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## Osmotic Stress Activates Two Reactive Oxygen Species Pathways with Distinct Effects on Protein Nanodomains and Diffusion

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1 **Short title: ROS production effects protein nano-organisation**

2 **Osmotic stress activates two reactive oxygen species pathways with distinct**  
3 **effects on protein nanodomains and diffusion**

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16 **One-sentence summary** Hyperosmotic stress induces reorganization and increased diffusion  
17 of plasma membrane proteins by a specific ROS production pathway

18

## 19 **AUTHOR CONTRIBUTIONS**

20 AM, MS, CA, XD, KH and YJ preformed research; JBF and MN developed new analytic and  
21 computational tools; AM and SM design research; AM and CM wrote the paper.

22

23 **ABSTRACT**

24 Physiological acclimation of plants to an ever-changing environment is governed by complex  
25 combinatorial signaling networks that perceive and transduce various abiotic and biotic  
26 stimuli. Reactive oxygen species (ROS) serve as one of the second messengers in plant  
27 responses to hyperosmotic stress. The molecular bases of ROS production and the primary  
28 cellular processes that they target were investigated in the *Arabidopsis* (*Arabidopsis thaliana*)  
29 root. Combined pharmacological and genetic approaches showed that RESPIRATORY  
30 BURST OXIDASE HOMOLOG (RBOH) pathway and an additional pathway involving  
31 apoplastic ascorbate and iron can account for ROS production upon hyperosmotic stimulation.  
32 The two pathways determine synergistically the rate of membrane internalization, within  
33 minutes following activation. Live super-resolution microscopy revealed at single-molecule  
34 scale how ROS control specific diffusion and nano-organization of membrane cargo proteins.  
35 In particular, ROS generated by RBOHs initiated clustering of the PIP2;1 aquaporin and its  
36 removal from the plasma membrane. This process is contributed to by clathrin-mediated  
37 endocytosis, with a positive role of RBOH-dependent ROS, specifically under hyperosmotic  
38 stress.

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## 42 INTRODUCTION

43 Terrestrial plants can experience dramatic changes in water availability when exposed to  
44 environmental challenges as diverse as drought, soil salinity, and fluctuations in air humidity  
45 or even temperature. To maintain their water status and acclimate to these environmental  
46 constraints, plants have evolved numerous physiological or developmental responses,  
47 including short-term regulation of stomatal aperture and tissue hydraulics and, on a longer  
48 term, alteration of root system architecture and leaf abscission. Despite their central role, the  
49 early cellular events that lead to these adaptive responses are largely unknown.

50 A few molecules and mechanisms that contribute, at least partially, to osmotic stress  
51 perception have emerged from recent studies. As first described in bacteria and now in plants,  
52 osmotic shocks result in mechanical stimuli that activate a group of nonselective  
53 mechanosensitive ion channels and help the cell to counteract excessive membrane tensions  
54 (Martinac et al., 1987; Hamilton et al., 2015b). In addition, the transmembrane histidine  
55 kinase ATHK1 was suggested to act as an osmosensor, similar to its yeast homolog (Urao et  
56 al., 1999). Finally, a direct genetic screen identified cations channels of the Reduced  
57 Hyperosmolality, Induced Ca<sup>2+</sup> Increase 1/Calcium Permeable Stress-Gated  
58 Cation Channel 1 (OSCA/CSC1) gene family as mediating calcium influx during  
59 hyperosmotic stress (Hou et al., 2014; Yuan et al., 2014). Loss-of-function mutant plants  
60 showed a reduced stomata closure and altered root growth responses in stress conditions  
61 (Yuan et al., 2014).

62 Several types of second messengers are possibly involved, downstream of osmotic  
63 signal perception. For instance, a substantial apoplastic alkalization occurs concomitant to  
64 calcium influx in the first minutes following exposure of roots to salt (Choi et al., 2014; Guo  
65 et al., 2009; Gao et al., 2004; Stephan et al., 2016). Reactive oxygen species (ROS), which  
66 accumulate in the frame of tens of minutes following osmotic stress, also represent key  
67 second messengers during hyperosmotic signaling (Leshem et al., 2007). ROS signaling  
68 mediated by NADPH oxidases of the RESPIRATORY BURST OXIDASE HOMOLOG  
69 (RBOH) family are generally accepted as the dominant pathway in plants (Baxter et al., 2014;  
70 Mittler, 2017). By using the cytoplasmic pool of NADPH, these enzymes catalyze the  
71 production of apoplastic superoxide, which is further transformed into hydrogen peroxide by  
72 spontaneous dismutation or superoxide dismutase (SOD) activities (Figure 1A). Loss-of-  
73 function plants for *RBOHD* or *RBOHF* showed a reduced ROS accumulation in response to  
74 numerous environmental stimuli, including salt stress (Leshem et al., 2007). In addition,

75 *rbohD* or *rbohF* mutant plants displayed reduced proline accumulation in response to a long  
76 hyperosmotic treatment (Ben Rejeb et al., 2015). Thus, RBOHD or RBOHF represent good  
77 candidates for ROS production under osmotic stress

78

79 Osmotic stress exerts strong and rapid effects on cell membrane dynamics. Whereas  
80 membrane proteins should freely diffuse in the plane of the membrane due to thermal motion,  
81 a lot of plant plasma membrane (PM) proteins are essentially immobile (Martinière et al.,  
82 2012). This suggests an anchoring of these proteins to fix them in place. Within minutes  
83 following an osmotic or salt treatment, PLASMA MEMBRANE INTRINSIC PROTEIN 2;1  
84 (PIP2;1) was found to start diffusing within the PM (Li et al., 2011; Hosy et al., 2015). High  
85 salt and sorbitol concentrations also enhance exchanges between the PM and endosomes  
86 within the same time frame. In particular, a strong bulk membrane internalization was  
87 revealed by FM4-64 uptake (Leshem et al., 2007; Zwiewka et al., 2015). In addition, all cargo  
88 proteins tested so far, among which the PIP2;1 aquaporin, PIN-FORMED 2 (PIN2) auxin  
89 transporter, and BRASSINOSTEROID INSENSITIVE 1 (BRI1) brassinosteroid receptor are  
90 depleted from the PM (Zwiewka et al., 2015; Li et al., 2011). Thus, osmotically induced bulk  
91 membrane internalization is thought to drive the removal of cargo proteins from the PM.  
92 Wudick et al. (2015) recently demonstrated that external application of hydrogen peroxide on  
93 root cells enhances PIP2;1 lateral diffusion and endocytosis, thereby mimicking the effects of  
94 a salt or hyperosmotic treatment (Wudick et al., 2015). A link between membrane dynamics  
95 and ROS signaling has also been demonstrated upon cryptogenin elicitation of tobacco  
96 (*Nicotiana tabacum*) BY2 cells. Cryptogenin induced clathrin-dependent endocytosis, and  
97 cells silenced for *NtRboh* had less clathrin foci at the PM than control ones (Leborgne-Castel  
98 et al., 2008). The exact mechanisms by which ROS act on membrane and cargo protein  
99 dynamics are not yet known.

100 In this work, we used *Arabidopsis* (*Arabidopsis thaliana*) roots to investigate early cell  
101 responses to osmotic stress. We focused on the molecular mechanisms of stress-induced  
102 production of ROS and their subsequent impact on PM dynamics. We identified, besides  
103 RBOH family members, another source of ROS involving ascorbate and iron (Fe). Whereas  
104 ROS play a general role in bulk endocytosis, we report on the behavior of individual  
105 membrane protein molecules and show that PIP2;1 nanodomain organization and endocytosis  
106 are strictly under the control of RBOH-dependent ROS. Super-resolution microscopy was  
107 used to further investigate the role of the clathrin machinery in these specific membrane  
108 dynamic responses.

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## 112 RESULTS

### 113 RBOHs contribute only partially to osmotically induced ROS production.

114 Production in plant tissues of ROS, and superoxide ( $O_2^-$ ) in particular, can be monitored  
115 through oxidation and enhanced fluorescence of dehydroethidium (DHE) dye (Figure 1B)  
116 (Tsukagoshi et al., 2010; Chen et al., 2013). A steady increase in fluorescence for up to 120  
117 min was observed in 5-day-old plantlets incubated in the presence of DHE (Supplemental  
118 Figure 1). When plantlets were concomitantly treated with 300 mM sorbitol, the rate of DHE  
119 fluorescence increase was enhanced 2-fold (Supplemental Figure 1), within all cell types  
120 (Figure 1B). Hydroxyphenyl fluorescein (HPF), another ROS-sensitive dye that is mostly  
121 reactive with hydroxyl radicals (Tsukagoshi et al., 2010), revealed an essentially similar  
122 osmotic-stress-induced staining pattern (Supplemental Figure 2A). These observations  
123 conform to the idea that osmotic stress rapidly (i.e. within minutes) induces an  
124 overaccumulation of ROS in Arabidopsis roots.

125 Because the role of RBOH proteins was never formally tested in the context of osmotic stress,  
126 we used single (*rbohD* and *rbohF*) and double (*rbohD/F*) loss-of-function mutants. With  
127 respect to Col-0 plants, ROS accumulation in these genotypes was not altered in resting  
128 conditions. In contrast, the ROS response to sorbitol was partially reduced in each of the  
129 single mutants, with no further decrease in the *rbohD/F* double mutant (Figure 1C). The  
130 residual response of the latter plants suggested that other RBOH isoforms may be involved.  
131 Therefore, we used diphenylene iodonium (DPI), which inhibits all RBOHs by interacting  
132 with their flavin adenine dinucleotide (FAD) binding domain. In these experiments, plants  
133 were pretreated for 30 min with the inhibitor prior to staining with DHE for 15 min  
134 (Supplemental Figure 3). DPI slightly reduced ROS accumulation in Col-0 roots under resting  
135 conditions and partially inhibited the sorbitol-induced ROS response (compare Figure 1C and  
136 1D). In contrast, the ROS response to sorbitol was fully insensitive to DPI in the *rbohF/D*  
137 double mutant (Figure 1F). These results show that RBOHD and F both contribute to  
138 osmotically induced ROS accumulation, though in a nonadditive manner. The data also show  
139 that an additional pathway, independent of RBOH, contributes to the osmotic-stress-induced  
140 ROS response.

### 141 Osmotically induced ROS production also involves reduced transition metals

142  $O_2^-$  can also be produced, independently of NADPH oxidases, by a Haber-Weiss reaction  
143 whereby reduced transition metals transfer one electron to dioxygen (Figure 1E). To

144 investigate the contribution of this mechanism to ROS accumulation under osmotic stress, we  
145 treated plants with bathophenanthrolinedisulfonic acid (BPDS), a membrane-impermeant  $\text{Fe}^{2+}$   
146 chelator that depletes free  $\text{Fe}^{2+}$  in the cell apoplasm. Whereas BPDS had no effect on DHE  
147 fluorescence in control conditions, it induced a significant reduction of ROS accumulation  
148 upon sorbitol treatment (Figure 1G).

149 Concomitant treatment of plants with DPI and BPDS resulted in a slight decrease in ROS  
150 accumulation in resting conditions and totally abolished the sorbitol effects (Figure 1G).  
151 When treated with BPDS, the *rbohD/F* double mutant also lost its sorbitol-induced ROS  
152 accumulation (Figure 1H). When HPF staining was used, a single pretreatment with DPI or  
153 BPDS totally inhibited the sorbitol-induced ROS response (Supplemental Figure 2B). These  
154 slight differences in response between DHE and HPF staining are likely due to their distinct  
155 sensitivity to the various ROS species (Tsukagoshi et al., 2010). Nevertheless, the overall data  
156 indicate that two ROS production pathways, mediated by RBOH and reduced iron,  
157 respectively, contribute to most of, if not all, the overaccumulation of ROS in Arabidopsis  
158 roots under osmotic stress.

### 159 **A role of apoplastic ascorbate in ROS accumulation**

160 Since reduced iron is considered to be almost absent in cells due to its very high toxicity, an  
161 iron reduction mechanism must be active during osmotic stress. We hypothesized that  
162 ascorbate could represent an alternative reducing agent (Grillet et al., 2014). Treatment of  
163 roots with purified ascorbate oxidase (AOX) did not alter DHE staining in resting conditions,  
164 whereas it reduced the sorbitol-induced response by around 20% (Figure 1G). When AOX  
165 was coincubated with DPI, the residual sorbitol-induced ROS accumulation was totally  
166 abolished (Figure 1G). These results suggest that, complementary to RBOHs, ascorbate is  
167 necessary to trigger ROS accumulation, probably in association with iron. To investigate this  
168 point further, we used a *vtc2.4* mutant that, with respect to Col-0, shows a reduction in  
169 ascorbate accumulation by two-thirds, without any major growth defect in standard condition  
170 (Grillet et al., 2014). Compared to Col-0, *vtc2.4* accumulated less ROS after osmotic  
171 stimulation; this response did not occur when plants were cotreated with DPI (Figure 1I). To  
172 confirm the limiting role of ascorbate in ROS accumulation, we applied 100  $\mu\text{M}$  ascorbate  
173 onto roots and found a significant increase of ROS accumulation after 15 min (Figure 1J).

174 The results presented above were obtained after treatment with 300 mM sorbitol, which  
175 induces cell incipient plasmolysis (Supplemental Figure 4A). Under milder stress conditions

176 (100 mM sorbitol), cells experience a reduction in turgor with no major change in volume  
177 (Supplemental Figure 4A). Yet, this sorbitol treatment induced a ROS accumulation, of lower  
178 amplitude than with 300 mM, but with an essentially similar pharmacological inhibition  
179 profile. In particular, cotreatments with DPI and BPDS or DPI and AOX fully inhibited the  
180 osmotic response (Supplemental Figure 4B). These results indicate that the RBOH and  
181 iron/ascorbate pathways can be activated under a wide range of mild to pronounced osmotic  
182 stresses.

183 Due to their plasma membrane localization, RBOHD and F are expected to produce  
184 superoxide in the apoplast. In addition, exogenously supplied AOX, which is marginally  
185 permeable to cell membranes, inhibited ROS accumulation (Figure 1G). Thus, the pool of  
186 ascorbate that contributes to osmotically induced ROS accumulation also seems to be  
187 localized in the apoplast. To assess ROS accumulation in this compartment, we investigated  
188 DHE staining with confocal microscopy. In control conditions, oxidized DHE was localized  
189 in small dotted structures (Supplemental Figure 5, arrowheads) and in the nucleus (star).  
190 When cells were incubated with 300 mM sorbitol for 15 min, some staining was observed in  
191 the apoplast (Supplemental Figure 5, arrows). These observations suggest that at least a part  
192 of the ROS accumulation triggered by osmotic stress takes place in this compartment.

### 193 **The RBOH and iron/ascorbate pathways both contribute to activation of membrane** 194 **internalization, but with distinct impacts on cargo proteins**

195 Plant cells react to osmotic stress by modifying the rate of endocytosis. To investigate this  
196 process, we cotreated plants with the lipophilic fluorescent endocytic tracer FM4-64 and the  
197 fungal toxin Brefeldin A (BFA) and quantified the number of FM4-64-labeled BFA bodies  
198 per cell (Geldner et al., 2003). As expected, an increase in FM4-64 labeling of intracellular  
199 structures was observed in osmotically challenged plants (Figures 2A and 2B). In Col-0 plants  
200 treated with DPI or *rbohD/F* plants, intracellular labeling responses similar to those of Col-0  
201 were observed under both control and sorbitol treatment conditions (Figures 2B and 2C). By  
202 comparison, Col-0 plants treated with BPDS showed a small decrease in osmotically induced  
203 FM4-64 labeling (Figure 2B). Moreover, a full inhibition of this response was observed when  
204 a BPDS treatment was coupled to pharmacological (Figures 2A and 2B) or genetic (Figure  
205 2C) ablation of RBOH, using DPI or a *rbohD/F* genotype, respectively. Cell viability was  
206 checked after the experiment with fluorescein diacetate (FDA), with no visible effect of the  
207 inhibitors (Widholm, 1972) (Supplemental Figure 6). When the FM4-64 endocytotic index

208 from our whole set of measurements (Figure 2B and C) was plotted against corresponding  
209 DHE signals, a significant correlation was found ( $R^2=0.71$ ) (Figure 2D). These results support  
210 the idea that ROS activate endocytosis (Leshem et al., 2007; Wudick et al., 2015). More  
211 specifically, they indicate that ROS, produced by both the RBOH and the ascorbate/Fe  
212 pathways, quantitatively contribute to the enhancement of cell endocytosis under osmotic  
213 stress.

214 While FM4-64 labeling reports on bulk membrane lipid internalization, we wondered whether  
215 cargo proteins follow the same route. We first investigated the P-type  $H^+$ -ATPase, PLASMA  
216 MEMBRANE PROTON ATPASE 2 (AHA2), as one of most abundant PM proteins (Gaxiola  
217 et al., 2007). For this, we used plants expressing a pro35S:GFP-AHA2 construct and counted  
218 the average number of GFP-labeled BFA compartments per cell as a proxy of AHA2  
219 internalization. Surprisingly, this number was not enhanced but rather slightly decreased by a  
220 sorbitol treatment (Figures 2E and 2F). In agreement with previous observations in plants  
221 treated with salt or hydrogen peroxide (Li et al., 2011; Luu et al., 2012), root cells expressing  
222 a proPIP2;1:PIP2;1-GFP construct showed a higher number of GFP-labeled BFA bodies after  
223 a sorbitol treatment (Figures 2G and 2H). It was previously demonstrated that this is due to a  
224 higher endocytosis rate (Zwiewka et al., 2015). Interestingly, a DPI treatment fully suppressed  
225 the effect of osmotic stress on BFA body formation, while BPDS had no effects (Figure 2H).  
226 Thus, osmotic stress results in the selective endocytosis of certain cargos, which, in the case  
227 of PIP2;1, is fully dependent on RBOH-induced ROS accumulation.

### 228 **Lateral diffusion of PIP2;1 and AHA2 is enhanced by osmotic stress but negatively** 229 **controlled by ROS**

230 Major changes in the mobility and distribution of PM proteins are thought to underlie their  
231 endocytosis. For instance, a hyperosmotic treatment can induce a substantial increase in  
232 PIP2;1 diffusion (Li et al., 2011; Hosy et al., 2015). Here, we wondered whether this process  
233 is under the control of ROS signaling, as suggested by previous experiments using hydrogen  
234 peroxide treatments (Wudick et al., 2015). A super-resolution microscopy approach called  
235 single-particle-tracking photoactivated localization microscopy (sptPALM) was used to  
236 visualize individual PIP2;1-mEOS molecules under total internal reflection fluorescence  
237 (TIRF) illumination in living cells (Hosy et al., 2015). In brief, the localization and  
238 displacement of several thousands of single molecules were reconstructed and used to assess  
239 the protein instantaneous diffusion constant ( $D$ ) with a spatial resolution of around 30 nm and

240 temporal resolution of 20 ms (Figure 3A). For each reconstructed track, the mean square  
241 displacement (MSD) was computed and instantaneous diffusion coefficient was estimated  
242 with the help of homemade analysis software. In agreement with previous observations (Hosy  
243 et al., 2015), the diffusion of PIP2;1-mEOS within the PM was increased 2-fold after sorbitol  
244 treatment ( $\log D_{\text{ctrl}}=-2.9$ ,  $\log D_{\text{sorbitol}}=-2.6$ , Figures 3B and 3C). While DPI or BPDS had no  
245 individual effects on AtPIP2;1-mEOS diffusion, both in the absence or presence of sorbitol,  
246 the combination of the two inhibitors markedly enhanced this parameter, specifically after a  
247 sorbitol treatment (Figure 3C). The overall results (Figure 2H and Figure 3C) suggest an  
248 opposite ROS dependency of PIP2;1 diffusion and endocytosis. At variance with PIP2;1  
249 internalization, its diffusion under osmotic stress was negatively regulated by both the  
250 RBOHs and iron/ascorbate pathways

251 We used a similar sptPALM approach with transgenic lines expressing proUBQ10:mEOS2-  
252 AHA2 or proUBQ10:LTI6b-mEOS constructs to monitor diffusion within the PM of AHA2  
253 and LOW TEMPERATURE INDUCED PROTEIN 6B (LTI6b), two proteins that are  
254 typically immobile or highly mobile, respectively (Figure 3 and Supplemental Figure 7). No  
255 change in diffusion of the already fast-diffusing LTI6b-mEOS molecules was observed after a  
256 sorbitol treatment (Supplemental Figure 7A and 7B). In contrast, diffusion of mEOS-AHA2,  
257 similar to that of PIP2;1-mEOS, was enhanced in these conditions (Figures 3D and 3E).  
258 Diffusion of mEOS-AHA2 was further enhanced by DPI and BPDS treatments, indicating  
259 that ROS accumulation generated by RBOHs or by the iron/ascorbate dependent pathway is  
260 acting as a negative regulator of mEOS2-AHA2 diffusion in sorbitol-treated plants (Figure  
261 3F). Overall, the data show that increased diffusion of cargo proteins can be clearly  
262 dissociated from their endocytic behavior, questioning their causal relation.

263 **PIP2;1, but not AHA2, shows enhanced RBOH-dependent clustering in response to**  
264 **osmotic stress.**

265 Super-resolution imaging revealed that, in control conditions, PIP2;1-mEOS displayed a  
266 heterogeneous and sparse localization at the PM (Figure 4A). To test if this spatial  
267 organization corresponds to a random distribution or not, movies with a comparable density  
268 ( $3.20 \text{ tracks}/\mu\text{m}^2$ ) were generated by simulation (Supplemental Figure 8A). The presence of  
269 clusters was assessed by calculating the local density of each track using a Voronoi  
270 tessellation and comparing it to the one obtained in cells expressing PIP2;1-mEOS (Levet et  
271 al., 2015) (Supplemental Figures 8B and 8C). Whereas randomly generated data yielded a

272 narrow repartition of local densities peaking at 3.5 tracks/ $\mu\text{m}^2$ , PIP2;1-mEOS local density  
273 distribution was broader (Supplemental Figures 8B and 8C), reflecting the coexistence of  
274 sparse to very dense clusters (from 0.3 to 100 tracks/ $\mu\text{m}^2$ ). After a sorbitol treatment, PIP2;1-  
275 mEOS showed a shift towards higher local densities (Figure 4B and Supplemental Figure 8B),  
276 which was even appreciable from the super-resolution intensity images (Figure 4A). These  
277 results show that PIP2;1-mEOS molecules have a higher local density in response to a  
278 hyperosmotic treatment, but a similar number of PIP2;1-mEOS tracks per cell (Supplemental  
279 Figure 8A). To investigate how this phenomenon relates to other aspects of PIP2;1 dynamics  
280 (*e.g.* endocytosis and diffusion), we tested its dependency on ROS. Although the basal level  
281 of PIP2;1-mEOS clustering was sensitive to DPI, this inhibitor, but not BPDS, was able to  
282 prevent the enhancement of PIP2;1-mEOS local density by a hyperosmotic treatment (Figure  
283 4C). When expressed in a *rbohD* background, which has a similarly impaired ROS production  
284 compared to *rbohF* or *rbohD/F* (Figure 1C), the pPIP:PIP2;1-mEOS construct yielded a  
285 reduced particle clustering and, most importantly, had become insensitive to osmotic  
286 treatment (Figure 4D). This inhibition profile matches the one obtained for PIP2;1  
287 endocytosis (Figure 2H) and strongly suggests a causal chain linking, during osmotic stress,  
288 RBOH-dependent ROS accumulation, clustering of PIP2;1, and its eventual endocytosis.

289 mEOS2-AHA2 was also organized in small particle clusters, sparsely distributed in the PM  
290 (Figure 4E). Voronoi tessellation indicated this clustering to be similar to the one obtained  
291 with pPIP:PIP2;1-mEOS-expressing plants (Figure 4F and Supplemental Figure 8B and 8C).  
292 In contrast, the local density of mEOS2-AHA2 was insensitive to a sorbitol treatment (Figures  
293 4E and 4F). LTI6b-mEOS-expressing lines showed a higher local density of fluorescent  
294 particles that, however, were also insensitive to a sorbitol treatment (Supplemental Figure  
295 7C). The data indicate that some cargo proteins, such as PIP2;1, at variance with others  
296 (AHA2 and LTI6b), are clustered in the PM and endocytosed upon a hyperosmotic treatment.

### 297 **RbohD controls CLC2 diffusion in response to osmotic treatment**

298 Previous reports indicate that part of PIP2;1 constitutive cycling occurs through clathrin-  
299 mediated endocytosis (CME) (Li et al., 2011; Luu et al., 2012; Ueda et al., 2016; Dhonukshe  
300 et al., 2007). For instance, PIP2;1-GFP showed a partial colocalization at the PM with  
301 CLATHRIN LIGHT CHAIN2 (CLC2) fused to mCherry (Li et al., 2011). Here, we expressed  
302 a pro35S:PIP2;1-GFP construct in proITAM>UAS-HUB1 plants (Dhonukshe et al., 2007). In  
303 the presence of tamoxifen, this line overaccumulates HUB1, a dominant-negative mutant form

304 of CLATHRIN HEAVY CHAIN1 (CHC1), thereby compromising clathrin coat assembly.  
305 BFA bodies labeled by GFP-PIP2;1 were rarely observed in tamoxifen-treated plants, whether  
306 they were treated or not by sorbitol (Figure 5A). Thus, PIP2;1-GFP is internalized mostly  
307 through CME, in both control and osmotic stress conditions. To estimate how a hyperosmotic  
308 treatment acts on CME, we generated Col-0 plants expressing proUBQ10:CLC2-mEOS used  
309 here as a core marker of the CME machinery (Konopka et al., 2008). Super-resolution  
310 imaging revealed that CLC2-mEOS was organized in clusters at the PM (Figure 5B) and that  
311 its local density was insensitive to a sorbitol treatment (Figure 5C). We also investigated the  
312 diffusion of individual CLC2-mEOS particles at the PM and its dependency on a sorbitol  
313 treatment. Tracks generated from sptPALM movies revealed two populations of CLC2  
314 particles, with a low ( $\sim 0.001 \mu\text{m}^2/\text{sec}$ ) and high ( $\sim 0.1 \mu\text{m}^2/\text{sec}$ ) mean diffusion constant,  
315 respectively (Figure 5D). The repartition between these so-called mobile and immobile  
316 fractions was not altered after a sorbitol treatment (Figures 5E and 5G). In *rbohD* plants,  
317 however, a significant decrease of the CLC2-mEOS mobile fraction was induced by a  
318 hyperosmotic treatment (Figures 5F and 5G). These results suggest that CLC2-mEOS  
319 molecular dynamics are enhanced by RBOHD, specifically under osmotic stress. These  
320 effects may support the specific endocytosis of some PM-localized proteins, such as PIP2;1.

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## 326 **DISCUSSION**

327 Upon perception of an osmotic imbalance, plant cells generate multiple secondary  
328 messengers, such as H<sup>+</sup>, Ca<sup>2+</sup>, and ROS, which in turn trigger a cascade of cell responses  
329 leading to physiological acclimation (Feng et al., 2016). In this work, we focused on ROS  
330 signaling, deciphered the molecular bases of their generation, and described their role in  
331 membrane dynamics and protein sorting at high resolution.

### 332 **Two ROS-producing pathways contribute to ROS accumulation during osmotic** 333 **signaling**

334 RBOH proteins have emerged as central players of ROS signaling in plants. Here, we used  
335 pharmacological (DPI) and genetic tools (*rbohD* and *rbohF* mutants) to show their activation  
336 in the few minutes following hyperosmotic stimulation (Figure 6). We further show that  
337 RBOHD and RBOHF individually contribute to ROS accumulation, but in a nonadditive  
338 manner. These findings support previous work on the role of ROS and RBOH in long-term  
339 accumulation of proline in Arabidopsis roots under hyperosmotic stress (Ben Rejeb et al.,  
340 2015). More surprising was the finding that osmotically induced ROS accumulation is only  
341 partially dependent of the presence of functional RBOHs (Figures 1C and 1D), indicating the  
342 contribution of an additional mechanism for ROS production.

343 In these respects, our work points to a significant, but counterintuitive, role of ascorbate. As a  
344 reducing agent, ascorbate should primarily diminish ROS accumulation. This is typically the  
345 case when ascorbate fuels cytoplasmic peroxidases, like ASCORBATE PEROXIDASE 1  
346 (APX1) (Davletova et al., 2005; Koussevitzky et al., 2008). Here, we found ascorbate to  
347 positively regulate ROS accumulation. Exogenous application of ascorbate on roots almost  
348 doubled the DHE signal; conversely, its genetic or pharmacological depletion decreased the  
349 fluorescent signal (Figures 1F-I). Since in the latter approach most of the exogenously  
350 supplied ascorbate oxidase (AOX) likely remains confined to the cell apoplasm, we propose  
351 that ascorbate mainly acts in this compartment during the hyperosmotic response. The finding  
352 that specific chelation of metals with BPDS reduced the DHE signal indicates that ascorbate  
353 would reduce transition metals, such as iron. The newly formed ferric iron would in turn  
354 promote the formation of superoxide from dioxygen through a Haber-Weiss reaction (Figure  
355 1E). However, how can significant ascorbate levels be maintained in the cell apoplasm to  
356 sustain ROS production during osmotic treatment? An ascorbate efflux system at the cell PM,  
357 as was previously described in the context of iron nutrition in seeds (Grillet et al., 2014),

358 represents one possibility (Figure 6). Alternatively, cytoplasmic reducing power may be  
359 transferred through the cell membrane, by cytochrome proteins for instance, to regenerate the  
360 apoplastic dehydroascorbate (DHA) pool (Figure 6). Finally, an osmotically induced efflux of  
361 DHA can also be hypothesized (Figure 6). Interestingly, the balance between ascorbate and  
362 DHA in the apoplast has to be tightly controlled since misregulation of apoplastic ascorbate  
363 oxidases in tobacco plants altered hormonal and plant pathogen responses (Pignocchi et al.,  
364 2006). In line with this work, we also note that a polyamine oxidase was recently shown to act  
365 in a feedforward loop with RBOH proteins to regulate ROS production in stomata (Gémes et  
366 al., 2016). Thus, multiple ROS enzymes or generating mechanisms seem to work in concert  
367 during plant stress signaling. Whether these mechanisms are functionally redundant or convey  
368 some kind of response specificity remains largely unknown. This question was addressed here  
369 by analyzing membrane dynamics and protein sorting in root cells under hyperosmotic stress.

### 370 **The two ROS-producing pathways contribute in synergy to lipid membrane** 371 **internalization**

372 Within seconds or tens of seconds, hyperosmotic conditions induce a loss of cell water and  
373 consequently a decrease in turgor. This results in instantaneous changes in cell shape, as well  
374 as PM tensions and invaginations (Oparka, 1994). Membrane cycling between the cell surface  
375 and intracellular compartments is simultaneously enhanced (Leshem et al., 2007; Luu et al.,  
376 2012; Zwiewka et al., 2015). Consistent with this model, we observed that an enhanced  
377 internalization of FM4-64 occurs in osmotically challenged root cells. We further  
378 demonstrated that lipid membrane internalization was correlated to superoxide accumulation  
379 (Figure 2) and that both inhibition of RBOH activities and iron chelation were necessary for  
380 total suppression of this process (Figures 2 B-C and Figure 6). While the data identify the two  
381 major sources of ROS involved in membrane reshaping, the mode of action of ROS on lipid  
382 membrane dynamics remains an intriguing question. ROS exert direct effects on phospholipid  
383 structure through peroxidation and can induce lipid-lipid cross-linking. These chemical effects  
384 could translate into changes in membrane biophysical properties, such as viscosity or  
385 curvature, and act on membrane internalization (Eichenberger et al., 1982; Bruch and Thayer,  
386 1983). In addition, it cannot be excluded that ROS accumulation triggers formation of ordered  
387 microdomains at the PM to promote endocytosis, as was described in cells exposed to  
388 pathogen elicitors (Sandor et al., 2016).

389 **Osmotically induced PIP2;1 endocytosis specifically controlled by ROS produced by**  
390 **RBOHs**

391 While lipid membranes are largely internalized in response to a hyperosmotic challenge, we  
392 wondered whether protein cargoes would show the same movements. This seems to be case  
393 for the PIP2;1 aquaporin, which is internalized in response to a salt stress (Li et al., 2011; Luu  
394 et al., 2012), and showed here a similar response to a sorbitol hyperosmotic challenge. By  
395 contrast, endocytosis of the AHA2 H<sup>+</sup>-ATPase was insensitive to the same treatment. Since  
396 AHA2 cycling is, along with AHA1, responsible for the maintenance of a proton gradient  
397 across the cell PM (Haruta and Sussman, 2012; Haruta et al., 2018), we speculate that this  
398 function has to be critically maintained for energizing turgor regulation under hyperosmotic  
399 conditions. Also unknown is the mechanism that allows selection of specific cargoes for either  
400 internalization or retention.

401 Nevertheless, the most intriguing observation remained that, although RBOH and the  
402 ascorbate/iron pathway generate the same end product (superoxide), PIP2;1 internalization is  
403 selectively dependent on the former pathway (Figure 2H, Figure 4 C-D and Figure 6). Since  
404 ROS show short life spans (ms to sec for superoxide), limiting their diffusion to a restricted  
405 area, we propose that RBOHs create a ROS microenvironment in their vicinity, thereby acting  
406 on a specific protein subpopulation. Thus, the ROS-producing machinery rather than the  
407 produced species (superoxide) itself can determine signal specificity.

408 A recent study showed that in the Arabidopsis hypocotyl, RBOHD is depleted from the PM  
409 and degraded at 30 min after a 100 mM NaCl treatment (Hao et al., 2014). Since this response  
410 seems to be triggered by activation of RBOHD itself, a similar PM depletion might happen  
411 during osmotic stress. In addition, AtPIP2;1, which acts as a facilitator of hydrogen peroxide  
412 diffusion through the PM (Rodrigues et al., 2017), could undergo co-endocytosis with  
413 RBOHD. This putative complex between aquaporins and NADPH oxidases might participate  
414 in regulation of cytoplasmic ROS.

415 **Linking protein diffusion, clustering, and cycling**

416 Previous studies, including ours, have investigated lateral protein diffusion at the PM, with  
417 the implicit idea that this phenomenon supports subsequent endocytosis (Wudick et al., 2015).  
418 Using a sptPALM approach, we investigated protein clustering as an additional prerequisite

419 for cellular endocytosis and tried to relate these different parameters with an unprecedented  
420 time and space resolution.

421 Most intrinsic PM proteins of plants, and PIPs especially, have a very low lateral diffusion  
422 (around  $0.001 \mu\text{m}^2/\text{sec}$ ). Yet, two recent reports have demonstrated that hyperosmotic and salt  
423 treatments can induce a substantial increase in PIP2;1 diffusion (Li et al., 2011; Hosy et al.,  
424 2015). In this work, we confirmed these results and extended them to AHA2, showing that,  
425 under a hyperosmotic treatment, both PIP2;1 and AHA2 shift to another organization through  
426 increased diffusion. In contrast, LTI6b diffusion and distribution were not modified in the  
427 same conditions. Thus, a hyperosmotic challenge can alter the lateral diffusion of some but  
428 not all PM proteins. Second, we found that neither RBOH nor the ascorbate/Fe ROS-  
429 generating system was required for cargo mobilization. In contrast, their inhibition enhanced  
430 protein diffusion under hyperosmotic conditions, showing that ROS rather act as negative  
431 regulators of their diffusion, maybe by lipid peroxidation (Figure 3).

432 This finding seems at variance with previous experiment showing that exposure of root cells  
433 to exogenous  $\text{H}_2\text{O}_2$  enhanced PIP2;1 diffusion ((Wudick et al., 2015); our unpublished  
434 sptPALM data). Since production of apoplastic ROS is spatially regulated (see above) and  
435 involves complex interspecies conversion, we believe that these processes cannot be properly  
436 mimicked by treatment with exogenous  $\text{H}_2\text{O}_2$ . Overall, our comparative study of PIP2;1,  
437 AHA2, and LTI6b, together with ROS inhibition experiments, provides compelling evidence  
438 that an increase in protein diffusion is not linked to its subsequent endocytosis. More  
439 generally, protein diffusion may be corralled by PM /cell wall connections rather than being  
440 controlled by cellular ROS accumulation (Martiniere et al., 2011, Martiniere et al., 2013).  
441 These ideas led us to consider in closer detail protein clustering as another marker of protein  
442 behavior at the cell surface. The super-resolution microscopy approach indeed indicated that  
443 PIP2;1, AHA2, and LTI6b were not evenly distributed at the cell surface but were rather  
444 concentrated in small domains. After a hyperosmotic treatment, PIP2;1, but not AHA2 or  
445 LTI6b, showed higher clustering values, and this process was strictly RBOH dependent.  
446 These data perfectly match the endocytosis data, pointing to a causal link between higher  
447 clustering and enhanced endocytosis.

448 Our next question was about the mechanisms that drive PIP2;1 internalization, a process  
449 known to involve canonical clathrin-mediated endocytosis (CME) (Li et al., 2011). BFA  
450 experiments in plants expressing HUB1, a dominant-negative mutant form of CHC1,

451 confirmed this notion in the context of cell responses to hyperosmolarity (Figure 5A). Yet,  
452 inhibition of BFA compartment staining was only partial. Although we cannot exclude that  
453 conditional *HUB1* expression was not able to fully suppress CME, these partial effects may  
454 reflect, in agreement with previous work (Li et al., 2011; Wudick et al., 2015), an additional  
455 pathway for PIP2;1 internalization, independent of CME. Next, we investigated the effects of  
456 an osmotic stress on the molecular dynamics of CLC2, another major component of CME.  
457 CLC2-mEOS molecules showed two types of kinetic behavior, with either a high or a low  
458 diffusion. In animals, adaptor complex proteins (AP2) are known to bind to the PM by  
459 stochastic association with phosphatidylinositol two phosphates (PIP2), prior to clathrin-  
460 coated vesicle (CCV) initiation, growth, and maturation (Kadlecova et al., 2016). This binding  
461 induces an allosteric activation of the AP2 complex, thereby allowing interaction with clathrin  
462 triskelion (Jackson et al., 2010; Kelly et al., 2014). At this stage, cargo proteins can bind to  
463 AP2, and clathrin polymerization is primed. Alternatively, AP2/clathrin can dissociate from  
464 the PM. Due to its large size, the diffusion of CLC2-mEOS2 associated within CCV is  
465 supposed to be highly restricted. In contrast, when associated with PIP2/AP2/CLC complexes,  
466 CLC2-mEOS likely has a much higher diffusion coefficient. Thus, CLC2 diffusion can be  
467 used to reveal CCVs and the proportion of clathrin stochastic assemblies at the PM. In our  
468 experiments, the fraction of diffusible CLC2-mEOS was specifically reduced in *rbohD* under  
469 osmotic treatment, suggesting that clathrin stochastic assembly is perturbed in this context.  
470 Thus, the association between PIP2/AP2/CLC complexes and the PM might be supported by  
471 RBOHD-mediated ROS production, specifically in hyperosmotic conditions (Figure 6).

472 In conclusion, we have shown that PM dynamics are tightly controlled by cell signaling  
473 processes involving ROS, allowing the cell to respond to its osmotic environment. In our  
474 model, a hyperosmotic constraint activates RBOHs and a redox system coupling ascorbate  
475 and transition metals. The resulting ROS trigger overall cell membrane internalization.  
476 However, the nano-organization, lateral diffusion, and endocytosis of cargo proteins reveal  
477 highly specific behaviors and can be independently modulated depending on the cargo. While  
478 we demonstrate that an increase in protein lateral diffusion is not sufficient for triggering  
479 clustering and endocytosis, it will be interesting to see to what extent this upregulation  
480 remains necessary.

481

## 482 MATERIALS AND METHODS

### 483 Plant materials

484 The cDNAs of At4g30190 (*AHA2*) and At2g40060 (*CLC2*) were amplified by PCR and  
485 cloned into pENTR-D-TOPO (Invitrogen). The resulting constructs were used to generate N-  
486 or C-terminal fusions with mEOS, under the control of the *UBQ10* promoter (*ProUBQ10*), by  
487 cloning into pUBNEosFP and pUBCEosFP vectors, respectively (Grefen et al., 2010). The  
488 cDNA of At3g05890 (*LTI6b-RCi2B*) was cloned into pDONR207 by BP cloning. Multisite  
489 Gateway was then used to clone 2x35Sprom::*LTI6b-mEos* in pB7m34GW by LR  
490 recombination, using 2x35Sprom/pDONRP4RP1, *LTI6bnoSTOP/pDONR207*, and  
491 mEOS/pDONRP2RP3 as donor vectors (Karimi et al., 2007). mEOS/pDONRP2RP3 was  
492 obtained by amplifying mEOS and subsequent BP cloning into pDONRP2RP3 (Jaillais et al.,  
493 2011). Stable transformation of *Arabidopsis* (*Arabidopsis thaliana*) accession Col-0 was  
494 performed according to the floral dip method (Clough and Bent, 1998). proPIP2;1:PIP2;1-  
495 mEOS2 (Hosy et al., 2015) and proUBQ:CLC2-mEOS were also introduced into *rbohD*  
496 (Torres et al., 2002) by crossing. *Arabidopsis* plants expressing proPIP2;1:PIP2;1-GFP,  
497 proITAM>>HUB1, and mutants lines *RbohD*, *RbohF*, *RbohDxRbohF*, and *vtc2-4* were  
498 described elsewhere (Torres et al., 2002; Grillet et al., 2014; Wudick et al., 2015; Zwiewka et  
499 al., 2015).

### 500 Plant growth

501 Seeds were surface sterilized by agitation for 10 min in a solution containing 3.4 g/L  
502 BayroChlore and 0.02% Tween 20 detergent. Seeds were then rinsed 3 times with sterile  
503 water and sown on square plates containing half-strength Murashige and Skoog medium  
504 (MS/2) complemented with 2.5 mM 4-morpholineethanesulfonic acid (MES)-KOH, pH 6, and  
505 1% sucrose. Plates were placed vertically in a growth chamber with 16 h light ( $200\mu\text{mol m}^{-2} \text{s}^{-1}$ )  
506 and 8 h dark cycles at 21°C and 70% of relative humidity for 5 days.

### 507 Osmotic and pharmacological treatments

508 Five-day-old *Arabidopsis* plantlets were bathed in a liquid MS/2 medium to allow recovery  
509 from transplanting. When indicated, 20  $\mu\text{M}$  diphenyleneiodonium (DPI), 50  $\mu\text{M}$   
510 baptholphenanthrolinesulfonate (BPDS), 100  $\mu\text{M}$  ascorbate, or 1.5 units/ml ascorbate oxidase  
511 (AOX) was included. After 30 min, plantlets were gently transferred for an additional 15 min

512 into a control MS/2 medium, in the absence or presence of 100 mM (mild osmotic stress) or  
513 300 mM (severe osmotic stress) sorbitol, and with or without the corresponding inhibitors.

#### 514 ROS measurements

515 The accumulation of  $O_2^-$  or hydroxyl radicals ( $\cdot OH$ ) was probed using 5  $\mu M$  dihydroethidium  
516 (DHE) or 10  $\mu M$  hydroxyphenyl fluorescein (HPF), respectively. The two dyes show  
517 increased fluorescence when oxidized. Observations were performed on the root elongation  
518 zone using a Zeiss Axiovert 200M inverted fluorescence microscope (20x/0.8 objective), with  
519 512/25-nm excitation and 600/50 emission filters for DHE, and 475/28 nm excitation and  
520 535/30 emission filters for HPF. Exposure time was 500 ms and 200 ms, for DHE and HPF,  
521 respectively. Images were acquired using a CCD camera (Cooled SNAP HQ, Photometrics),  
522 controlled by fluorescence imaging software (MetaFluor®, Molecular device). To quantify  
523 the intensity of the fluorescence signal, the images were analyzed using ImageJ software.  
524 After subtraction of the background noise, an average mean grey value was calculated from  
525 epidermal and cortical cells.

#### 526 Endocytosis assay

527 For estimation of bulk endocytosis, seedlings were first pretreated in liquid MS/2 medium for  
528 30 min in the absence or presence of the appropriate inhibitors. Seedlings were then  
529 transferred in 1  $\mu M$  N-(3-triethylammoniumpropyl)-4[6-(4-  
530 (diethylamino)phenyl)hexatrienyl]pyridinium dibromide (FM4-64) in MS/2. After 7 min, the  
531 seedlings were washed in a liquid MS/2 medium deprived of FM4-64. They were then treated  
532 with 25  $\mu M$  Brefeldin A (BFA) for 1 h, in the presence or absence of the appropriate  
533 inhibitors. To monitor the internalization of PIP2;1-GFP or GFP-AHA2, seedlings were  
534 treated for 45 min with 25  $\mu M$  BFA. The number of BFA bodies per cell stained with FM4-64  
535 or GFP was manually counted from images taken with a confocal microscope.

#### 536 Confocal laser scanning microscopy

537 A Leica SP8 microscope with a 40x/1.1 water objective and the 488-nm line of its argon laser  
538 was used for live-cell imaging. Fluorescence emission was detected at 600-650 nm for FM4-  
539 64 and at 500-540 nm for GFP. To explore the full volume of epidermal cells, z-stacks of  
540 epidermal cell layers were made within 7 steps per acquisition, covering a 15- $\mu m$  depth. For  
541 quantitative measurements of BFA bodies, the laser power, pinhole, and gain settings of the  
542 confocal microscope were identical among different treatments or genotypes.

## 543 sptPALM

544 Seedlings were transferred from vertically oriented plates to a petri dish containing MS/2 for  
545 30 minutes, to allow recovery prior to incubation for 40 min in MS/2 complemented or not  
546 with 300 mM sorbitol. This last step was shortened to 15 min in the case of CLC2  
547 experiments. Root cells were observed with a homemade TIRF microscope equipped with an  
548 emCCD camera (Andor iXON XU\_897) and a 100x oil immersion objective (Apochromat  
549 NA = 1.45, Zeiss). Laser angle was selected to be close to supercritical angle and generate  
550 evanescent waves and to give the maximum signal-to-noise ratio (Konopka and Bednarek,  
551 2008; Johnson and Vert, 2017). To ensure PALM localization of proteins, a continuous low-  
552 intensity illumination at 405 nm (OBIS LX 50mW Coherent) was used for photoactivation,  
553 while 561 nm (SAPPHIRE 100mW Coherent) with 600/50 (Chroma) emission filter was  
554 selected for image acquisition. The acquisition was steered by LabView software in streaming  
555 mode at 50 images/sec (20 msec exposure time). Ten thousand images were recorded per  
556 region of interest (ROI). From 10 to 20 ROIs were collected out of three biological replicates.

## 557 Single-particle tracking and Voronoi tessellation

558 Individual single molecules were localized and tracked using a MTT software (Sergé et al.,  
559 2008). Dynamic properties of single emitters in root cells were then inferred from the tracks  
560 using homemade analysis software written in Matlab. From each track, the mean square  
561 displacement (MSD) was computed. In order to reduce the statistical noise while keeping a  
562 sufficiently high number of trajectories per cell, tracks of at least 5 steps (*i.e.*  $\geq 6$   
563 localizations) were used. Missing frames due to mEOS blinking were allowed up to a  
564 maximum of three consecutive frames. The diffusion coefficient  $D$  was then calculated by  
565 fitting the MSD curve using the first four points. For the clustering analysis, the centroid of  
566 each individual track was calculated and used to implement SR-Tesseler software (Levet et  
567 al., 2015). Local densities of each track were calculated as the invert of their minimal surface.

## 568 Statistical analysis

569 For each condition or treatment, 9-12 cells were analyzed from at least 5-7 different seedlings.  
570 All experiments were independently repeated 2-3 times. Data are expressed as mean  $\pm$  SE.  
571 Statistical analyses were performed in GraphPad Prism, using ANOVA followed by a  
572 Tukey's post hoc test or Student's  $t$ -test depending of the needs ( $p < 0.05$  was considered  
573 significant). Different letters in histograms indicate statistically different values.

574 Accession Numbers

575 Sequence data from this article can be found in the Arabidopsis Genome Initiative or  
576 GenBank/EMBL databases under the following accession numbers: *PIP2.1*, *At3G53420*;  
577 *AHA2*, *At4G30190*; *LTI6-b*, *At3G05890*; *CLC2*, *At2G40060*.

578 Supplemental Data

579 Supplemental Figure 1: Kinetics of DHE staining after a hyperosmotic treatment.

580 Supplemental Figure 2: ROS accumulation in root cells after a sorbitol treatment as revealed  
581 by hydroxyphenyl fluorescein (HPF)

582 Supplemental Figure 3: Diagram of experimental design.

583 Supplemental Figure 4: Effects of various sorbitol concentrations on cell plasmolysis and  
584 ROS accumulation.

585 Supplemental Figure 5: Localization using confocal microscopy of DHE fluorescence induced  
586 after 15 min of a mock or sorbitol treatment.

587 Supplemental Figure 6: Cell viability of root cells after a multiple inhibitor treatment (DPI  
588 plus BPDS).

589 Supplemental Figure 6: Lateral diffusion and local density of LTI6b-mEOS in response to a  
590 hyperosmotic sorbitol treatment.

591 Supplemental Figure 7: Effect of a hyperosmotic sorbitol treatment on PIP2;1-mEOS2 and  
592 mEOS-AHA2 cluster formation.

594 **FIGURE LEGENDS**

595 Figure 1: ROS accumulation in root cells after a sorbitol treatment. A, Schematic  
 596 representation of ROS production as mediated by NADPH oxidases. B, ROS imaging using  
 597 DHE fluorescence. Roots were incubated for 15 min with 5  $\mu$ M DHE in the presence or  
 598 absence of 300 mM sorbitol and subsequently observed under an epifluorescence microscope.  
 599 The figure shows the red fluorescent signal induced upon oxidation of DHE. C, Quantification  
 600 of DHE fluorescence in Col-0, *rbohD*, *rbohF*, and *rbohD/F* plants subjected or not to the  
 601 sorbitol treatment. Effects on DHE fluorescence of a 30-min pretreatment, with either control  
 602 (DMSO) or DPI, prior to incubation for 15 min with 5  $\mu$ M DHE followed by a mock (no  
 603 sorbitol) or 300 mM sorbitol treatment on Col-0 (D) or *rbohD/F* (F) plants. E, Schematic  
 604 representation of ROS production by the putative ascorbate/iron pathway. G, Effects on DHE  
 605 fluorescence of a 30-min pretreatment with BPDS ( $\text{Fe}^{2+}$  chelation) or AOX (ascorbate  
 606 depletion), alone or in combination with DPI (RBOH inhibition). H, Effect of a 30-min  
 607 pretreatment with BPDS on DHE fluorescence in the *rbohD/F* double mutant. I, Averaged  
 608 DHE fluorescence intensity in Col-0 or *vtc2.4* roots incubated for 15 min in the presence or  
 609 absence of sorbitol. DMSO represents a mock condition for comparison to a DPI  
 610 pretreatment. J, Averaged DHE fluorescence intensity in control plants or plants treated with  
 611 100  $\mu$ M ascorbate (Asc) for 15 min. Histograms show mean values  $\pm$  SE ( $n = 38$ -211 cells).  
 612 Different letters indicate statistically different values (ANOVA). Scale bars represent 20  $\mu$ m.

613 Figure 2: Effects of hyperosmotic sorbitol treatment on bulk membrane and protein cargo  
 614 internalization. A, Roots were pretreated with FM4-64 for 7 min followed by BFA for 1 h in  
 615 the absence or presence of 300 mM sorbitol. FM4-64-labeled BFA bodies (arrows) indicate  
 616 cellular bulk endocytosis. BFA bodies are more frequent in sorbitol-treated plants than in  
 617 untreated plants, or plants cotreated with sorbitol, BPDS, and DPI. B, Average number of  
 618 FM4-64-labeled BFA bodies per cells in control and sorbitol-treated plants. When indicated,  
 619 plants were pretreated with BPDS and DPI, alone or in combination. C, Average number of  
 620 FM4-64-labeled BFA bodies per cell in Col-0, *rbohD/F*, and *rbohD/F* plants treated with  
 621 BPDS. D, Fluorescence intensity values shown in Figures 1C, 1D, and 1F were plotted  
 622 against corresponding numbers of FM4-64-labeled BFA bodies per cell. A significant linear  
 623 correlation ( $R^2 = 0.71$ ) was observed. E and G, Confocal observation of root cells expressing  
 624 Pro35S:GFP-AHA2 (E) or ProPIP2;1:PIP2;1-GFP (G) after a 45-min BFA treatment in the

625 presence (S) or absence (NS) of sorbitol. Only the ProPIP2;1:PIP2;1-GFP cells show a  
626 sorbitol-induced increase in GFP-labeled BFA bodies (arrows). F and H, Average number of  
627 GFP-AHA2- (F) or PIP2;1-GFP-labeled (H) BFA bodies per cell under control or sorbitol  
628 treatment conditions. For PIP2;1-GFP, the experiments were performed in the absence (Ctrl)  
629 or presence of a pretreatment with DPI or BPDS, alone or in combination. Histograms show  
630 means values  $\pm$  SE (n =10-19). Scale bars represent 20  $\mu$ m.

631 Figure 3: Effects of hyperosmotic sorbitol treatment on lateral diffusion of PIP2;1-mEOS and  
632 mEOS-AHA2. A and D, Track reconstructions in plants expressing ProPIP2;1:PIP2;1-mEOS  
633 (A) or ProUBQ10:mEOS-AHA2 (D), in the absence or presence of 300 mM sorbitol for 40  
634 min. Each color represents a single molecule position over time. The right panels show close-  
635 up views of tracks in no sorbitol (NS) or sorbitol (S) conditions. B and E, Distribution of  
636 diffusion coefficients of PIP2;1-mEOS (B) and mEOS-AHA2 (E) in log scale. C and F, Mean  
637 diffusion coefficient values of PIP2;1-mEOS (C) and mEOS-AHA2 (F), in the absence or  
638 presence of sorbitol, and after treatment with the indicated inhibitors (DPI, BPDS, and  
639 DPI/BPDS). Histograms show mean values calculated from several thousands of individual  
640 tracks with SE (n = 12-30). Scale bars represent 1  $\mu$ m.

641 Figure 4: Effects of hyperosmotic sorbitol treatment on local density of PIP2;1-mEOS and  
642 mEOS-AHA2. A and E, Super-resolution intensity map of PM of plants expressing  
643 ProPIP2;1:PIP2;1-mEOS (A) or ProUBQ10:mEOS-AHA2 (E), in the absence or presence of  
644 300 mM sorbitol for 40 min. B and F, Local density distribution of PIP2;1-mEOS (B) or  
645 mEOS-AHA2 (F). C, Effects of DPI, BPDS, or their combination on the mean log value of  
646 local density of PIP2;1-mEOS, in the absence or presence of sorbitol. D, Effects of sorbitol on  
647 the mean log value of the local density of PIP2;1-mEOS in Col-0 or *rbohD* plants. Histograms  
648 show mean values calculated from several thousands of individual tracks with SE (n = 12-30).  
649 Scale bars represent 1  $\mu$ m.

650 Figure 5: Effects of clathrin-mediated endocytosis on PIP2;1-GFP internalization and  
651 molecular dynamics of CLC2-mEOS in response to a hyperosmotic sorbitol treatment. A,  
652 Root cells coexpressing ProITAM>>HUB and Pro35S:PIP2;1-GFP in the presence of BFA  
653 for 45 min. PIP2;1-GFP labels BFA bodies in control conditions (left), but to a much lesser  
654 extent after induction of HUB with tamoxifen (Tam) (12 h), in the absence (center) or  
655 presence (right) of sorbitol. B, Super-resolution intensity map of PM of a plant expressing  
656 ProUBQ10:CLC2-mEOS, in the absence or presence of 300 mM sorbitol for 20 min. C, Local

657 density distribution of CLC2-mEOS. D, Track reconstruction in a ProUBQ10:CLC2-mEOS-  
658 expressing plant. Each color represents the displacement of a single molecule over time.  
659 Particles with a high (yellow) and low (blue) diffusion coefficient can be observed. E-F,  
660 Diffusion coefficient distribution of CLC2-mEOS, in the absence or presence of sorbitol, and  
661 in Col-0 (E) or *rbohD* (F). The gray box corresponds to the CLC2-mEOS mobile fraction. G,  
662 Corresponding graph showing the average percentage of CLC2-mEOS mobile fraction in Col-  
663 0 or *rbohD*, in the absence or presence of sorbitol. Histograms show mean values calculated  
664 from several thousands of individual tracks with SE ( $n > 15-31$ ). Scale bars represent 1  $\mu\text{m}$ .

665 Figure 6: Diagram of ROS signaling and its impact on protein dynamics after a hyperosmotic  
666 treatment. In control conditions, RBOHD/F (green) and putative efflux machinery (pink) for  
667 reducing power are inactive. A basal level of membrane internalization exists. CLC2 (brown)  
668 is either associated with AP2 (purple) in its diffusible form or is associated with CCVs,  
669 yielding nondiffusible forms. PIP2;1 (blue) and AHA2 (red) are organized in clusters and are  
670 mostly immobile. After a hyperosmotic treatment, activation of RBOHD/F and the efflux  
671 machinery (pink) for reducing power leads to enhanced production of superoxide. In the case  
672 of the reducing power efflux, this is achieved either by a direct efflux of cytoplasmic  
673 ascorbate (Asc) or an efflux or regeneration of dehydroascobate (DHA). The resulting  
674 reducing power reduces apoplastic transition metals, which in turn react with oxygen to  
675 generate ROS. The accumulation of ROS enhances lipid membrane internalization by an  
676 unknown mechanism. In parallel, ROS produced by RBOH facilitate plasma membrane  
677 association of CLC2/AP2 complexes by promoting interactions of AP2 with lipid at the  
678 membrane. An excess of these complexes can bind to PIP2;1, thereby facilitating its  
679 incorporation in CCVs. As a consequence, PIP2;1 clustering and endocytosis are enhanced.  
680 The rate of CLC2 dissociation from the CCV is intrinsically enhanced by the hyperosmotic  
681 stress, and this effect is compensated for by RBOH-dependent ROS and the above-mentioned  
682 effects on CL2/AP2 complex formation.

683

## 684 SUPPLEMENTAL FIGURE LEGENDS

685 Supplemental Figure 1: Kinetics of DHE staining after a hyperosmotic treatment. Roots were  
686 incubated with 5  $\mu$ M DHE in the absence (empty symbols) or presence (filled symbols) of  
687 300 mM sorbitol and observed for up to 120 min. The fluorescent signal shows a steady  
688 increase over time, which was always higher after a sorbitol treatment. Mean fluorescence  
689 intensity values  $\pm$  SE ( $n > 26$ ).

690 Supplemental Figure 2: ROS accumulation in root cells after a sorbitol treatment as revealed  
691 by hydroxyphenyl fluorescein (HPF). A, Root cells stained with HPF for 15 min in the  
692 presence or absence of 300 mM sorbitol. B, Mean HPF fluorescence in response to sorbitol in  
693 roots pretreated for 30 min with DPI, BPDS, or their respective mock treatments (DMSO or  
694 ctrl). Histograms show mean fluorescence intensity values  $\pm$  SE ( $n > 18$ ). Scale bar represents  
695 20  $\mu$ m.

696 Supplemental Figure 3: Diagram of experimental design. Five-day-old seedlings were  
697 transferred in a liquid medium (MS/2, MES-KOH, pH 6, 1% sucrose) in the absence or  
698 presence of the indicated inhibitors. After 30 min, plants were incubated in the same liquid  
699 medium, but supplemented with DHE, and with or without sorbitol. Microscopy observation  
700 was performed after 15 min of this second treatment.

701 Supplemental Figure 4: Effects of various sorbitol concentrations on cell plasmolysis and  
702 ROS accumulation. A, Confocal observation of root cells expressing p35s:PIP2;1-GFP to  
703 reveal the PM and stained with propidium iodide (PI) to label the cell wall. In contrast to the  
704 400 mM sorbitol treatment (see arrow heads), no plasmolysis was detected in control and 100  
705 mM sorbitol conditions. In the presence of 300 mM sorbitol, an incipient plasmolysis can be  
706 observed (arrows). B, DHE fluorescence intensity (mean  $\pm$  SEM) in plants incubated in the  
707 absence or presence of 100 mM sorbitol, without (Ctrl) or with DPI, BPDS, or AOX, alone or  
708 in combination (BPDS/DPI, AOX/DPI). WF, wide field. Scale bar represents 10  $\mu$ m.

709 Supplemental Figure 5: Localization using confocal microscopy of DHE fluorescence induced  
710 after 15 min of a mock or sorbitol treatment. DHE stains the dotted structure (arrowheads)  
711 and nucleoli (star) in control cells and also in the apoplast (arrows) after sorbitol treatment.

712 Supplemental Figure 6: Cell viability of root cells after a multiple inhibitor treatment (DPI  
713 plus BPDS). Plants were treated as explained for FM4-64 endocytosis test (Figure 3A, B, and

714 C) followed by an incubation with 3  $\mu$ M FDA for 2 min. (A) FDA-induced fluorescent  
715 labeling of root cells. (B) Quantification of FDA fluorescence.

716 Supplemental Figure 6: Lateral diffusion and local density of LTI6b-mEOS in response to a  
717 hyperosmotic sorbitol treatment. A, Track reconstruction of ProUBQ10:LTI6b-mEOS. Each  
718 color represents the successive positions of one single molecule over time. The right panels  
719 show close-up views of tracks in the absence (NS) or presence (S) of sorbitol. B, Distribution  
720 of LTI6b-mEOS diffusion coefficient. C, Distribution of LTI6b-mEOS local density.

721 Supplemental Figure 7: Effect of a hyperosmotic sorbitol treatment on PIP2;1-mEOS2 and  
722 mEOS-AHA2 cluster formation. A, Average track density from simulated data or  
723 measurements in PIP2;1-mEOS2- or mEOS2-AHA2-expressing plants, in the absence or  
724 presence of 300 mM sorbitol. B, Voronoi tessellation calculated from the position of each  
725 track centroid for simulated data or measurements in ProPIP2;1-PIP2;1-EOS- or  
726 ProUBQ10:mEOS2-AHA2-expressing cells, in the absence or presence of sorbitol. The  
727 smaller the polygons, the higher the local density of the detected molecule. C, Local density  
728 distribution of simulated data or particles detected in ProPIP2;1-PIP2;1-EOS- or  
729 ProUBQ10:mEOS2-AHA2-expressing plants.

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733

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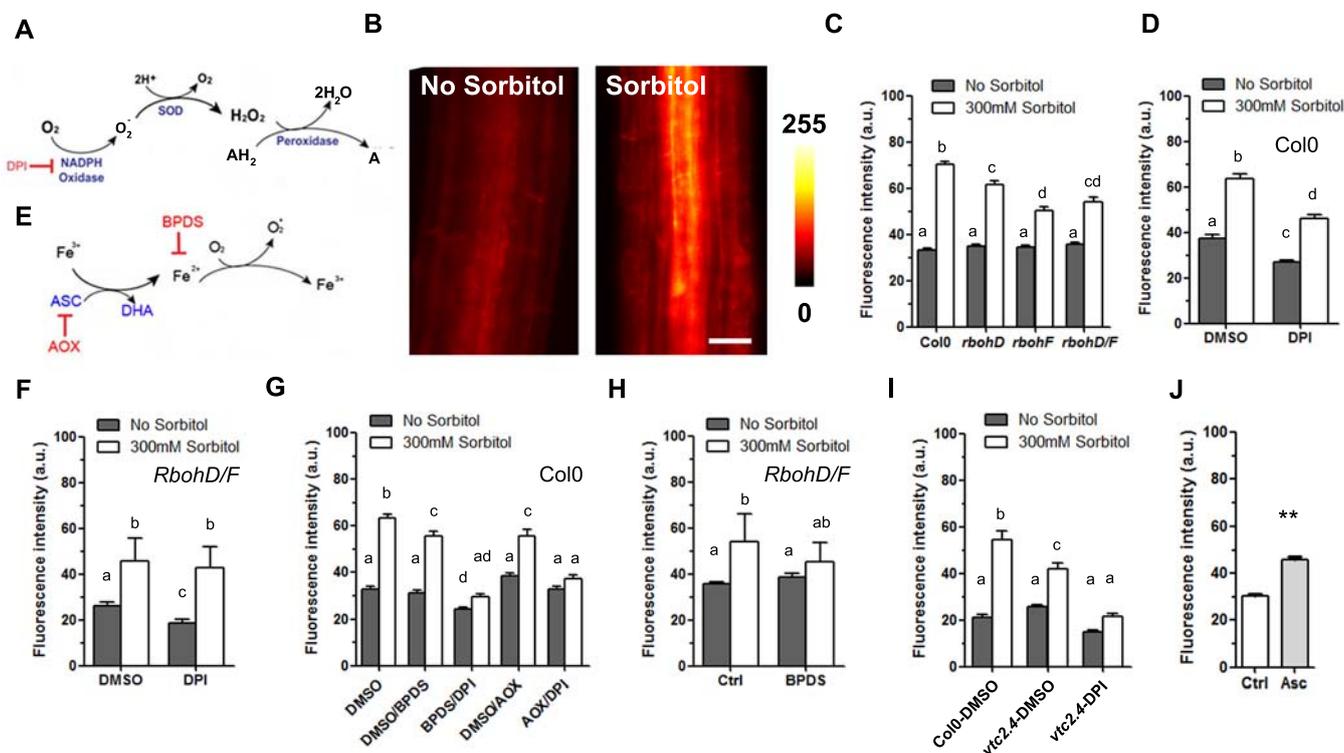


Figure 1: ROS accumulation in root cells after a sorbitol treatment. A, Schematic representation of ROS production as mediated by NADPH oxidases. B, ROS imaging using DHE fluorescence. Roots were incubated for 15 min with 5  $\mu$ M DHE in the presence or absence of 300 mM sorbitol and subsequently observed under an epifluorescence microscope. The figure shows the red fluorescent signal induced upon oxidation of DHE. C, Quantification of DHE fluorescence in Col-0, *rbohD*, *rbohF* and *rbohD/F* plants subjected or not to the sorbitol treatment. Effects on DHE fluorescence of a 30 min pretreatment, either control (DMSO) or with DPI, prior to incubation for 15 min with 5 $\mu$ M DHE followed by a mock (no sorbitol) or 300 mM sorbitol treatment on Col0 (D) or *rbohD/F* (F) plant. E, Schematic representation of ROS production by the putative ascorbate/iron pathway. G, Effects on DHE fluorescence of a 30 min pretreatment with BPDS (Fe<sup>2+</sup> chelation), or AOX (ascorbate depletion), alone or in combination with DPI (RBOH inhibition). H, Effect of a 30 min pretreatment with BPDS on DHE fluorescence in a *rbohD/F* double mutant. I, Averaged DHE fluorescence intensity in Col-0 or *vtc2.4* roots incubated for 15 min in the presence or absence of sorbitol. DMSO represents a mock condition for comparison to a DPI pretreatment. J, Averaged DHE fluorescence intensity in control plants or plants treated with 100  $\mu$ M ascorbate (Asc) for 15min. Histograms show mean values  $\pm$ SEM (n = 38-211 cells). Different letters indicate statistically different values (ANOVA). Scale bars represent 20  $\mu$ m.

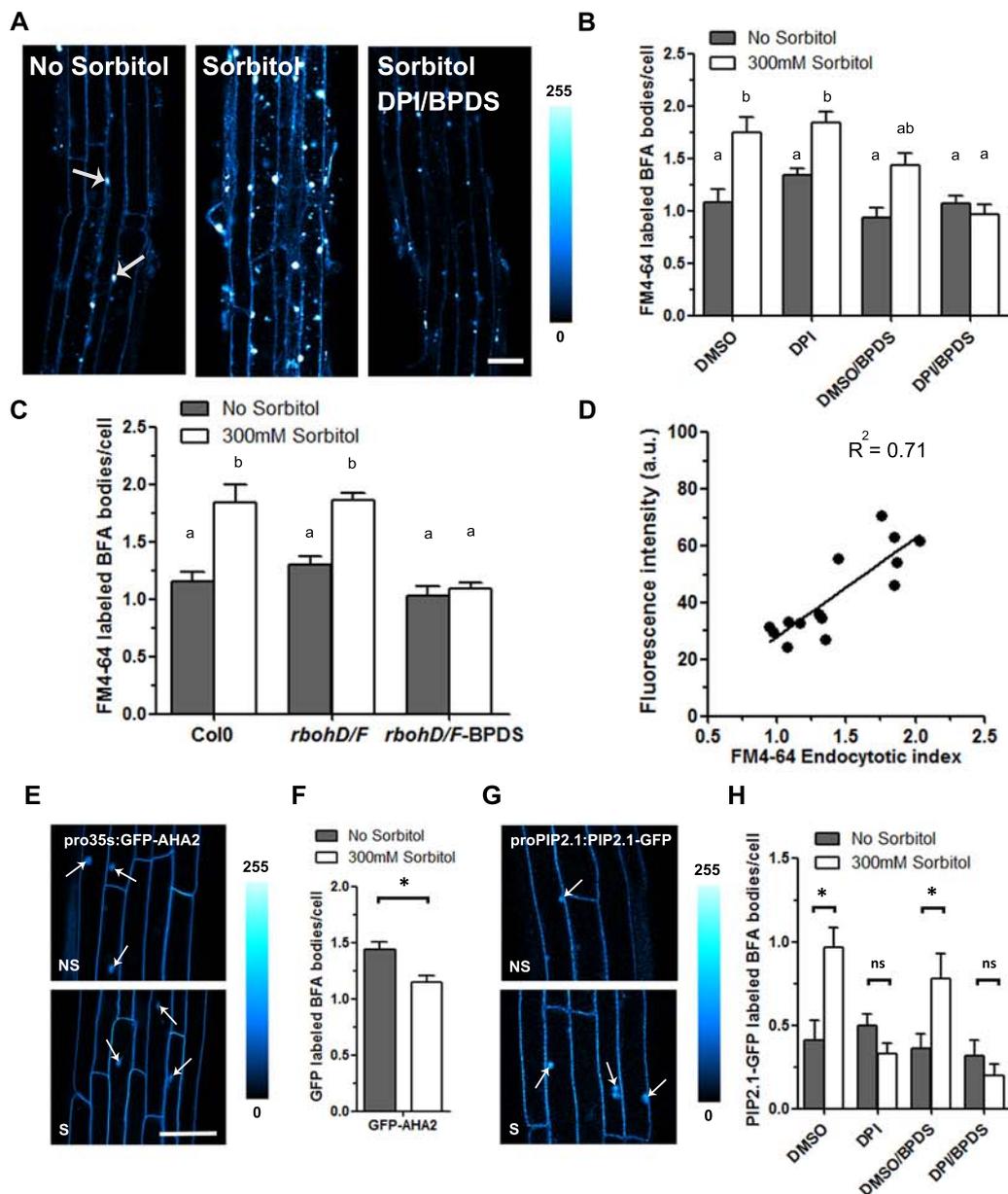


Figure 2: Effects of a hyperosmotic sorbitol treatment on bulk membrane and protein cargo internalization. A, Roots were pre-treated with FM4-64 for 7 min followed by BFA for 1 h in the absence or presence of 300 mM sorbitol. FM4-64 labeled BFA bodies (arrows) indicate cellular bulk endocytosis. BFA bodies are more frequent in sorbitol treated plants than in untreated plants, or plants co-treated with sorbitol, BPDS and DPI. B, Averaged number of FM4-64 labeled BFA bodies per cells in control and sorbitol treated plants. When indicated, plants were pretreated with BPDS and DPI, alone or in combination. C, Averaged number of FM4-64 labeled BFA bodies per cells in Col-0, *rbohD/F* and *rbohD/F* plants treated with BPDS. D, Fluorescence intensity values shown in Figures 1C, 1D and 1F were plotted against corresponding numbers of FM4-64 labeled BFA bodies per cells. A significant linear correlation ( $R^2 = 0.71$ ) can be observed. E and G, Confocal observation of root cells expressing Pro35S:GFP-AHA2 (E) or ProPIP2.1:PIP2.1-GFP (G) after a 45 min BFA treatment in the presence (S) or absence (NS) of sorbitol. Only the ProPIP2.1:PIP2.1-GFP cells show a sorbitol-induced increase in GFP-labeled BFA bodies (arrows). F and H, Averaged number of GFP-AHA2 (F) or PIP2.1-GFP (H) labeled BFA bodies per cell under control or sorbitol treatment conditions. For PIP2.1-GFP the experiments were performed in the absence (Ctrl) or presence of a pretreatment with DPI, BPDS, alone or a combination. Histograms show means values  $\pm$  SEM ( $n = 10-19$ ). Scale bars represent 20  $\mu$ m.

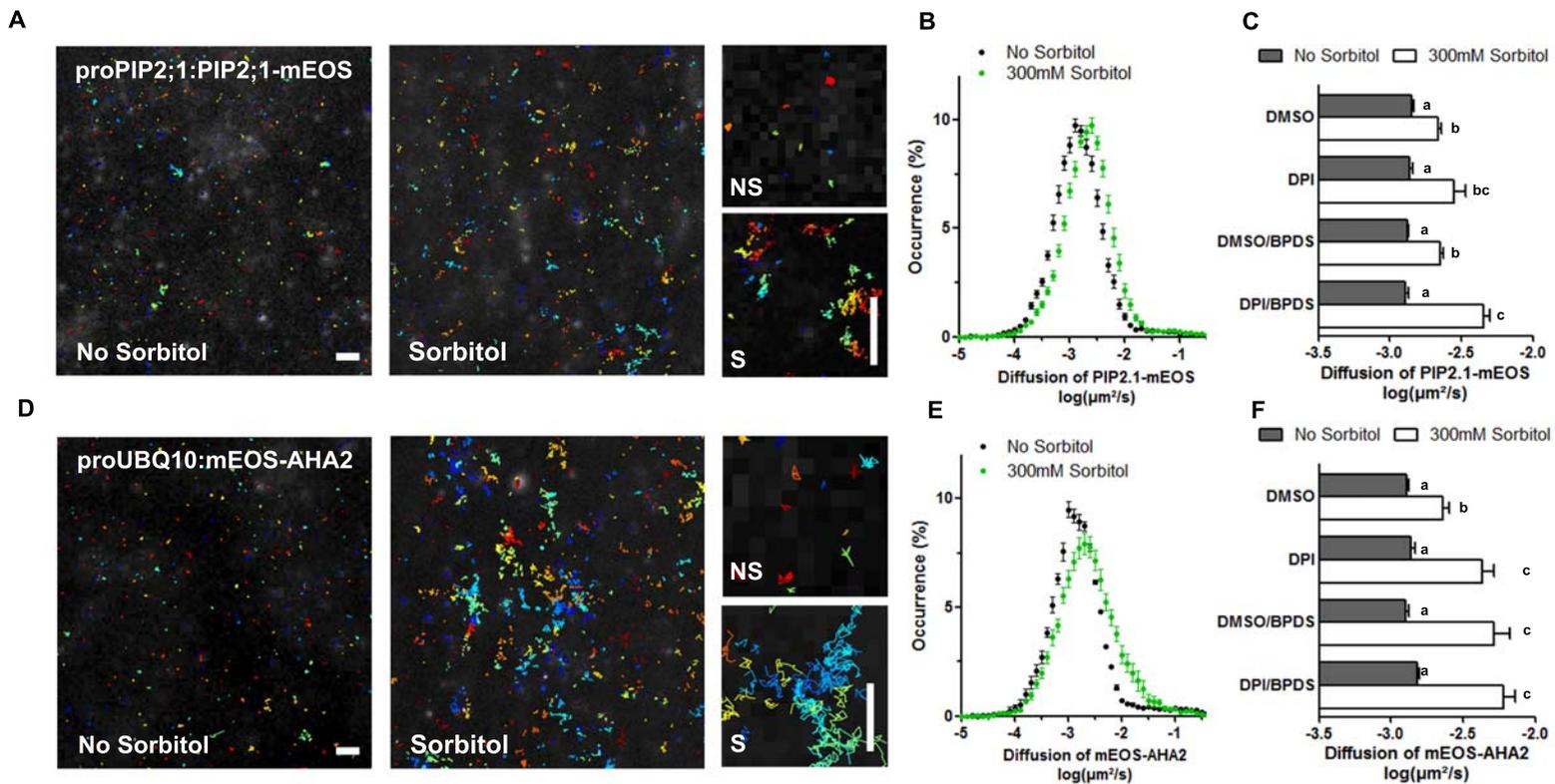


Figure 3: Effects of a hyperosmotic sorbitol treatment on lateral diffusion of PIP2;1-mEOS and mEOS-AHA2. A and D, Track reconstructions in plants expressing ProPIP2;1:PIP2;1-mEOS (A) or ProUBQ10:mEOS-AHA2 (D), in the absence or presence of 300mM sorbitol for 40min. Each color represents single molecule position over time. The right panels show close up views of tracks in no sorbitol (NS) or sorbitol (S) conditions. B and E, Distribution of diffusion coefficients of PIP2;1-mEOS (B) and mEOS-AHA2 (E) in log scale. C and F, Mean diffusion coefficient values of PIP2;1-mEOS (C) and mEOS-AHA2 (F), in the absence or presence of sorbitol, and after treatment with the indicated inhibitors (DPI, BPDS and DPI/BPDS). Histograms show mean values calculated from several thousands of individual tracks with SEM ( $n = 12-30$ ). Scale bars represent  $1 \mu\text{m}$ .

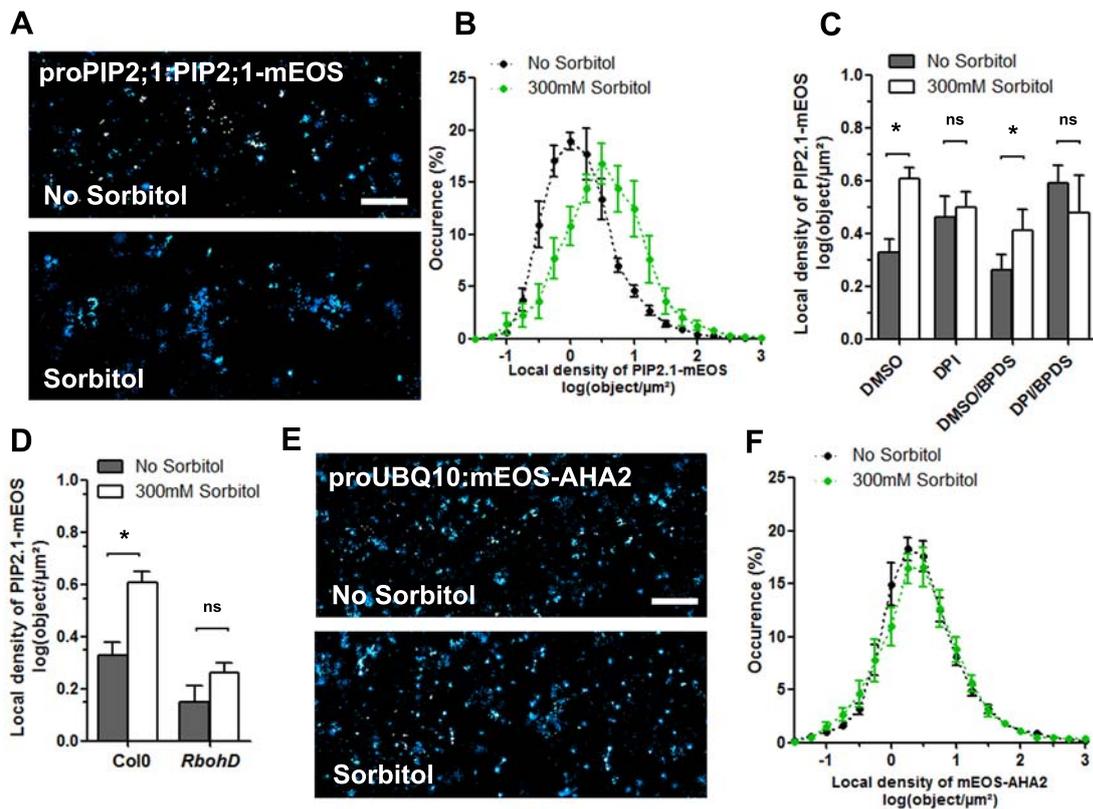


Figure 4: Effects of a hyperosmotic sorbitol treatment on local density of PIP2;1-mEOS and mEOS-AHA2. A and E, Super resolution intensity map of PM of plants expressing ProPIP2;1:PIP2;1-mEOS (A) or ProUBQ10:mEOS-AHA2 (E), in the absence or presence of 300mM sorbitol for 40 min. B and F, Local density distribution of PIP2;1-mEOS (B) or mEOS-AHA2 (F). C, Effects of DPI, BPDS or their combination on the mean log value of local density of PIP2;1-mEOS, in the absence or presence of sorbitol. D, Effects of sorbitol on the mean log value of the local density of PIP2;1-mEOS in Col-0 or *rbohD* plants. Histograms show mean values calculated from several thousands of individual tracks with SEM ( $n = 12-30$ ). Scale bars represent 1  $\mu\text{m}$ .

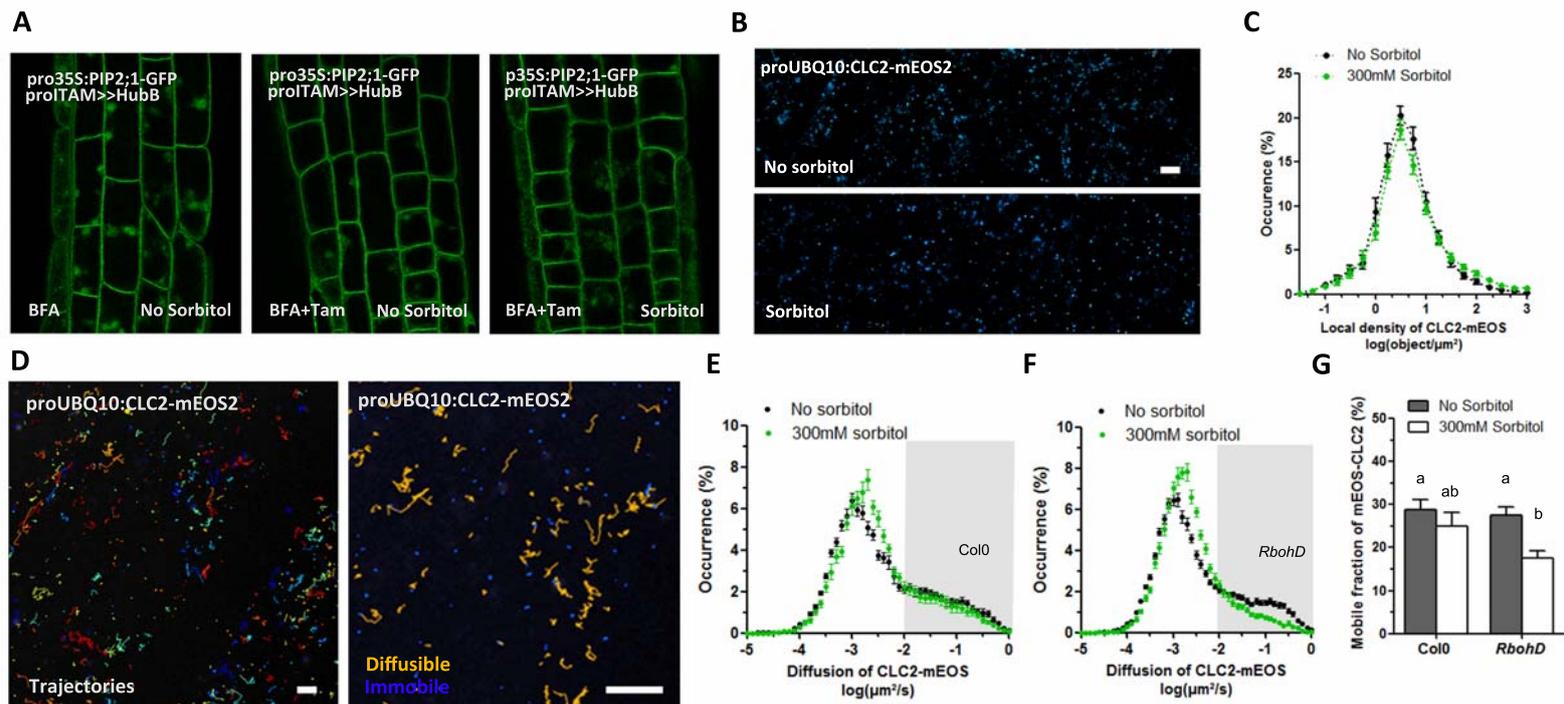


Figure 5: Effects of clathrin-mediated endocytosis on PIP2;1-GFP internalization and molecular dynamics of CLC2-mEOS in response to a hyper-osmotic sorbitol treatment. A, Root cells co-expressing ProITAM>>HUB and Pro35S:PIP2;1-GFP in the presence of BFA for 45 min. PIP2;1-GFP labels BFA bodies in control conditions (left), but to a much lesser extent after induction of HUB with tamoxifen (Tam) (12 h), in the absence (center) or presence (right) of sorbitol. B, Super resolution intensity map of PM of a plant expressing ProUBQ10:CLC2-mEOS, in the absence or presence of 300mM sorbitol for 20 min. C, Local density distribution of CLC2-mEOS. D, Track reconstruction in a ProUBQ10:CLC2-mEOS expressing plant. Each color represents the displacement of a single molecule over time. Particles with a high (yellow) and low (blue) diffusion coefficient can be observed. E-F, Diffusion coefficient distribution of CLC2-mEOS, in the absence or presence of sorbitol, and in Col-0 (E) or rbohD (F). The grey box corresponds to the CLC2-mEOS mobile fraction. G, Corresponding graph showing the average percentage of CLC2-mEOS mobile fraction in Col0 or rbohD, in the absence or presence of sorbitol. Histograms show mean values calculated from several thousands of individual tracks with SEM ( $n > 15-31$ ). Scale bars represent 1  $\mu\text{m}$ .

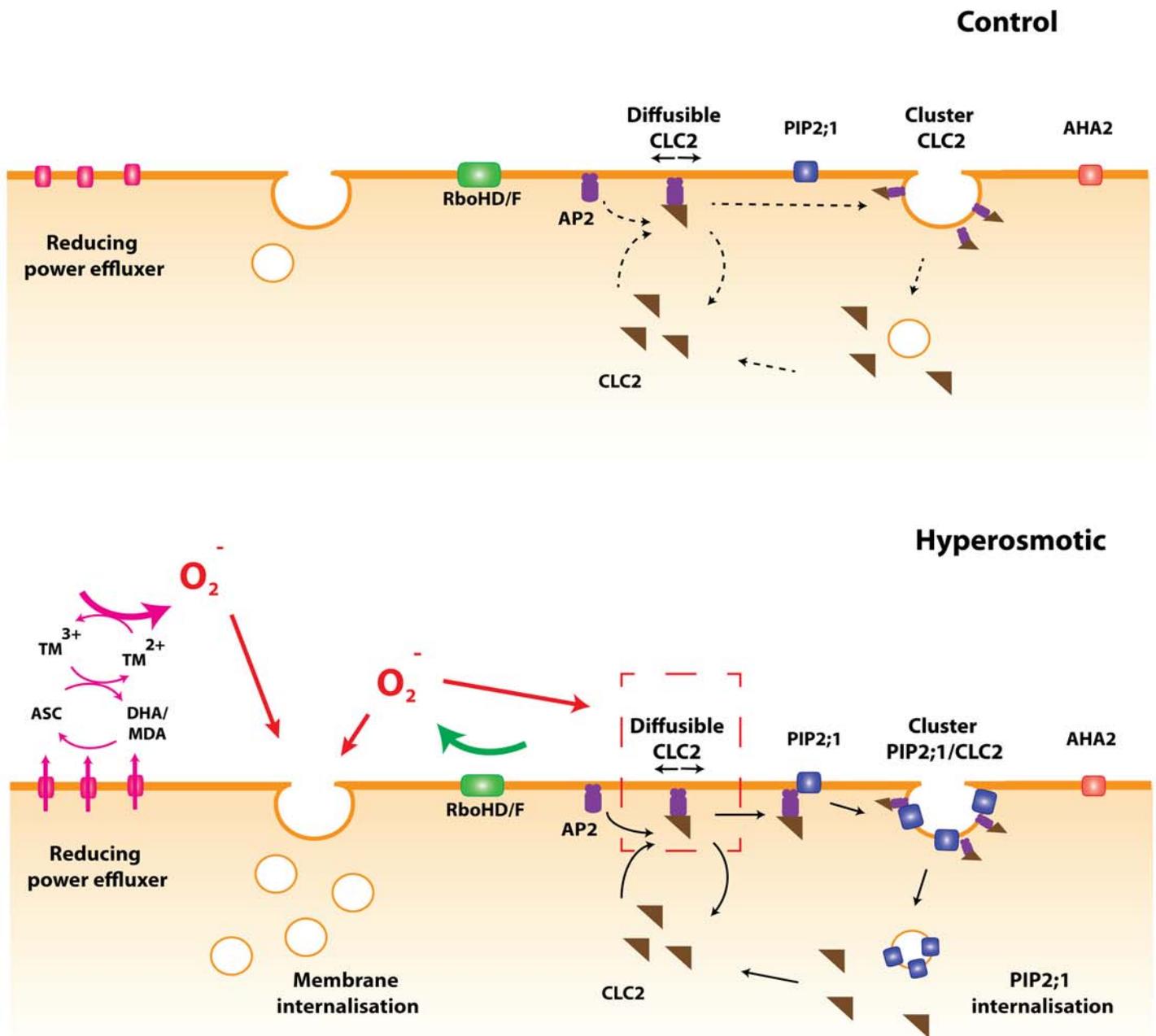


Figure 6: Diagram of ROS signaling and its impact on protein dynamics after a hyperosmotic treatment. In control conditions, RBOHD/F (green) and a putative efflux machinery (pink) for reducing power are inactive. A basal level of membrane internalization exists. CLC2 (brown) is either associated with AP2 (purple) in its diffusible form or is associated with CCVs, yielding non-diffusible forms. PIP2;1 (blue) and AHA2 (red) are organized in clusters and are mostly immobile. After a hyperosmotic treatment, activation of RBOHD/F and the efflux machinery (pink) for reducing power leads to enhanced production of super oxide. In the case of the reducing power efflux, this is achieved either by a direct efflux of cytoplasmic ascorbate (Asc), or an efflux or regeneration of dehydroascobate (DHA). The resulting reducing power reduces apoplasmic transition metals, which in turn react with oxygen to generate ROS. The accumulation of ROS enhances lipid membrane internalization by an unknown mechanism. In parallel, ROS produced by RBOH facilitate plasma membrane association of CLC2/AP2 complexes by promoting interactions of AP2 with lipid at the membrane. An excess of these complexes can bind to PIP2;1, thereby facilitating its incorporation in CCVs. As a consequence, PIP2;1 clustering and endocytosis is enhanced. The rate of CLC2 dissociation from the CCV is intrinsically enhanced by the hyperosmotic stress, this effect being compensated by RBOH-dependent ROS and the above mentioned effects on CL2/AP2 complex formation.

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