were shown to interact [28].

289 Because *ROP6* and RBOHs physically interact and *ROP6* forms nanodomains that are
290 necessary for ROS accumulation, we hypothesized that RBOHs could also be organized in
291 nanodomains in the cell PM. Arabidopsis lines overexpressing GFP-tagged *RBOHD* and
292 *RBOHF* were generated. Under TIRF illumination, GFP-*RBOHD* showed a uniform
293 localization in control condition, while 2 minutes after -0.75 MPa treatment, cells had
294 clearly visible spots (Figure 4 C and D). By using GFP-*RBOHD*xRFP-*ROP6* plants, we
295 observed that *ROP6* accumulated in the same structure as *RBOHD* after osmotic
296 stimulation (Figure 4 E and F). As *rbohF* and *rbohD* mutant plants display similarly
297 reduced ROS accumulation in response to osmotic stimulation, we tested if *RBOHF* would
298 form stimuli-dependent clusters in the PM, like *RBOHD* does [12]. Eventhough there was
299 a substantial number of detectable clusters in control condition, GFP-*RBOHF*
300 overexpressing plants showed an increased cluster density minutes after -0.75 MPa
301 treatment, though less than in the case of *RBOHD* (Figure S5 E and F). This last result
302 suggests that to some extent both *RBOHD* and *RBOHF* have a re-localisation behaviour in
303 response to osmotic stimulation. Then, to analyse whether RBOH domains formation is a
304 consequence of *ROP6* activation or is triggered through an independent pathway, we
305 crossed Col0 GFP-*RBOHD* line with RFP-*ROP6*-CA or *rop6.2*. The density of GFP-*RBOHD*
306 clusters is much higher when the constitutively active form of *ROP6* is present in cells,
307 even in the absence of any stimulation (Figure 4G and H). In the case of *ROP6* loss of
308 function plants, GFP-*RBOHD* is observed in clusters in control condition, and its density
309 did not change after cell stimulation (Figure 4 G and I). Since GFP-*RBOHD* clusters are
310 present in the absence of *ROP6*, and since an active *ROP6* is likely required for RBOH
311 function in response to osmoticum, these last results suggest that GFP-RBOH cluster
312 formation is not strictly associated to ROS production.

313 To confirm that *RBOHD*/*ROP6* nanodomains are acting as a functional unit for ROS
314 production in the plant cell, we tested whether *ROP6* nanodomain formation is caused
315 and is not a consequence of ROS production. Neither inhibition of ROS by DPI/BPDS nor
316 H₂O₂ treatment have any impact on *ROP6* nanodomain formation (Figure S5 G, H and I).
317 Most importantly, we also tested if an osmotic signal can trigger the interaction between
318 *ROP6* and *RBOHD* in Arabidopsis roots. FLIM experiment was performed in Arabidopsis
319 roots expressing GFP-*RBOHD* and wild type or constitutive active *ROP6*. A significant
320 decrease of life time was observed 5 minutes after osmotic treatment with the GFP-
321 *RBOHD*xRFP-*ROP6* plant, but not when GFP-*RBOHD* is alone nor with GFP-*RBOHD*xRFP-
322 *ROP6*-CA which display constitutive low FLIM as expected (Figure 4 J and K). Thus, a

323 RBOHD/ROP6 complex is formed in membrane nanodomains upon cell stimulation. This
324 structure is necessary but not sufficient for osmotically-induced accumulation of ROS in
325 cells.

326 **Can ROP6 nanodomain formation mediate independent signaling**
327 **events?**

328 ROP6 is necessary for several plant signaling responses including to the phytohormone
329 auxin [14,18,27]. The correct targeting of the auxin transport efflux carrier PIN2 is
330 mediated by ROP6 and therefore participates in root gravitropic response [27,18].
331 Recently, ROP6 nanodomain formation, mediated by the anionic lipid phosphatidylserine
332 (PS), was described in response to auxin [19]. Together with our results on osmotic
333 signaling, this suggests that nanodomain formation is a general feature of ROP6 signaling
334 pathways in plants (Figure 5 A and B). We addressed whether RBOHD clustering is also
335 induced in response to auxin stimulation, as it happens after the induction of osmotic
336 signaling pathway. No increase of GFP-RBOHD clusters density was observed in such
337 condition, whereas ROP6 clearly show, as expected, numerous dotted structure in the PM
338 (Figure 5 A and B). As it was previously described, roots exposed to auxin for a short
339 time (60 min) failed to accumulate ROS, which contrasts with osmotic stimulation (Figure
340 5 C) [31–34]. These results show that ROP6 nanoclusters formed after auxin or osmotic
341 stimulations can differ in their constituent and consequently encode, to a certain extent,
342 for signal specificity.

343

DISCUSSION

By combining genetic and super resolution live imaging, we showed that ROP6 forms osmotic specific nanodomains within the PM that are required to trigger secondary messenger in cells. The role of this specific ROP isoform is central for osmotic signaling since *rop6.2* has a totally abolished osmotically-induced ROS production. In contrast, ROP2 and ROP4 which are also highly expressed in roots are dispensable for osmotic signalling [34]. In addition, we found that ROP6 controls some terminal plant responses to osmotic stress. Indeed, loss of function plants for *ROP6* exhibit less osmotically-induced lignin deposition in their roots. Lignin polymerisation requires cellular ROS. This was for example demonstrated in the case of Casparian strip formation, where the NADPH oxidase, RBOHF, is localised in specific membrane domains to produce ROS that permit a spatially targetted polymerisation of monolignol [21,35]. It is therefore likely that osmotically-induced lignin deposition is also mediated by ROS but from ROP6/RBOHs nanodomains. This enhancement of lignin synthesis in response to hyperosmotic stimulation could participate in plant acclimation to stress conditions. Indeed, lignin increases cell wall stiffness and may protect cells from deformation due to turgor loss. Secondary walls are also known to counteract mineral and water leakage in roots [36,37]. Enhanced lignin deposition may participate in such phenonena during long term osmotic treatment. In addition, we observed that cells under elongation form spheres rather than cylinders when exposed to a hyperosmotic treatment. This tendency to isotropic cell expansion could minimize tension and consequently could prevent cell bursting. ROPs are known regulators of cell polarity in pavement cells or tip growing cells. In particular, it was demonstrated that they can participate in cytoskeleton remodelling throught interaction with RIC1 and katanin [38]. Here, the need for ROP6 to ensure cell isotropic expansion and modified root elongation in response to stress condition not necessarily involves ROS production but could be mediated by other types of effector proteins. Thus, we believe that ROP6 is an important factor for plant osmotic signaling, likely acting right after cell osmotic perception, as ROP6 nanodomain formation happens only minutes after cell stimulation. Its role in long term adaptation is likely more indirect.

We also demonstrated that, upon cell activation by osmotic stimulation, enhanced ROS accumulation is associated with the formation of a ROP6/RBOHD complex within the PM. Plants expressing a GTP-lock form of ROP6 show a higher cellular accumulation of ROS. In this genetic background, ROP6 nanoclustering and its colocalization with RBOHD happen without any cell stimulation. These results fit with our FLIM experiment, where RBOH interacts preferentially with ROP6 GTP-locked form. On the other hand, *rop6* plants complemented with mutated ROP6 that are unable to be acylated, loose both the osmotically-induced nanodomain formation and consequently the ROS accumulation after hyperosmotic stimulation. But how can ROP6/RBOH complex get into nanodomains? The constitutive active ROP6 (ROP6-CA) was shown to be associated with detergent resistant membranes together with a slower diffusion [17,19]. This behaviour is mediated firstly by acylation of C23 and C158 residues of the protein with palmitic and/or stearic acids and secondly by the direct binding between lysine residues in ROP6 hypervariable tail and phosphatidylserine (PS) [17,19]. These results suggest, like for their animal and yeast counterpart, plant small GTPases have a greater affinity for specific lipid environment when they are activated, which then determine their nanoclustering [39–41]. Because activated ROP6 is interacting with RBOHs, we think that the former might drag and/or retain RBOHs protein to ROP6 nanodomains. This is supported by the fact that ROP6-CA can alone induce RBOH clustering and that in ROP6 loss-of-function mutants, GFP-RBOHD clustering is not inducible by osmotic stimuli. Nevertheless, we also observed that the basal level of RBOH clusters in *rop6.2* is higher than in control plant. Thus, RBOH can make clusters in the absence of ROP6. In this case, however, the clusters are not associated with ROS production and suggesting that ROP6 could act as a negative regulator.

401 Our group has recently described that two ROS machineries are under action in response
402 to osmotic stimulation, one of these involving two isoforms of NADPH oxidase, RBOHD
403 and F [12]. Our results suggest that ROP6 is an upstream regulator of both ROS
404 generating pathways (Figure1 C and E). However, we also found that ROP6-DN
405 expressed at its native level is able to partially rescue the ROS-release phenotype of
406 *rop6.2* under high stress conditions. Since ROP6-DN cannot interact with PAK1 nor
407 RBOHD and F, we believe that ROP6-DN may act as a scaffold for the ROS producing
408 machinery that is independent of RBOH. On the other hand, the role of ROP6 on RBOH-
409 dependant ROS production is rather associated with nanodomain formation. However,
410 how recruitment of RBOHs in ROP6 nanodomains can regulate ROS accumulation is still
411 unclear. Because of its ability to generate potentially harmful oxygen radicals, RBOH
412 activity is tightly controlled in cells. This is particularly well described for pathogen
413 elicitors, whereby several protein kinases including BIK1 and CPK5 are necessary for PTI
414 mediated ROS accumulation and can directly phosphorylate RBOHD N-terminus [42,43].
415 The change in RBOH PM localization, as mediated by ROP6, could participate in RBOH
416 interaction with protein kinases and consequently alter RBOH
417 phosphorylation/dephosphorylation kinetic. Also, RBOHD and F contain EF-hands that can
418 directly bind calcium and are essential for RBOH activity [44,45]. Within the cell
419 membrane, calcium gradients might exist in the vicinity of calcium membrane
420 transporters [46]. Therefore, recruitment of RBOH proteins in ROP6-containing
421 nanodomains that would also harbour these calcium transporters could alter RBOH micro-
422 environment, thereby regulating its activity. In addition, RBOH dimerization was
423 observed from purified OsRBOHB N-terminus but was also suggested from step bleaching
424 experiment done *in vivo* [7,47]. Interestingly, we observed an epistatic interaction
425 between *rbohD* and *rbohF* for osmotically induced ROS, suggesting that RBOHD and F
426 might form heteromers [12]. Similar observations were recently described for ROS
427 triggered upon cell ablation [48]. We speculate that co-clustering of RBOHD and RBOHF
428 in ROP6-containing nanodomains could increase their probability to form functional
429 heteromers.

430
431 Rho GTPases are generally seen as the neck of an hourglass for signal integration at the
432 cell surface. Indeed, multiple input pathways converge on a single Rho GTPase, leading
433 to various downstream cellular outputs, which are often specific to the upstream signal.
434 How signaling specificity is achieved in this context is an outstanding unresolved
435 question. In our work, we found that a single ROP isoform could, in response to different
436 stimuli e.g. auxin and osmotic stimulus, generate very similar nanodomains in terms of
437 shape, cellular density or even size [19]. Nevertheless, we also found that these
438 nanoclusters differ in their composition, in at least RBOH proteins. Therefore, the
439 segregation of signaling components in distinct plasma membrane nanodomains can
440 generate signal specificity downstream of a single small GTPase. How this discrimination
441 happens still remains an open question. It could be because of specific lipid environment
442 or/and recruitment of additional proteins that will participate in the stabilization of
443 ROP6/RBOH complexes.

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451 **AUTHOR CONTRIBUTIONS**

452 M.S. and A.M. conceived the study; M.S., C.F., M.P.P., C.A., X.D., N.P. and V.B.
453 performed experiments; M.S., A.M., J.B.F. and P.N. analyzed data; J.B.F. and M.N.
454 designed the TIRF microscopes and the sptPALM analysis pipeline. C.A. gave advice about
455 the FLIM imaging procedure; C.M., Y.J. and A.M. prepared figures and wrote the manu-

457 script with input from all co-authors; and all authors read and approved the final version
458 of the manuscript

459

460 **DECLARATION OF INTERESTS**

461 The authors declare no competing interests

462

463 **FIGURE LEGENDS**

464

465 **Figure 1: ROP6 activation is necessary and sufficient to trigger osmotically**

466 **driven ROS accumulation in Arabidopsis root cells.** (A) Drawing of Arabidopsis

467 plantlets, where the red square highlights the part of root under study. (B)

468 Dihydroethidium (DHE) stained root cell of Col0, *rop6.2* and *rop6.2xmCit-ROP6* in control

469 condition (0 MPa) or after 15 min of -0.75 MPa treatment. (C-D) DHE fluorescence

470 quantification after 15 min treatment with 0, -0.26 or -0.75 MPa solution in different

471 genetic materials: Col(0), *rop6.2*, ROP6 over expresser line (GFP-ROP6ox) and *rop6.2*

472 lines expressing under ROP6 endogenous promotor, either ROP6 (mCit-ROP6

473 (*rop6.2xmCit-ROP6*), the constitutive active ROP6 (*rop6.2xmCit-ROP6-CA*) or the

474 dominant negative (*rop6.2xmCit-ROP6-DN*). (E) ROS quantification (DHE fluorescence) in

475 root cells expressing the constitutive active ROP6 (mCit-ROP6-CA) in control or after mild

476 or high osmotic stimulus (respectively 0, -0.26 and -0.75 MPa) supplemented or not with

477 ROS enzyme inhibitors. DPI was used for inhibition of NADPH oxidase activity and BPDS

478 to inhibit ROS produced from ferric iron. Error bars correspond to a confidence interval at

479 95%. ANOVA followed by Tukey test, letters indicate significant differences among means

480 (p -value<0.001). $n > 49$ from 4-6 independent biological replicates. Scale bar 20 μm .

481

482 See also Figure S1, S2 and S3.

483

484 **Figure 2: Osmotic stimulus triggers ROP6 molecular nanoclustering at the PM**

485 (A) TIRFM micrograph of oxGFP-ROP6 expressing cells after 2 min incubation with

486 solutions at either 0 MPa, -0.26 or -0.75 MPa. (B) Quantification of ROP6 cluster density.

487 (C) Kymograph image of oxGFP-ROP6 clusters from cells exposed to 0.75 MPa. Clusters

488 at initial time point are labelled with arrows. (D) Image reconstruction of around 5 000

489 single mEOS2-ROP6 molecule trajectories in two control cells. (E) Close-up view of cell

490 expressing mEOS2-ROP6, where trajectories with high or low diffusion instantaneous

491 coefficient labelled in orange or blue respectively. (F) Mean square displacement curves

492 of the highly or lowly diffusible molecules in control (0 MPa) or treatment (-0.75 MPa)

493 conditions. (G) Bimodal distribution of molecule instantaneous diffusion coefficients in

494 control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions. (H)

495 Close up view of the PM of cells expressing mEOS2-ROP6 2 minutes after a -0.75 MPa

496 treatment. (I) Histogram represents the percentage of molecules with an instantaneous

497 diffusion below $0.01 \mu\text{m}^2 \cdot \text{s}^{-1}$ in control (0 MPa) or after treatment (-0.75 MPa). (J)

498 Voronoï tessellation of mEOS2-ROP6 molecules localization map from the exact same two

499 control cells of (D). Top right inset is a close up view, showing a mEOS2-ROP6

500 nanodomain. (K) Distribution of molecules local density in control (0 MPa, green curve)

501 and treatment (-0.75 MPa, purple curve) conditions. (L) Percentage of molecules with a

502 $\log(\text{local density})$ higher than 3. (M) Distribution of the mEOS2-ROP6 nanodomains

503 diameter in control (0 MPa) and treatment (-0.75 MPa) conditions. (N) Relative

504 occurrence of mEOS2-ROP6 in nanodomains in control (0 MPa) and treatment (-0.75

505 MPa) conditions. (O) Nanodomain density in control (0 MPa) or after 2 minutes treatment

506 with -0.75 MPa solution. Error bars correspond to a confidence interval at 95%. For (B)

507 an ANOVA followed by Tukey test was done, letters indicate significant differences among

508 means (p -value<0.001). * p -value below 0.01 T-Test. $n > 12$ from 3 independent

509 biological replicates. Scale Bar $10 \mu\text{m}$, except for E and H where it is 1 μm .

510

511 See also Figure S3, S4 and Video S1

512

513 **Figure 3: ROP6 nanoclustering is required for ROS accumulation.** (A) Bimodal
514 distribution of mEOS2-ROP6 instantaneous diffusion (green curve) or mEOS2-ROP6-CA
515 (purple curve) in control condition (0 MPa). (B) Histogram represents the percentage of
516 molecules with an instantaneous diffusion below $0.01 \text{ } \mu\text{m}^2.\text{s}^{-1}$ in mEOS2-ROP6 and
517 mEOS2-ROP6-CA expressing lines in control (green) or after osmotic stimulation
518 (purple). (C) Percentage of molecules with a $\log(\text{local density})$ higher than 3 in mEOS2-
519 ROP6 and mEOS2-ROP6-CA expressing lines in control (green) or after osmotic
520 stimulation (purple). (D) Bimodal distribution of mEOS2-ROP6^{C21S/C158S} instantaneous
521 diffusion coefficients in control (0 MPa, green curve) and treatment (-0.75 MPa, purple
522 curve) conditions. (E) Histogram represents the percentage of mEOS2-ROP6^{C21S/C158S}
523 molecules with an instantaneous diffusion below $0.01 \text{ } \mu\text{m}^2.\text{s}^{-1}$ in control (0 MPa) and
524 treatment (-0.75 MPa) conditions. (F) Percentage of mEOS2-ROP6^{C21S/C158S} molecules
525 with a $\log(\text{local density})$ higher than 3. (G) Quantification of ROS accumulation by DHE
526 staining in *rop6.2xmCit-ROP6* or *rop6.2xmCit-ROP6*^{C21S/C158S} expressing cells after 15 min
527 treatment with 0, -0.26 or -0.75 MPa solution. (H) Plasma membrane localization of
528 mCit-ROP6 and mCit-ROP6^{C21S/C158S} and its relative amount at the PM (I). Error bars
529 correspond to a confidence interval at 95%. * p-value below 0.01 t-test. ns. Non-
530 significant. n>9 from 3 independent biological replicates. Scale bar 10 μm .
531

532 **Figure 4: ROP6 interacts and forms nanoclusters with RBOHD at the PM.** (A) GFP-
533 RBOHD fluorescence lifetime when co-expressed with dominant negative (RFP-ROP6-DN)
534 or constitutive active ROP6 (RFP-ROP6-CA) in transient expression in tobacco leaf
535 epidermal cells and its quantification (B). (C) TIRF micrograph of cell expressing GFP-
536 RBOHD in control or after 2 minute treatment with -0.75 MPa solution and quantification
537 of clusters density (D). (E, F) Cell co-expressing GFP-RBOHD with RFP-ROP6 in control
538 (E) or after -0.75 MPa treatment (F). Graphs below represent the pixel intensity along
539 the dotted line in each of the conditions. (G) TIRFM micrograph of GFP-RBOHD signal in
540 GFP-RBOHDxRFP-ROP6, GFP-RBOHDxRFP-ROP6-CA and *rop6.2xGFP-RBOHD* plant in
541 control or after -0,75 MPa incubation and their respective quantification (H and I). (J)
542 GFP-RBOHD fluorescence lifetime when expressed alone or co-expressed with RFP-ROP6
543 or RFP-ROP6-CA in root cells and its quantification (K). Error bars correspond to a
544 confidence interval at 95%. For (B) an ANOVA followed by Tukey test was done, letters
545 indicate significant differences among means (p-value<0.001). * p-value below 0.01 T-
546 Test. n>12 from 3 independent biological replicates. Scale bar 10 μm .
547

548 See also Figure S5

550 **Figure 5: Auxin-stimulated ROP6 nanodomains are free of RBOHD.** (A) TIRFM
551 micrograph of cell expressing GFP-ROP6 or GFP-RBOHD in control condition (DMSO) or
552 after 10 μM NAA for 1 hour. (B) Cluster density quantification after NAA or -0,75 MPa
553 treatment. (C) Quantification of ROS accumulation by DHE staining in control (DMSO) or
554 after 15 min treatment with 10 μM NAA. Error bars correspond to a confidence interval at
555 95%. For (B) an ANOVA followed by Tukey test was done, letters indicate significant
556 differences among means (p-value<0.001). * p-value below 0.01 T-Test. n>11 from 3
557 independent biological replicates. Scale bar 10 μm .
558

559

560 **STAR METHODS**

561 Detailed methods are provided in the online version of this paper and include the
562 following

563 **CONTACT FOR REAGENT AND RESOURCE SHARING**

564 Further information and requests for resources and reagents should be directed to and
565 will be fulfilled by the Lead Contact, Alexandre Martiniere (alexandre.martiniere@cncrs.fr)

566 **MATERIEL AVAILABILITY STATEMENT**

567 Arabidopsis lines and plasmids generated in this study are available upon request to the
568 Lead Contact, Alexandre Martiniere (alexandre.martiniere@cncrs.fr).

569 **DATA AND CODE AVAILABILITY STATEMENT**

570 This study did not generate any code and the published article includes all dataset
571 analyzed during this study.

572 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

573 Plant material

574 Arabidopsis thaliana accession Col-0 was used as wild type in this study. The following
575 lines were published before: *rop6.2* [29], *rop2.1* [49], *rop4.1* [50], *rop6.2xrop2.1* [51],
576 *rop6.2xrop2.1xROP4RNAi* [51], *rop6.2xpROP6:mCit-ROP6g* [19], *p35S:mEOS-ROP6g*
577 [19], *p35S:mEOS-ROP6g-CA* [19], *pRBOHD:nls-GUS-GFP* [19], *pRBOHF:mcherry-*
578 *RBOHFg* [21] and *bik1* [52], *bik1xpbl1* [53], *cpk5/6/11* [54] and *ost1.2*[55]. Information
579 on all genes referenced in this work, including mutant alleles and sources is provided in
580 Table S1.

581 **METHOD DETAILS**

582 Growing conditions and plant materials:

583 Plants were stratified for 2 days at 4°C and grown vertically on agar plates containing
584 half-strength Murashige and Skoog (½ MS) medium supplemented with 1% (w/v)
585 sucrose and 2.5mM MES-KOH pH6 for 5 days at 22°C in a 16-h light/8-h dark cycle with
586 70% relative humidity and a light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, prior to use. *Nicotiana*
587 *tabacum* used for transient expression were grown in soil at 22°C in a 8-h light/16-h dark
588 cycle with 70% relative humidity and a light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For root
589 architecture analyses, seedlings were grown on vertical square 12x12 cm Petri dishes in
590 a self-contained imaging unit equipped with a 16M pixel linear camera, a telecentric
591 objective and collimated LED backlight. Plants were grown in the imaging automat
592 dedicated growth chamber at 23°C in a 16-h light/8-h dark cycle with 70% relative
593 humidity and a light intensity of 185 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Vegeled Floodlight, Colasse Seraing –
594 Belgium). Plates were imaged every four hours allowing fine kinetic analysis.

595 Cloning and plant transformation

596 The vector ROP6g/pDONRP2RP3, which includes the full ROP6 genomic sequence from
597 ATG to the end of its 3'UTR (ROP6g – At4g35020) [19] was amplified with the
598 overlapping primers to generate either ROP6g-CA/pDONRP2R (G15V) or ROP6g-
599 DN/DONRP2RP3 (T20N). ROP6g-CA/pDONRP2R-P3 and ROP6g-DN/pDONRP2R-P3 were

600 then recombined by LR multisite reaction with ROP6prom/pDONRP4P1R [19],
601 mCITRINEnoSTOP/pDONR221 [56] and pB7m34GW [57] to generate pROP6:mCit-
602 ROP6g-CA and pROP6:mCit-ROP6g-DN vectors, respectively. ROP6g/pDONRP2R3
603 was amplified with overlapping primers to generate ROP6gC21S-C158S/pDONRP2R3.
604 ROP6gC21S-C158S/pDONRP2R-P3 was then recombined by LR multisite reaction with
605 2x35Sprom/pDONRP4P1R [58], mEOS2noSTOP/pDONR221[19] and pB7m34GW [57] to
606 generate p35S:mEOS2-ROP6C21S-C158S. ROP6gC21S-C158S/pDONRP2R-P3 was also
607 recombined by LR multisite reaction with ROP6prom/pDONRP4P1R [19],
608 mCITRINEnoSTOP/pDONR221 [56] and pB7m34GW [57] to generate
609 ROP6prom:mCITRINE-ROP6C21S-C158S. The coding sequence of RBOHD (At5g47910),
610 RBOHF (At1g64060), ROP6 (At4g35020), ROP6-CA (G15V) and ROP6-DN (T20N) were
611 PCR amplified and inserted into pENTR/D-TOPO. pB7WGF2 and pB7WGR2vector were
612 used as destination vector for respectively GFP and RFP fusion. The unimolecular FRET
613 sensor with intact C-terminus was designed based on RhoA biosensors [59]. The CRIB
614 domain of hsPAK1 is known to interact with GTP bound form of ROP [60,61]. We used it
615 as a genetic probe for ROP6 GTP conformation. We ordered a synthetic gene coding for
616 PAK1-mCherry-mVenus-ROP6 (iROP) and cloned it into *pDONR221*. The different
617 binary were used either for transient expression in tobacco [62] or to generate stable
618 Arabidopsis plants by floral dip method in Col-0 and then crossed with *rop6.2* line [63].
619 Additional information on all constructs vectors and oligonucleotides is provided in Table
620 S2 and S3 respectively.

621 Osmotic and Pharmacological Treatments

622 Plantlets were bathed in a liquid MS/2 medium for 30 min to allow recovery from
623 transplanting. When indicated, a pre-treatment with DPI (30min, 20 μ M), BPDS (50 μ M,
624 30min), flg22 (1 μ M, 30 min), ABA (1 μ M, 1 hour), NAA (10 μ M, 1 hour) or H2O2 (1mM,
625 3mins for TIRF imaging and 1h for ligning quantification) was applied. Then, plantlets
626 were gently transferred for an additional 15 min with 5 μ M of ROS dye dehydroethidium
627 (DHE) , with or without the corresponding inhibitors, into MS/2 medium (0 MPa), MS/2
628 medium plus 100 mM sorbitol (-0.26 MPa) for mild stress or MS/2 medium plus 300 mM
629 sorbitol (-0.75 MPa) for severe osmotic stress. 300g/l PEG8000 was also used to mimic
630 severe osmotic stress. The osmotic potential of each solution was verified by point
631 freezing osmometer (WESCOR, VAPRO 5520).

632 Western blot

633 Tissues from 5 days old Col-0, *rop6.2xmCit-ROP6g* and GFP-ROP6 plantlets were grinded
634 with liquid nitrogen to a fine powder and resuspended in 1 mL/g powder of RIPA
635 extraction buffer (150 mM NaCl, 50mM Tris-HCl, pH- 8, 0.1% SDS, 0.5% Na
636 deoxycholate, 1% Triton X-100, 2mM leupeptin, 1mM PMSF and 5mM DTT). Western blot
637 analysis was performed with antibodies diluted in blocking solution (1% BSA in 0.1%
638 Tween-20 and PBS) at the following dilutions: α -GFP with conjugated HRP 1:2000. Whole
639 protein quantity was revealed with Commasie blue stain.

640 Sample clarification and phloroglucinol staining

641 Seedlings were vertically grown in half-strength MS-agar plates for 5 days and transfered
642 on control (MS/2) or 300mM sorbitol plates for 24 hours. Plantlets were treated
643 accordingly to Malamy et al., 1997 [64]. In brief, they were incubated in 0.24 M of HCl
644 prepared in 20% ethanol, at 80°C for 15 minutes, and then transferred in a solution of
645 7% NaOH in 60% ethanol for another 15 minutes at room temperature. The incubated
646 seedlings are rehydrated in subsequent baths for 5 minutes in 40%, 20% and 10%
647 ethanol and infiltrated thereafter in 5% ethanol/25% glycerol for 15 minutes.
648 Alternatively, root samples were stained with phloroglucinol as in Prajakta Mitra et al.,
649 2014 [22].

650 ROS and autofluorescence quantification

651 Observations were performed on the root elongation zone using an Axiovert 200M
652 inverted fluorescence microscope (20×/0.5 objective; Zeiss), with 512/25-nm excitation
653 and 600/50 emission filters for DHE staining and with 475/28 nm excitation and 530/25
654 nm emission for lignin stained samples. Exposure time was 500 ms. Images were
655 acquired using a CCD camera (Cooled SNAP HQ; PhotoMetrics), controlled by imaging
656 software (MetaFluor; Molecular Devices). To quantify the intensity of the fluorescence
657 signal, the images were analyzed using ImageJ software. After subtraction of the
658 background noise, an average mean grey value was calculated from epidermal and
659 cortical cells.

660 Confocal laser scanning microscopy

661 Signal from *rop6.2xmCit-ROP6g*, *rop6.2xmCit-ROP6g-CA*, *rop6.2xmCit-ROP6g-DN* and
662 *rop6.2xmCit-ROP6g^{C21S/C158S}* was imaged using Leica SP8 microscope with a 40×/1.1
663 water objective and the 488-nm line of its argon laser was used for live-cell imaging.
664 Fluorescence emission was collected from 500–540 nm for GFP and from 600–650 nm by
665 sequential acquisition when sample where stained 10 min with 2 μM of FM4-64. To detect
666 FRET from the different iROP variants, images were taken with the 488-nm line of its
667 argon laser and simultaneous detection between 515-540nm (mVenus detection) and
668 625-650nm (FRET channel). The ratio of FRET/Venus images was calculated with a Fiji
669 software. Mean grey value of each cells present in the field of view was measured
670 independently by drawing specific ROI.

671 TIRF microscopy

672 For cluster density analysis, Total Internal Reflection Fluorescence (TIRF) microscopy was
673 done using an inverted Zeiss microscope and a 100×/1.45 oil immersion. Images were
674 acquired with 50ms exposure time at 50 gain, with 475 nm excitation and 530/25 nm
675 emission. Acquisitions were recorded for 0.5 seconds. Images were Z stacked by average
676 intensity and object detection of GFP-ROP6, GFP-ROP6CA, RbohD-GFP and RbohF-GFP
677 was made using machine learning-based segmentation with Elastik [65]. For
678 colocalization study, TIRF microscopy was done using an inverted Nikon microscope
679 (Eclipse) equipped with azimuthal-TIRFiLas2 system (Roper Scientific) and a 100×/1.49
680 oil immersion. One hundred images were acquired with 100ms exposure time using
681 sequentially 488nm laser illumination with 425/20 emission filters and 561nm laser with
682 600/25.

683 FRET-FLIM

684 FRET-FLIM measurements were performed by multiphoton confocal microscopy (ZEISS
685 LSM 780) with the method of measuring the lifetime of photons (TCSPC: Time correlated
686 single photon counting) and under a 40×/1.3 oil immersion objective (Peter and Ameer-
687 Beg, 2004). The GFP (donor GFP-RBOHD or GFP-RBOHF) was excited with 920 nm by a
688 pulsating infra-red laser Ti:Saphir (Chameleon ULTRA II, COHERENT) during 90 seconds
689 and the emitted fluorescence was collected by HPM-100 Hybrid detector. The decreasing
690 fluorescence curve was obtained with the SPCImage (Becker-Hickl) software for each
691 zone of interest. The lifetime of the GFP was estimated based on a regression curve,
692 either mono-exponential when the donor was expressed alone and bi-exponential when
693 the donor was expressed in the presence of the acceptor proteins (RFP-ROP6, RFP-ROP6-
694 CA and RFP-ROP6-DN). Three biological repetitions were done and for every biological
695 replicate, 5 cells were analyzed.

696 sptPALM

697 Root cells were observed with a homemade total internal reflection fluorescence
698 microscope equipped with an electron-multiplying charge-coupled device camera (Andor
699 iXON XU_897) and a 100×/1.45 oil immersion objective. The coverslips (Marienfeld 1.5H)
700 were washed sequentially with 100% ethanol, acetone and water. Then, they were
701 bathed with a 1M KOH solution and then ultra-sonicated for 30 min. After several wash-
702 outs with MilliQ water, they were dried under Bunsen burner flame. The laser angle was
703 adjusted so that the generation of the evanescence waves give a maximum signal-to-
704 noise ratio. The activation of the photoconvertible tagged mEOS-ROP6, mEOS-ROP6-CA
705 and mEOS-ROP6^{C21A/C158S} was done by a low-intensity illumination at 405 nm (OBIS LX
706 50mW; Coherent), and 561 nm (SAPPHIRE 100mW; Coherent) emission combined with a
707 600/50 (Chroma) emission filter was used for image acquisition [12]. Ten-thousand
708 images were recorded per region of interest and streamed into a LabVIEW software
709 (National Instruments) at 20ms exposure time. Ten to 20 cells/ treatment were analysed
710 out of three biological replicates.

711 Single-Particle Tracking and Voronoi Tessellation

712 Individual single molecules were localized and tracked using the MTT software [66].
713 Dynamic properties of single emitters in root cells were then inferred from the tracks
714 using a homemade analysis software written in MatLab (The MathWorks) [12]. From each
715 track, the MSD was computed. To reduce the statistical noise while keeping a sufficiently
716 high number of trajectories per cell, tracks of at least five steps (i.e. ≥ 6 localizations)
717 were used. Missing frames due to mEOS blinking were allowed up to a maximum of three
718 consecutive frames. The diffusion coefficient D was then calculated by fitting the MSD
719 curve using the first four points. For the clustering analysis, the positions returned by
720 MTT of each mEOS detection were used as input to the SR-Tesseler software [27].
721 Correction for multiple detection was made based on recommendation from Levet et al.,
722 2015 [27]. The local densities of each track were calculated as the invert of their minimal
723 surface. Then, nanocluster size, relative number of ROP6 molecules in nanodomains and
724 density of nanoclusters were calculated after defining region of interest (ROI) where the
725 local density was 50 times higher than the average. Only ROI with at least 25 detections
726 were considered.

727 Statistical Analysis

728 For each condition or treatment, 9–12 cells were analyzed from at least 5–7 different
729 seedlings. All experiments were independently repeated 2–3 times. Data are expressed
730 as mean \pm 95% confidence interval. ANOVA followed by Tukey test was done, letters
731 indicate significant differences among means (pvalue<0.001). * p-value below 0.01
732 Student T-Test. Statistical analyses were performed in GraphPad Prism (GraphPad
733 Software).

734 VIDEO S1 LEGEND

735
736 **VIDEO S1: Dynamic of mEOS-ROP6 single molecule at the plasma membrane.**
737 **Related to figure 2.**

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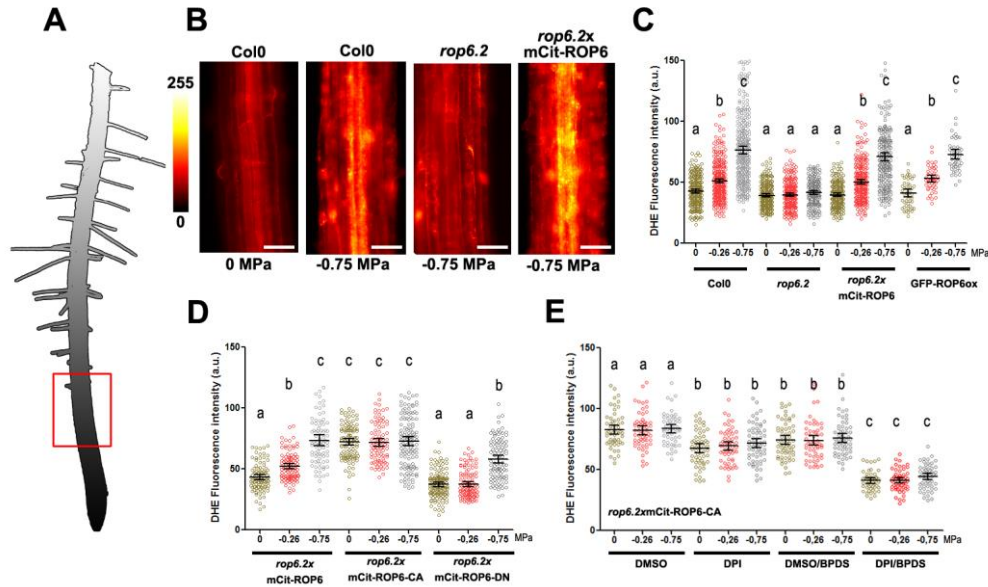


Figure 1: ROP6 activation is necessary and sufficient to trigger osmotically driven ROS accumulation in Arabidopsis root cells. (A) Drawing of Arabidopsis plantlets, where the red square highlights the part of root under study. (B) Dihydroethidium (DHE) stained root cell of Col0, *rop6.2* and *rop6.2xmCit-ROP6* in control condition (0 MPa) or after 15 min of -0.75 MPa treatment. (C-D) DHE fluorescence quantification after 15 min treatment with 0, -0.26 or -0.75 MPa solution in different genetic materials: Col(0), *rop6.2*, ROP6 over expresser line (GFP-ROP6ox) and *rop6.2* lines expressing under ROP6 endogenous promotor, either ROP6 (*rop6.2xmCit-ROP6*), the constitutive active ROP6 (*rop6.2xmCit-ROP6-CA*) or the dominant negative (*rop6.2xmCit-ROP6-DN*). (E) ROS quantification (DHE fluorescence) in root cells expressing the constitutive active ROP6 (*mCit-ROP6-CA*) in control or after mild or high osmotic stimulus (respectively 0, -0.26 and -0.75 MPa) supplemented or not with ROS enzyme inhibitors. DPI was used for inhibition of NADPH oxidase activity and BPDS to inhibit ROS produced from ferric iron. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p -value <0.001). $n>49$ from 4-6 independent biological replicates. Scale bar 20 μ m.

See also Figure S1, S2 and S3.

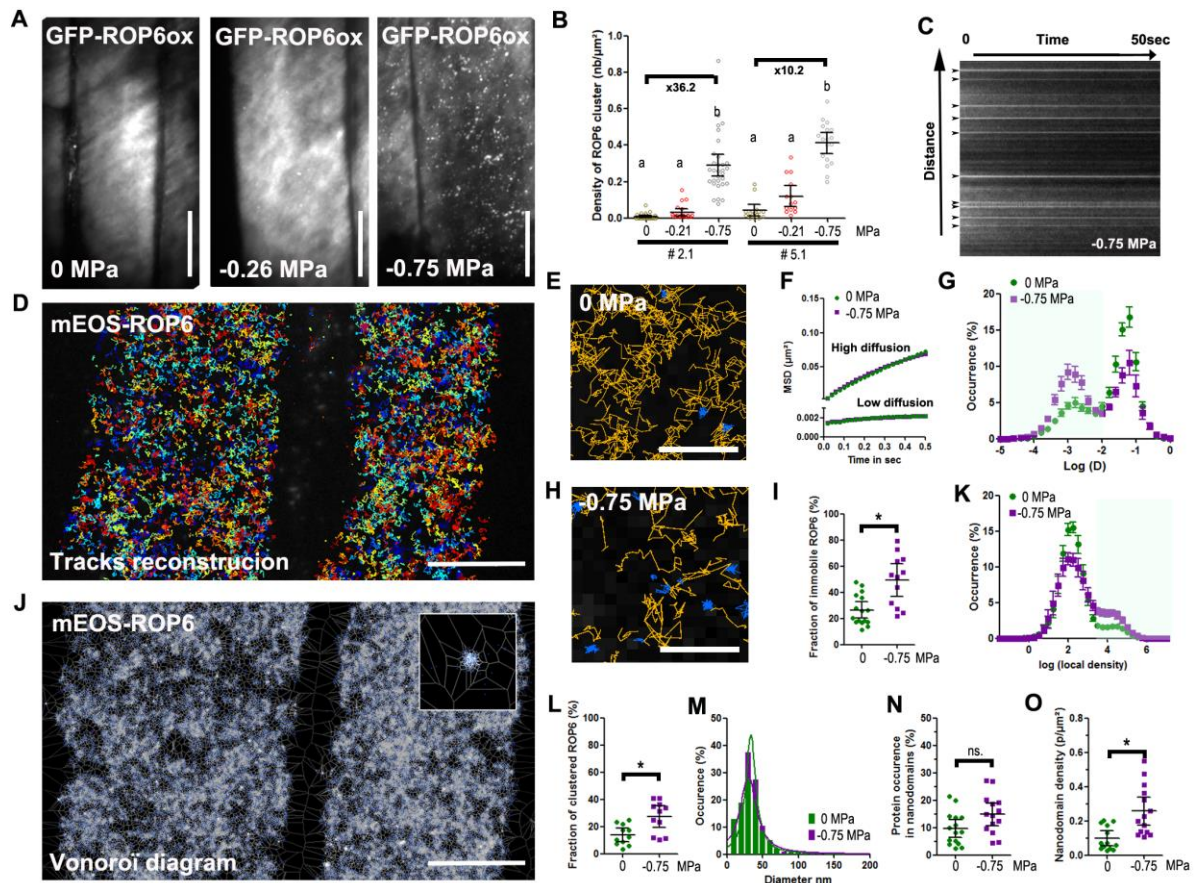


Figure 2: Osmotic stimulus triggers ROP6 molecular nanoclustering at the PM

(A) TIRFM micrograph of oxGFP-ROP6 expressing cells after 2 min incubation with solutions at either 0 MPa, -0.26 or -0.75 MPa. (B) Quantification of ROP6 cluster density. (C) Kymograph image of oxGFP-ROP6 clusters from cells exposed to 0.75 MPa. Clusters at initial time point are labelled with arrows. (D) Image reconstruction of around 5 000 single mEOS2-ROP6 molecule trajectories in two control cells. (E) Close-up view of cell expressing mEOS2-ROP6, where trajectories with high or low diffusion instantaneous coefficient labelled in orange or blue respectively. (F) Mean square displacement curves of the highly or lowly diffusible molecules in control (0 MPa) or treatment (-0.75 MPa) conditions. (G) Bimodal distribution of molecule instantaneous diffusion coefficients in control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions. (H) Close up view of the PM of cells expressing mEOS2-ROP6 2 minutes after a -0.75 MPa treatment. (I) Histogram represents the percentage of molecules with an instantaneous diffusion below $0.01 \text{ } \mu\text{m}^2 \cdot \text{s}^{-1}$ in control (0 MPa) or after treatment (-0.75 MPa). (J) Voronoi tessellation of mEOS2-ROP6 molecules localization map from the exact same two control cells of (D). Top right inset is a close up view, showing a mEOS2-ROP6 nanodomain. (K) Distribution of molecules local density in control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions. (L) Percentage of molecules with a $\log(\text{local density})$ higher than 3. (M) Distribution of the mEOS2-ROP6 nanodomains diameter in control (0 MPa) and treatment (-0.75 MPa) conditions. (N) Relative occurrence of mEOS2-ROP6 in nanodomains in control (0 MPa) and treatment (-0.75 MPa) conditions. (O) Nanodomain density in control (0 MPa) or after 2 minutes treatment with -0.75 MPa solution. Error bars correspond to a confidence interval at 95%. For (B) an ANOVA followed by Tukey test was done, letters indicate significant differences among

means (p-value<0.001). * p-value below 0.01 T-Test. n>12 from 3 independent biological replicates. Scale Bar 10 μ m, except for E and H where it is 1 μ m.

See also Figure S3, S4 and Video S1

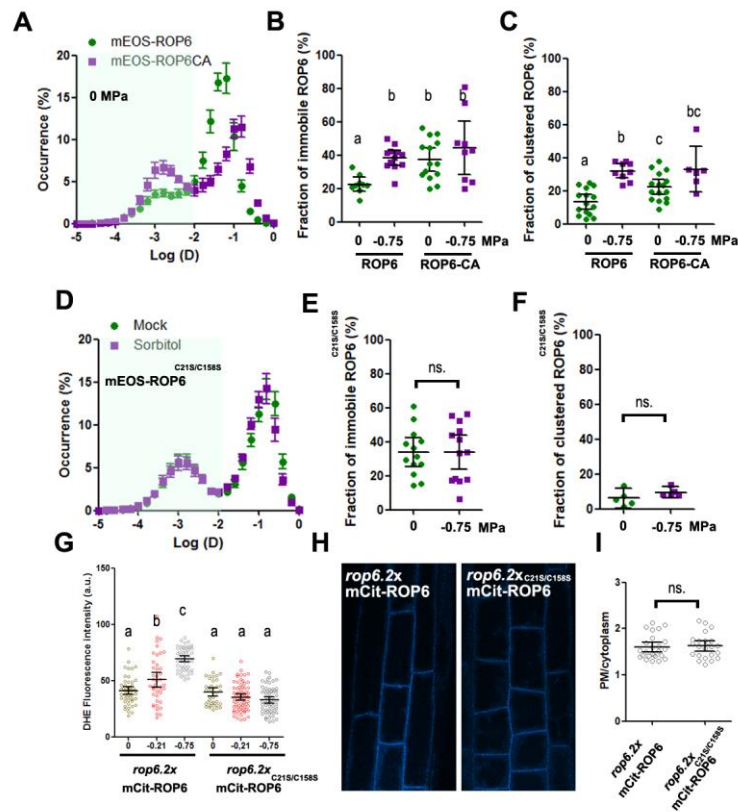


Figure 3: ROP6 nanoclustering is required for ROS accumulation. (A) Bimodal distribution of mEOS2-ROP6 instantaneous diffusion (green curve) or mEOS2-ROP6-CA (purple curve) in control condition (0 MPa). (B) Histogram represents the percentage of molecules with an instantaneous diffusion below $0.01 \text{ } \mu\text{m}^2.\text{s}^{-1}$ in mEOS2-ROP6 and mEOS2-ROP6-CA expressing lines in control (green) or after osmotic stimulation (purple). (C) Percentage of molecules with a $\log(\text{local density})$ higher than 3 in mEOS2-ROP6 and mEOS2-ROP6-CA expressing lines in control (green) or after osmotic stimulation (purple). (D) Bimodal distribution of mEOS2-ROP6^{C21S/C158S} instantaneous diffusion coefficients in control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions. (E) Histogram represents the percentage of mEOS2-ROP6^{C21S/C158S} molecules with an instantaneous diffusion below $0.01 \text{ } \mu\text{m}^2.\text{s}^{-1}$ in control (0 MPa) and treatment (-0.75 MPa) conditions. (F) Percentage of mEOS2-ROP6^{C21S/C158S} molecules with a $\log(\text{local density})$ higher than 3. (G) Quantification of ROS accumulation by DHE staining in *rop6.2xmCit-ROP6* or *rop6.2xmCit-ROP6*^{C21S/C158S} expressing cells after 15 min treatment with 0, -0.26 or -0.75 MPa solution. (H) Plasma membrane localization of mCit-ROP6 and mCit-ROP6^{C21S/C158S} and its relative amount at the PM (I). Error bars correspond to a confidence interval at 95%. * p-value below 0.01 *t*-test. ns. Non-significant. $n > 9$ from 3 independent biological replicates. Scale bar $10 \mu\text{m}$.

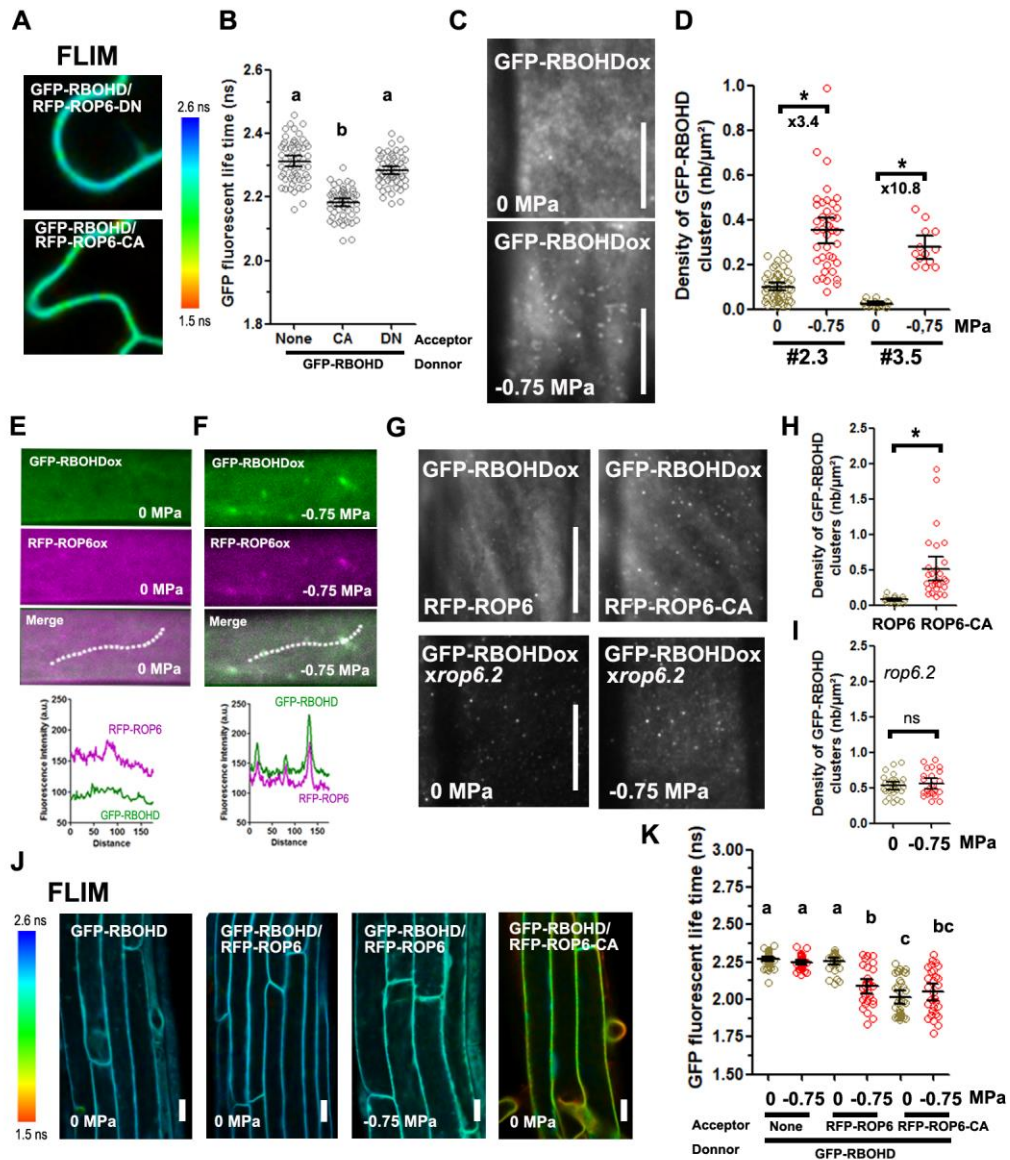


Figure 4: ROP6 interacts and forms nanoclusters with RBOHD at the PM. (A) GFP-RBOHD fluorescence lifetime when co-expressed with dominant negative (RFP-ROP6-DN) or constitutive active ROP6 (RFP-ROP6-CA) in transient expression in tobacco leaf epidermal cells and its quantification (B). (C) TIRF micrograph of cell expressing GFP-RBOHD in control or after 2 minute treatment with -0.75 MPa solution and quantification of clusters density (D). (E, F) Cell co-expressing GFP-RBOHD with RFP-ROP6 in control (E) or after -0.75 MPa treatment (F). Graphs below represent the pixel intensity along the dotted line in each of the conditions. (G) TIRFM micrograph of GFP-RBOHD signal in GFP-RBOHDxRFP-ROP6, GFP-RBOHDxRFP-ROP6-CA and *rop6.2*xGFP-RBOHD plant in control or after -0,75 MPa incubation and their respective quantification (H and I). (J) GFP-RBOHD fluorescence lifetime when expressed alone or co-expressed with RFP-ROP6 or RFP-ROP6-CA in root cells and its quantification (K). Error bars correspond to a confidence interval at 95%. For (B) an ANOVA followed by Tukey test was done, letters indicate significant differences among means (p -value <0.001). * p -value below 0.01 T-Test. $n>12$ from 3 independent biological replicates. Scale bar $10\mu\text{m}$.

See also Figure S5

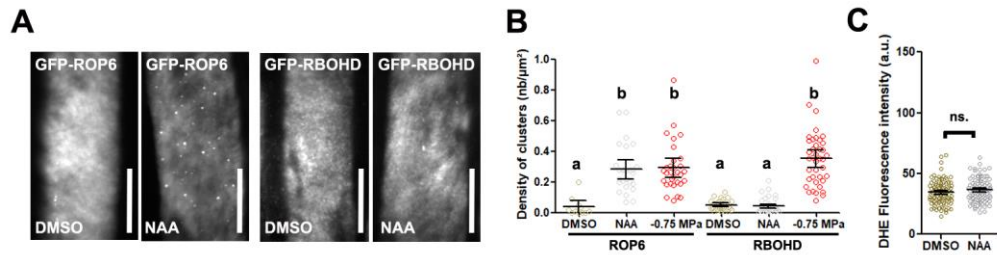


Figure 5: Auxin-stimulated ROP6 nanodomains are free of RBOHD. (A) TIRFM micrograph of cell expressing GFP-ROP6 or GFP-RBOHD in control condition (DMSO) or after 10 μM NAA for 1 hour. (B) Cluster density quantification after NAA or -0,75 MPa treatment. (C) Quantification of ROS accumulation by DHE staining in control (DMSO) or after 15 min treatment with 10 μM NAA. Error bars correspond to a confidence interval at 95%. For (B) an ANOVA followed by Tukey test was done, letters indicate significant differences among means (p -value <0.001). * p -value below 0.01 T-Test. $n>11$ from 3 independent biological replicates. Scale bar 10 μm .

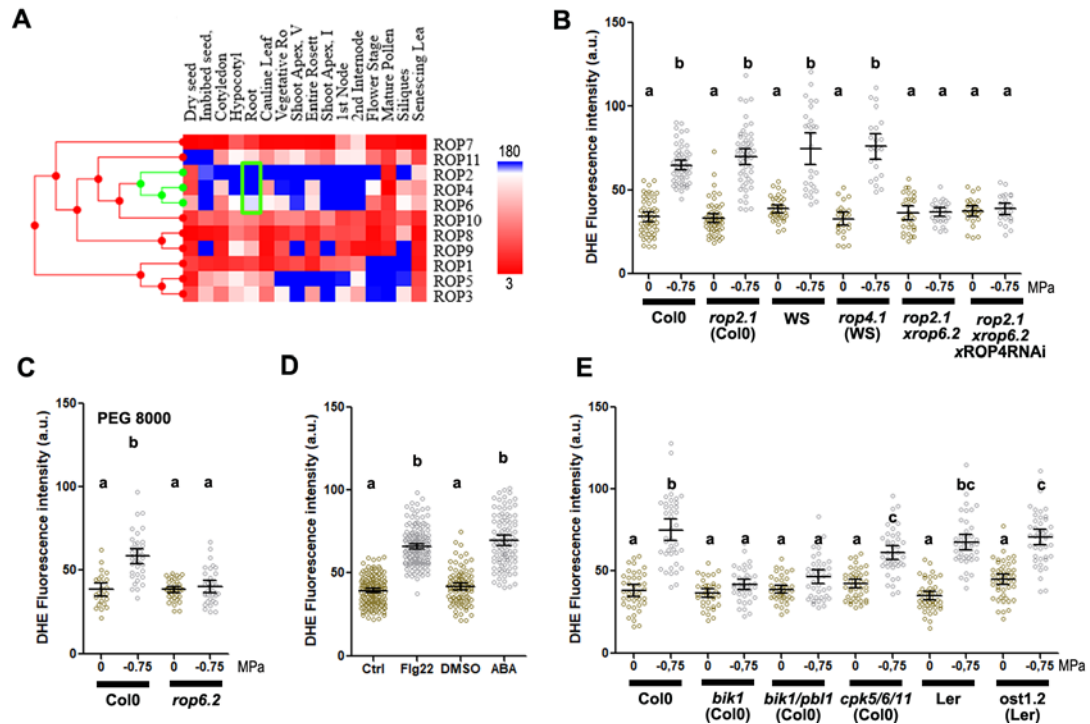


Figure S1: Expression pattern of different *ROP* isoforms and ROS production phenotypes of single and multiple mutants. Related to Figure 1.

(A) Gene expression clustering of the different *ROP* isoforms based on eFP-browser databases. Green square shows the three isoform highly expressed in root tissue (*ROP2*, *ROP4* and *ROP6*). (B-E) Quantification of ROS accumulation (DHE staining) in control or after 15 minutes of -0.75 MPa treatment in the indicated genotype, with either sorbitol (B and E), PEG8000 (C), or with 1 μ M flg22 for 30 minutes (D) or 1 μ M of ABA for 1 hour (D). Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test was done, letters indicate significant differences among means (p-value<0.001). n>21 from at least 2 independent biological replicates.

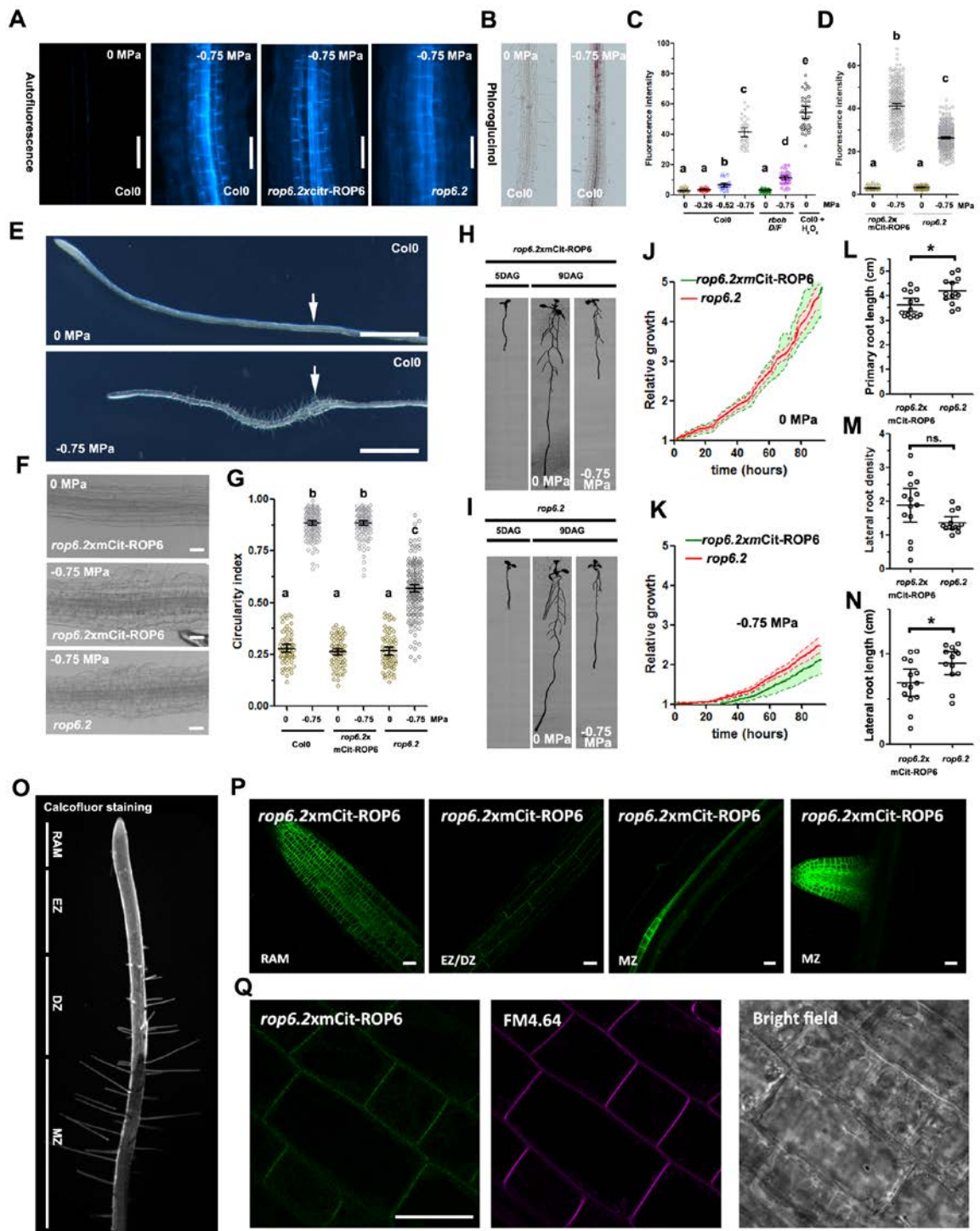


Figure S2: Expression pattern and implication of ROP6 in plant responses to osmotic stimulus. Related to Figure 1.

(A) Cell autofluorescence of *rop6.2* and complemented lines expressing mCit-ROP6 under ROP6 endogenous promoter in control plate or after -0.75 MPa treatment for 24 hours. (B) Phloroglucinol staining, that shows pink precipitates when in complex with lignin in control condition or after -0.75 MPa treatment for 24 hours. (C) Cell autofluorescence quantification of Col-0 plant exposed for 24 hours to control, -0.26, -0.5, -0.75 MPa. As a comparison, cell autofluorescence was also observed in *rbohDxrbohF* line exposed to -0.75 MPa and Col-0 treated for 1 hour with 1mM H₂O₂. (D) Cell autofluorescence quantification in *rop6.2* and *rop6.2xmCit-ROP6* in control or treated (-0.75 MPa) conditions. (E) 2 days after transfer on -0.75 MPa plate, root cells present inflated cells (arrow). The arrows are located at the point where the root tip was at the time of transfer. (F) Close up view of cells in this zone in control condition or after treatment (-0.75 MPa) for *rop6.2xmCit-ROP6* or *rop6.2*. (G) Quantification of cell circularity index. (H-N) the complemented line (*rop6.2xmCit-ROP6*) or the mutant *rop6.2* were grown 5 days on control plates and then transferred for 4 more days in either control condition or on plate supplemented with osmoticum to reach -0.75 MPa. Relative growth of *rop6.2xmCit-ROP6* or *rop6.2* in control (J) or in -0.75 MPa plate (K). Quantification of the primary root length (L), lateral density (M) and lateral root length (N) of *rop6.2xmCit-ROP6* or *rop6.2* grown on -0.75 MPa plates. (O) Arabidopsis control plant counterstained with calcofluor bright to illustrate the different root zone. Root apical meristem (RAM), elongation zone (EZ), differentiation zone (DZ) and mature zone (MS). (P) Representative micrograph of the fluorescent signal observed in *rop6.2* lines complemented with mCit-ROP6 under ROP6 endogenous promoter. (Q) mCit-ROP6 signal is mostly visible at the cell PM, as revealed by FM4-64 staining. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test was done, letters indicate significant differences among means (p value <0.001). Scale bar 20µm for (A, F, P and Q), 2 mm for (E). n>14 from 3 independent biological replicates.

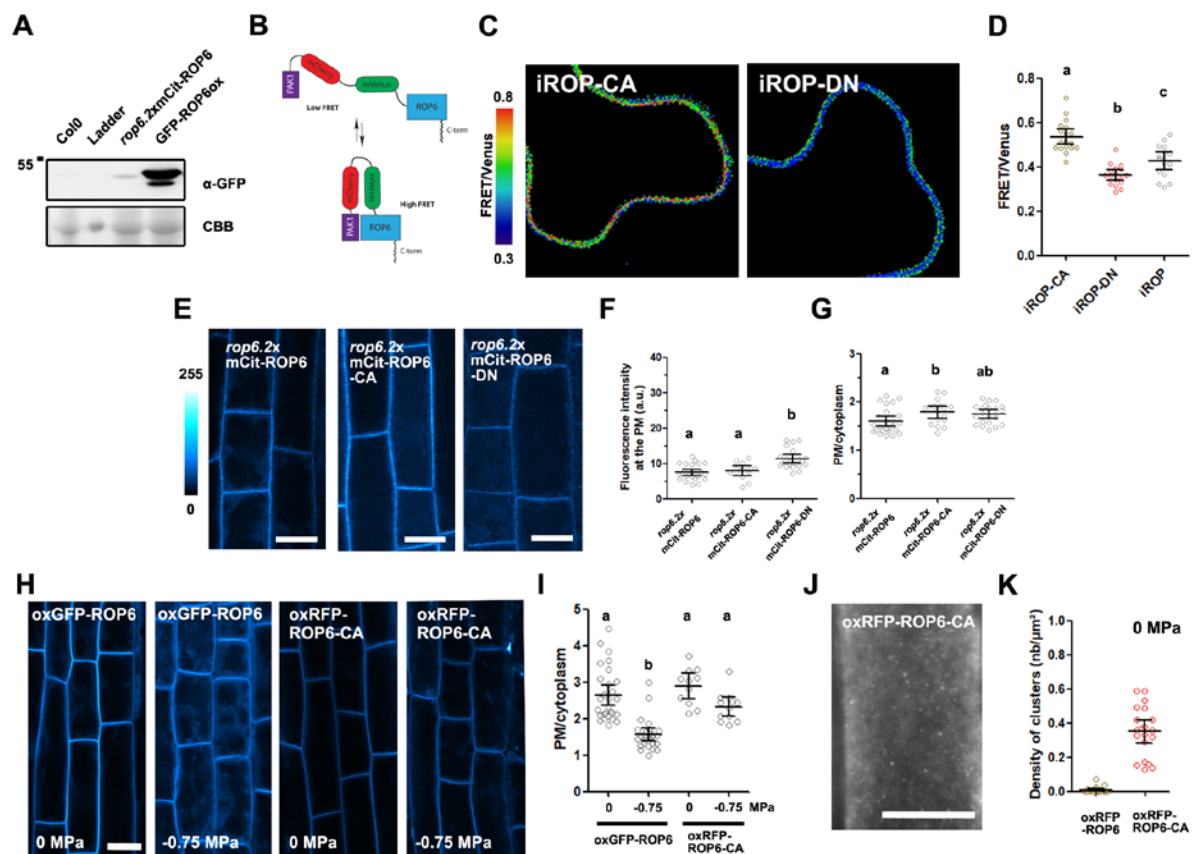


Figure S3: Characterization of GFP-ROP6 overexpressing line, detection of ROP6 activation with iROP sensor and localization of *rop6.2xmCit-ROP6*, *rop6.2xmCit-ROP6-CA* and *rop6.2xmCit-ROP6-DN*. Related to Figure 1 and 2.

(A) Western blot with antibody against GFP on plant protein extract from Col-0, ROP6 complemented line (*rop6.2xmCit-ROP6*) and ROP6 overexpressing line (oxGFP-ROP6). (B) Schematic view of iROP sensor. GTP-bound form of ROP6 interacts with PAK1, allowing FRET between Venus and mCherry. In contrast, if ROP6 is inactive in its GDP-bound form the distance between the two fluorescent proteins increases, thereby diminishing FRET efficiency. (C) Ratio images of transient expression of iROP sensors locked in GTP form (iROP-CA) or in GDP form (iROP-DN). (D) Relative variation of FRET between iROP-CA, iROP-DN and iROP. (E) Confocal micrograph showing the localization of wild type ROP6 (mCit-ROP6), constitutive active ROP6 (mCit-ROP6-CA) and dominant negative ROP6 (mCit-ROP6-DN). (F,G) Respective fluorescence (F) and relative amount at the PM (G) of the same forms. (H) Confocal micrograph showing the localization of oxGFP-ROP6 and oxRFP-ROP6-CA in control condition and after -0.75 MPa treatment and its respective quantification (I). (J) TIRFM micrograph of oxRFP-ROP6-CA expressing cells. (K) Quantification of clusters density of oxGFP-ROP6 and oxRFP-ROP6-CA in control condition. CBB, Coomassie brilliant blue. $n > 11$ from at least 2 independent biological replicates. Scale bar 10 μ m.

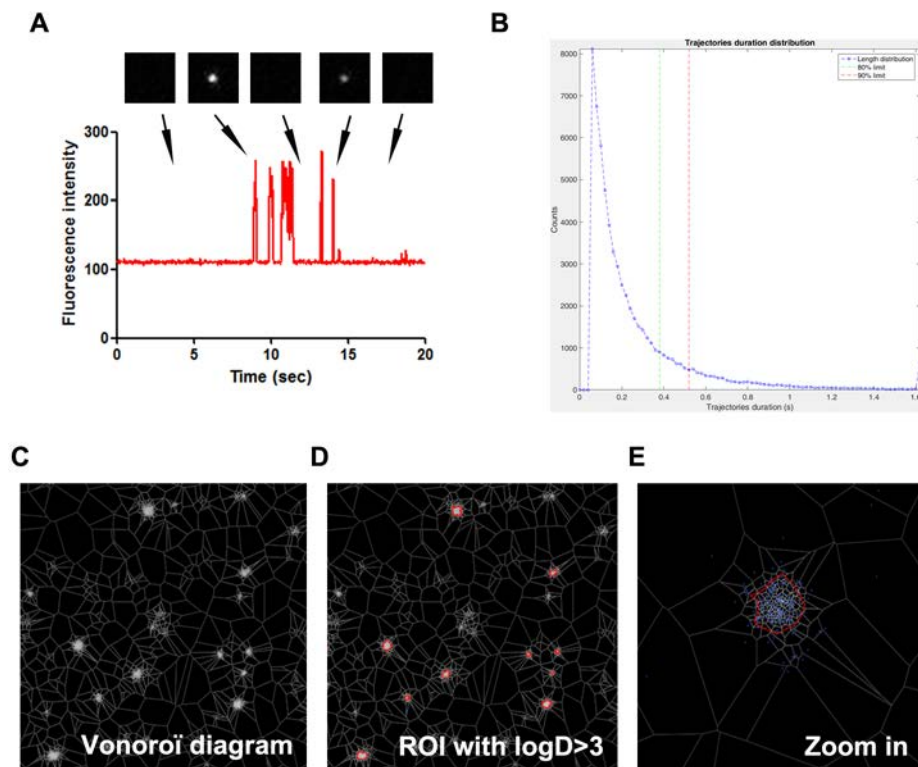


Figure S4: ROP6 single-molecule imaging and Voronoi tessellation. Related to Figure 2

(A) To verify that we are indeed recording single mEOS2-ROP6 molecules, we plot fluorescence intensity of a typical mEOS2-ROP6 sub-diffractive cluster along time. The signal intensity observed is not continuous and the OFF state vary in duration between seconds and milliseconds. This blinking behaviour is typical from single-molecule observation. We also quantify the track duration (B). As expected from single molecules, vast majority of the tracks do not last for more than 0.5 seconds. (C) Picture of Voronoi diagram, where each point/seeds corresponds to a mEOS2-ROP6 localization and edges of Voronoi cells are represented in white. (D) Segmented region of interest (ROI) with a particle local density greater than $\log(\text{local density}) > 3$ (ROI appear in red). (E) Close up view of a ROP6 nanodomain where each blue dot represents one mEOS2-ROP6 localization.

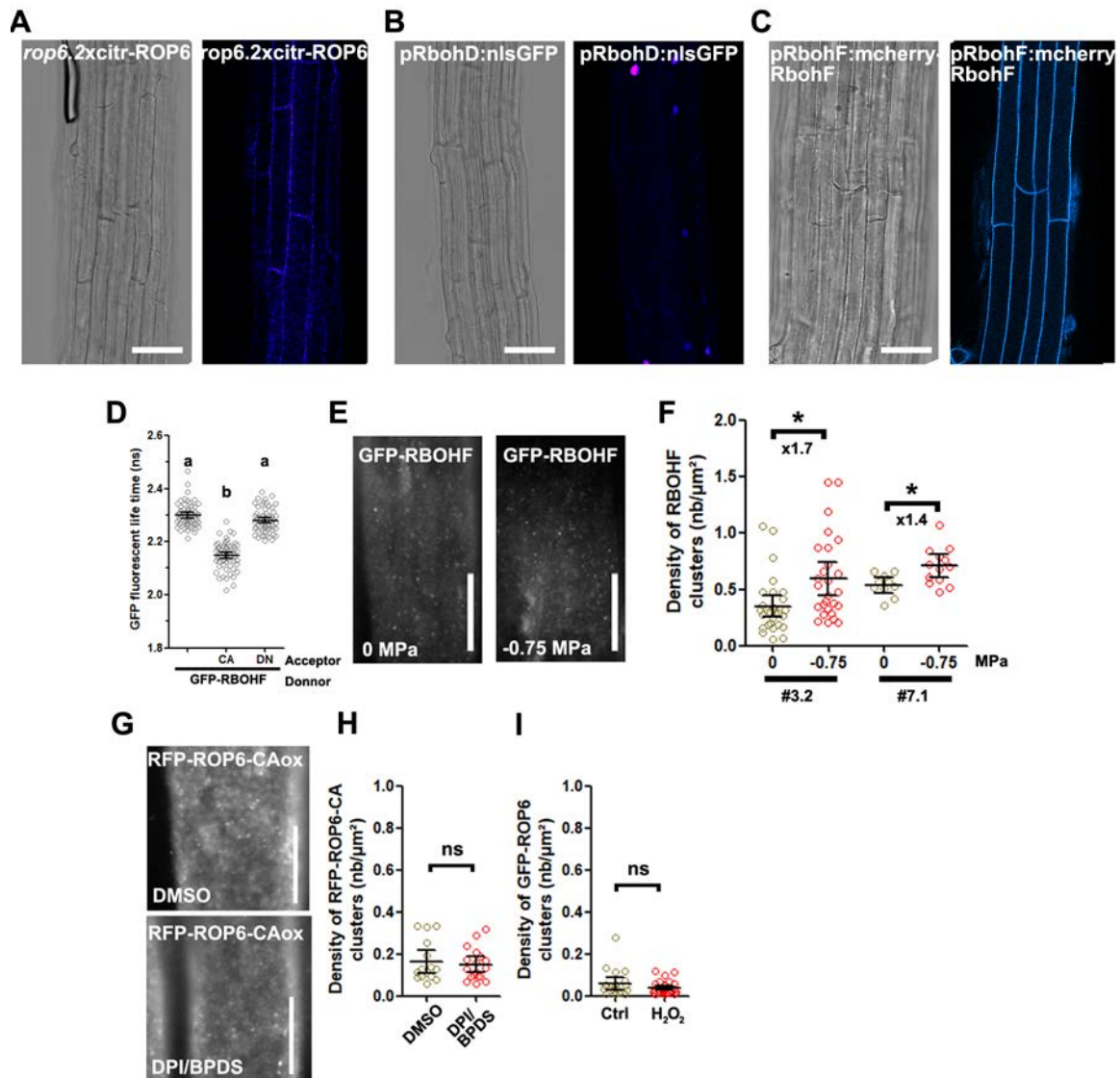


Figure S5: ROP6, RBOHD and RBOHF expression pattern, interaction and cluster formation. Related to Figure 4.

Expression pattern of the translational fusion pROP6:mCit-ROP6 (A) and pRBOHF:mCherry-RBOHF (B) and the transcriptional fusion pRBOHD:nls-GFP-GUS (C). (D) Quantification of GFP-RBOHF fluorescence life time expressed in transient expression in tobacco leaf epidermal cells, either alone, or co-expressed with the dominant negative (RFP-ROP6-DN) or the constitutive active ROP6 (RFP-ROP6-CA). (E) TIRFM micrograph of cell expressing GFP-RBOHF in control or after 2 minutes treatment with -0.75 MPa solution and quantification of cluster density (F). (G) TIRFM micrograph of RFP-ROP6-CA signal in control condition or after incubation for 30 min with ROS inhibitors and its quantification (H). DPI was used for inhibition of NADPH oxidase activity and BPDS was

used to inhibit ROS produced from ferric iron. (I) Quantification in GFP-ROP6 cluster density in control condition or after H₂O₂ treatment. Error bars correspond to a confidence interval at 95%. For (D and F), ANOVA followed by Tukey test was done, letters indicate significant differences among means (p-value<0.001). ns: p-value above 0.05 T-Test. n>9 from 3 independent biological replicates. Scale bar 5µm for A, B and C and 10µm for E and G.

Gene	Gene-ID	Mutant allele	Ecotype	Mutant ID
ROP6	At4g35020	<i>rop6-2</i>	Col-0	SALK_091737C [29]
ROP2	At1g20090	<i>rop2-1</i>	Col-0	SALK_055328 [49]
ROP4	At1g75840	<i>rop4-1</i>	WS	Wisconsin T-DNA line [50]
		<i>rop6-2xrop2-1</i>	Col-0	crossing parental single homozygous lines [51]
		<i>rop6-2xrop2-1xROP4RNAi</i>	Col-0	ROP4-RNAi was added on double homozygous <i>rop2-1</i> and <i>rop6-2</i> line [51]
BIK1	At2g39660	<i>bik1</i>	Col-0	SALK_005291 [52]
PBL1	At3g55450			
		<i>bik1xpbl1</i>	Col-0	cross between <i>bik1</i> (from Veronese et al., 2006) and SAIL_1236_D07 lines [53]
CPK5	At4g35310			
CPK6	At2g17290			
CPK10	At1g18890			
		<i>cpk5/6/11</i>	Col-0	<i>cpk5</i> :Sail_657C06 from Syngenta; <i>cpk6</i> :salk_025460; <i>cpk11</i> :salk_054495 [54]
OST1	At4g33950	<i>ost1-1</i>	Ler	EMS II-52, II-56, II-123 [55]
RBOHD	AT5G47910			
RBOHF	AT1G64060			

Table S1. List of identifiers for genes and single mutant alleles used in this study. Related to Star Methods

Gateway expression vectors

	Promoter	N-terminal Tag	ORF / CDS	C-terminal Tag	Plant selection marker	Bacterial selection marker	Binary vector
pROP6:mCit-ROP6g-CA	ROP6-Promoter	mCitrine vo STOP	mutated (G15V) ROP6 genomic sequence	/	Basta	Kana	pB7m34GW
pROP6:mCit-ROP6g-DN	ROP6-Promoter	mCitrine vo STOP	mutated (T20N) ROP6 genomic sequence	/	Basta	Kana	pB7m34GW
pROP6:mCITRINE-ROP6gC21S-C158S	ROP6-Promoter	mCitrine no STOP	mutated (C21S-C158S) ROP6 genomic sequence	/	Basta	Kana	pB7m34GW
p35S:mEOS2-ROP6gC21S-C158S	35S-Promoter	mEOS no STOP	mutated (C21S-C158S) ROP6 genomic sequence	/	Basta	Kana	pB7m34GW
p35S:GFP-RBOHD	35S-Promoter	GFP with linker	RBOHD / CDS	/	Basta	Spect	pB7WGF2
p35S:GFP-RBOHF	35S-Promoter	GFP with linker	RBOHF / CDS	/	Basta	Spect	pB7WGF2
p35S:GFP-ROP6	35S-Promoter	GFP with linker	ROP6 / CDS	/	Basta	Spect	pB7WGF2
p35S:RFP-ROP6	35S-Promoter	RFP with linker	ROP6 / CDS	/	Basta	Spect	pB7WGR2
p35S:RFP-ROP6-CA	35S-Promoter	RFP with linker	ROP6-CA (G15V)/ CDS	/	Basta	Spect	pB7WGR2
iROP	35S-Promoter	CRIB of PAK1	mcherry-mvenus	ROP6 / CDS	BASTA	Spect	pB7m34GW

Table S2. List of used expression vectors. Related to Star Methods

	Name	Sequence
Cloning of PCR fragments	ROP6-B2R	ggggacagctttctgtacaaagtggtatgagtgctcaaggttatcaagtg
	ROP6-B3w3'UTR	ggggacaactttgtataataaagttgccttaagacaattggtgtgaatctagg
	F-RBOHD	cttgcgccgcccccttcaaatgagacgagg
	R-RBOHD	gcaaggcgcgccacccttctagaagttctctttgtgg
	F-RBOHF	cttgcgccgcccccttcaaaccgttctcaaagaac
	R-RBOHF	gtcggcgcgccacccttttagaaatgctccttg
	R-ROP6 (CDS)	agcggccgccagtgctcaaggttatc
	F-ROP6 (CDS)	tggcgcgccctcagagtatagaacaacc
	ROP6prom-Rev	ttttgtacaaactgcctttctctccttctcaactc
	ROP6prom-Fw	gtatagaaaagtgtaacaagcttcagaaaagaggatg
Mutagenesis	ROP6-CA-(G15V)_F	gtcggcgacgttgctgttgaaagacttgc
	ROP6-CA-5G15V)_R	tccaacagcaacgtcgccgacagtgacacacttgataaacc
	ROP6-DN(T20N)-F	ggtgctgttgaaagaattgtctctcatctcctacactagc
	ROP6-DN(T20N)-R	atgagaagacaattcttccaacagcaccgtcgccgacagtg
	Mut-C152S_R	gagttttgactggattcgatataagcaggcgcgcccaatcagctcttagtc
	Mut-C152S_F	atcgaatccagtgcaaaaactcaacaggtattaacctgagagtcaatatctttatc
	Mut-C21S-WT_F	ggaaagacttctctctcatctcctacactagcaaacactttccccacggtagc
	Mut-C21S-WT_R	agatgagaagagaagctttccaacagcaccgtcgccgacagtgacacactg

Table S3. List of used oligonucleotides related to Star Methods