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Mehdi Doumane, Alexis Lebecq, Aurélie Fangain, Vincent Bayle, Frédérique Rozier, et al.. The Arabidopsis SAC9 Enzyme defines a cortical population of early endosomes and restricts PI(4,5)P 2 to the Plasma Membrane. 2021. hal-03377804

HAL Id: hal-03377804

<https://hal.science/hal-03377804>

Preprint submitted on 14 Oct 2021

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The Arabidopsis SAC9 Enzyme defines a cortical population of early endosomes and restricts PI(4,5)P₂ to the Plasma Membrane

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Short title: SAC9 prevents the intracellular accumulation of PI(4,5)P₂ in plant cells.

One-sentence summary: SAC9 prevents the accumulation of PI(4,5)P₂ along the endocytic pathway in plants and thereby contributes to the clathrin mediated endocytosis process at the plasma membrane via its interaction with SH3P2

Keywords: Arabidopsis, endocytosis, phosphoinositides, early endosome, plasma membrane

ABSTRACT ~200 words

1 Membranes lipids, and especially phosphoinositides, are differentially enriched within the
2 eukaryotic endomembrane system. This generates a landmark code by modulating the properties of each
3 membrane. Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] specifically accumulates at the plasma
4 membrane in yeast, animal and plant cells, where it regulates a wide range of cellular processes
5 including endocytosis. However, the functional consequences of mispatterning PI(4,5)P₂ in plants are
6 unknown. Here, we functionally characterized the phosphoinositide phosphatase *SUPPRESSOR OF*
7 *ACTIN9* (*SAC9*) in *Arabidopsis thaliana* (Arabidopsis). We found that *SAC9* depletion led to the
8 ectopic localization of PI(4,5)P₂ on cortical intracellular compartments, which depends on PI4P and
9 PI(4,5)P₂ production at the plasma membrane. *SAC9* localizes to a subpopulation of *trans*-Golgi
10 Network/early endosomes that are spatially restricted to a region close to the cell cortex and that are
11 coated with clathrin. Furthermore, it interacts and colocalizes with the endocytic component Src
12 Homology 3 Domain Protein 2 (SH3P2). In the absence of *SAC9*, SH3P2 localization is altered and the
13 clathrin mediated endocytosis rate is significantly reduced. Thus, *SAC9* is required to maintain efficient
14 endocytic uptake, highlighting the importance of restricting the PI(4,5)P₂ pool at the plasma membrane
15 for the proper regulation of endocytosis in plants.

16 INTRODUCTION

17 Phosphoinositides constitute a family of low abundant lipids differentially enriched in the
18 membranes of eukaryotic cells (Platre and Jaillais 2016; Balla 2013; Noack and Jaillais 2020). These
19 versatile lipids can be interconverted into one another. For example, Phosphatidylinositol 4,5-
20 biphosphate [PI(4,5)P₂] is synthesized from Phosphatidylinositol 4-phosphate (PI4P) by PI4P-5 kinases,
21 and is dephosphorylated into PI4P by PI(4,5)P₂ 5-phosphatase (Noack and Jaillais 2017). Furthermore,
22 PI(4,5)P₂ and PI4P are targeted by phospholipases C (PLC) into diacylglycerol and soluble
23 phosphorylated inositol (Balla 2013). PI(4,5)P₂ strictly localizes at the plasma membrane in plants and
24 animal cells (van Leeuwen et al. 2007; Carim et al. 2019; Del Signore et al. 2017; Simon et al. 2014;
25 Ben El Kadhi et al. 2011) despite the plasma membrane being constantly turned-over by endocytosis
26 and exocytosis. At the plasma membrane, PI(4,5)P₂ interacts with a variety of extrinsic membrane
27 proteins such as endocytic protein adaptors (Zhang et al. 2015) and actin-regulatory proteins (Paez
28 Valencia et al. 2016), which are recruited and/or activated by the binding to PI(4,5)P₂. Therefore,
29 PI(4,5)P₂ subcellular patterning likely is critical to regulate the recruitment of proteins that act at the
30 plasma membrane, and the cellular processes they mediate, including clathrin-mediated endocytosis.

31 Consistent with a critical role of PI(4,5)P₂ in the recruitment of the early clathrin-mediated endocytosis
32 factors, a *pip5k1 pip5k2* double mutant in *Arabidopsis thaliana* (*Arabidopsis*) lacking two ubiquitously
33 expressed PI4P-5 kinases, has abnormal auxin distribution and defective endocytic trafficking of the
34 transmembrane auxin efflux carriers PIN-FORMED 1 (PIN1) and PIN2 (Tejos et al. 2014; Ischebeck
35 et al. 2013; Mei et al. 2012). Furthermore, the *pip5k1 pip5k2* double mutant has an altered dynamic of
36 CLATHRIN LIGHT CHAIN2 (CLC2), with the density of CLC2 foci at the plasma membrane being
37 reduced in the mutant (Ischebeck et al. 2013). Overexpression of *Arabidopsis* PI4P-5 kinase 6 in tip-
38 growing pollen tubes induced massive aggregation of the plasma membrane in pollen tube tips due to
39 excessive clathrin-dependent membrane invagination, supporting a role for PI(4,5)P₂ in promoting early
40 stages of Clathrin-mediated endocytosis (Zhao et al. 2010). The inducible overexpression of a highly
41 active human PI4P-5 kinase leads to an increase PI(4,5)P₂ production, very strong developmental
42 phenotypes and heightened endocytic trafficking toward the vacuole (Gujas et al. 2017). In addition,
43 we recently showed that inducible depletion of the PI(4,5)P₂ from the plasma membrane using the
44 iDePP system leads to a decrease of the fraction of the clathrin adaptor protein AP2- μ and the Src
45 Homology (SH3)-domain containing protein 2 (SH3P2) at the plasma membrane (Doumane et al. 2021).
46 Furthermore, FM4-64 uptake experiment confirmed an impact of PI(4,5)P₂ depletion from the plasma
47 membrane on bulk endocytic flow.

48 In animal cells, several PI(4,5)P₂ phosphatases are required for late stages of clathrin-mediated
49 endocytosis (He et al. 2017; Pirruccello et al. 2014). Many PI(4,5)P₂ phosphatases belong to the 5-

50 Phosphatase enzyme family, including OCRL and synaptojanins (Syn1/2) in animals, and synaptojanin-
51 like proteins (Inp51p/Snj1p, Inp52p/Sjl2p and Inp53p/Sjl3p) in *Saccharomyces cerevisiae*. The
52 Arabidopsis genome contains 15 genes encoding 5-phosphatases, but only a few are characterized.
53 Mutation in the 5-phosphatases 9 leads to osmotic stress tolerance, with reduced reactive oxygen species
54 production and Ca^{2+} influx (Golani et al. 2013). The 5-phosphatases 6/COTYLEDON VASCULAR
55 PATTERN2 (CVP2) and 5-phosphatases 7/CVP2 LIKE 1 (CVL1) are specifically required for vascular
56 differentiation (Rodriguez-Villalon et al. 2015; Carland and Nelson 2009; Carland and Nelson 2004).
57 Finally, the 5-phosphatases 15/FRAGILE FIBER 3 (*FRA3*) is expressed in developing fibers and
58 vascular cells, which is consistent with the defective fiber and vessel phenotypes seen in the loss-of-
59 function *fra3* mutant (Zhong et al. 2004).

60 Proteins containing SUPPRESSOR OF ACTIN (or Sac1-like) domains constitute another family of
61 phosphoinositide phosphatases (Zhong and Ye 2003). In Arabidopsis, there are nine SAC proteins,
62 forming three clades (Zhong and Ye 2003). The first clade is composed of SAC1, a PI(3,5)P₂ 5-
63 phosphatase (Zhong et al. 2005), and its relatives SAC2 to 5, putative PI(3,5)P₂ 5-phosphatases
64 (Novakova et al. 2014). The second clade corresponds to SAC7/RHD4 a PI4P 4-phosphatase (Thole et
65 al. 2008) and its relatives SAC6 and SAC8 putative PI4P 4-phosphatases (Song et al. 2021). The third
66 clade is composed of a single member, a plant-specific protein called SAC9. SAC9 has a unique
67 structure, with a SAC phosphoinositide phosphatase domain at its N-terminus, immediately followed
68 by a putative protein/protein interaction domain (WW domain), and a long C-terminal region of 1104
69 amino acids where no putative domains are predicted by sequence homology analysis (Figure 1A,
70 (Zhong and Ye 2003). The *sac9* mutants is dwarf and it constitutively accumulates anthocyanins and
71 expresses genes from stress response pathways (Williams et al. 2005). Loss-of-function alleles of *SAC9*
72 display a three-fold increase in PI(4,5)P₂ content, together with a decrease of PI4P level, suggesting that
73 it acts as a PI(4,5)P₂ 5-phosphatase in planta (Williams et al. 2005).

74 In this study, we show that the phosphoinositide phosphatase activity of SAC9 is required for its
75 function. Using *in vivo* confocal microscopy, we found that SAC9 localizes in a subpopulation of trans-
76 Golgi network/early endosomes (TGN/EEs) spatially restricted to a region close to the cell cortex. Loss
77 of SAC9 results into PI(4,5)P₂ mis-patterning at the subcellular level, leading to the accumulation of
78 PI(4,5)P₂ in subcortical compartments associated with TGN/EEs. SAC9 interacts and colocalizes with
79 the endocytic component SH3P2. In the absence of SAC9, and therefore when the patterning of the
80 PI(4,5)P₂ is compromised, SH3P2 localization is affected and the CLC dynamics is significantly
81 reduced. Thus, SAC9 is required to maintain efficient endocytic uptake, highlighting the importance of
82 restricting the PI(4,5)P₂ pool to the plasma membrane for proper endocytosis in plants.

84 RESULTS

85 The cysteine 459 in the catalytic domain of SAC9 is required for its function

86 We investigated the root phenotype in the already described mutant alleles of SAC9 (Vollmer et al.
87 2011; Williams et al. 2005). As previously described, *sac9-1^{-/-}* and *sac9-3^{-/-}* knock-out mutants are two
88 times shorter compared to the Wild-type (WT) Col-0, at 12 days post-germination (dpg; Figure 1B and
89 1C). We also observed a three-time decrease in the lateral root density of *sac9-1^{-/-}* and *sac9-3^{-/-}*
90 compared to WT plants (Figure 1B to 1D). To confirm that the phenotype observed was due to the loss-
91 of-function of SAC9, we generated Arabidopsis lines expressing SAC9 fused to a fluorescent protein
92 (mCIT-SAC9 and TdTOM-SAC9) under SAC9 native promoter (*SAC9pro*) or the *UBIQUITIN10*
93 promoter (*UBQ10pro*), respectively (Figure 1). To assess the biological relevance of SAC9 phosphatase
94 activity, we also generated a mutated version of SAC9 in the C459 catalytic cysteine into an Alanine
95 (mCIT-SAC9^{C459A}) from C-x(5)-R-[T/S] conserved catalytic motif in the SAC domain (Hsu and Mao,
96 2015, 2013, Figure 1 and Supplemental Figure 1A). We found that both *SAC9pro:mCIT-SAC9* and
97 *UBQ10pro:TdTOM-SAC9* rescued *sac9-3^{-/-}* mutant root phenotype while *SAC9pro:mCIT-SAC9^{C459A}*
98 did not, indicating that i) the root phenotypes described above are indeed caused by SAC9 loss-of-
99 function, ii) N-terminally tagged SAC9 proteins are functional, and iii) SAC9 phosphatase activity is
100 required for SAC9 function (Figure 1B to 1D).

101

102 SAC9 localizes to a population of early endosomes close to the plasma membrane

103 Some phosphoinositide-phosphatases such as Metazoan Sac1 and yeast Sac1p, are able to
104 dephosphorylate *in vitro* several phosphoinositide species, but display a narrower specificity *in vivo*
105 (Hughes et al. 2000; Guo et al. 1999; Rivas et al. 1999). Also, some enzymes involved in
106 phosphoinositide metabolism, such as the yeast PI 4-kinases Stt4p and Pik1p, specifically impact
107 distinct pools of a given phosphoinositide species depending on their subcellular localization (Roy and
108 Levine 2004; Yoshida et al. 1994; Flanagan et al. 1993). Using stable transgenic lines expressing the
109 functional *SAC9pro:mCIT-SAC9* and *Ub10pro:TdTOM-SAC9* constructs, we assessed the subcellular
110 distribution of SAC9 in rescued *sac9-3^{-/-}* homozygous plant using live-cell fluorescence imaging. In
111 meristematic epidermal cells of Arabidopsis roots, mCIT-SAC9 and TdTOM-SAC9 were mainly
112 soluble and excluded from the nucleus (Supplemental Figure 1B). At the cortex of the cell, in close
113 vicinity to the plasma membrane, mCIT-SAC9 localized to a cortical population of mobile dotty
114 structures (Figure 2A, 2B Supplemental Movie 1). We observed that mCIT-SAC9^{C459A} was less soluble
115 with a three-fold increase in the number of mCIT-SAC9^{C459A} labeled dotty structures compared with
116 the signal collected for the functional fusion proteins mCIT-SAC9 and TdTOM-SAC9 (Figure 2C to

117 2E). This result suggests that the phosphoinositide phosphatase activity is required for the dynamic
118 interaction of SAC9 with intracellular membranes. Co-visualization of TdTOM-SAC9 and mCIT-
119 SAC9^{C459A} (Figure 2F) in Col-0 background demonstrated that all TdTOM-SAC9 dotted structures
120 colocalized with mCIT-SAC9^{C459A} at the cortex of the cell, indicating that the catalytic activity of SAC9
121 is only partially required for SAC9 localization. When imaging cells in their cortical part close to the
122 plasma membrane, the mCIT-SAC9^{C459A} signal was concentrated in intracellular compartments at the
123 close vicinity with the plasma membrane labelled by the hydrophobic protein Lti6b-2xmCH (Figure
124 2G). These results suggest a function for SAC9 in regulating phosphoinositide homeostasis either at or
125 in the close vicinity of the plasma membrane.

126 We next investigated the nature of the intracellular structures labeled by mCIT-SAC9. Both mCIT-
127 SAC9 and mCIT-SAC9^{C459A} colocalized with the amphiphilic styryl dye (FM4-64) stained endosomal
128 compartments at the cell's cortex, just beneath the plasma membrane (Figure 3A and 3B). We observed
129 that Brefeldin A (BFA) treatment led to the aggregation of mCIT-SAC9 and mCIT-SAC9^{C459A} into BFA
130 bodies (Figure 3C and 3D). Since early endosomes/TGN (EE/TGN) are sensitive to BFA while late
131 endosomes (LE/MVB) are not (Takagi and Uemura 2018), this result suggest that both functional and
132 catalytically dead SAC9 fusion proteins localize to endosomes and are likely part of the early steps of
133 endocytic trafficking pathway.

134 To get more precise insights into the SAC9's localization at the TGN, we crossed Arabidopsis lines
135 expressing fluorescent tagged SAC9 and SAC9^{C459A} proteins with endomembranes markers (Geldner et
136 al. 2009). Using live-cell imaging in root meristematic cells, we observed that mCIT-SAC9 colocalized
137 with TGN markers (CLC2-RFP >79% colocalization; mCH-RabA1g >91%, mCH-VTI12¹²⁶⁻²²² >67%;
138 Figure 3C, 3E and 3F). The colocalization of mCIT-SAC9 with these TGN markers was only partial
139 since mCIT-SAC9 was predominantly observed in the cortical section of the cell (Figure 2A), at the
140 close vicinity to the plasma membrane, whereas classical TGN markers are not restricted to the cell
141 cortex (Figure 3E and 3F). This was even more striking when localizing the fluorescent tagged SAC9
142 together with mCH-RabD1 (<26% colocalization, Figure 3E and 3F) that also localizes at TGN.
143 Eventually, mCIT-SAC9 showed very weak colocalization with late endosome (LE/MVB) marker
144 (mCH-RabF2a <17%, Figure 3E and 2F), and with the Golgi marker (mCH-Got1p <1%, Figure 3E and
145 3F). Similarly, mCIT-SAC9^{C459A} strongly colocalized with CLC2-RFP and mCH-RAB-A1g markers,
146 whereas it colocalized much weakly with mCH-RabD1 and did not colocalized with the Golgi marker
147 (Figure 3C, 3G and 3H). This confirmed that SAC9 and SAC9^{C459A} fusion proteins localized to the
148 TGN/EE, with the noticeable difference that SAC9 localization in endomembrane compartments was
149 restricted to the cortex of the cell, suggesting a role of SAC9 in PI(4,5)P₂ homeostasis between the
150 plasma membrane and early endosomal compartments.

151

152 SAC9 is required to maintain the pool of PI(4,5)P₂ at the plasma membrane

153 It was previously reported that *Arabidopsis sac9-1* loss-of-function mutant had a diminution of PI4P
154 and a three-fold accumulation of PI(4,5)P₂, suggesting that SAC9 has a PI(4,5)P₂ 5-phosphatase
155 catalytic activity (Williams et al. 2005). In order to visualize the effect of the perturbation of the PIP-
156 metabolism in the absence of SAC9, we introgressed two independent PI(4,5)P₂ biosensors (Simon et
157 al. 2014) into *sac9-3^{-/-}* genetic background. As expected, both PI(4,5)P₂ biosensors strictly labeled the
158 plasma membrane in wild-type cells (Figure 4A and 4C). In *sac9-3^{-/-}*, mCIT-2xPH^{PLC} and mCIT-
159 TUBBYc not only labeled the plasma membrane, but also decorated cortical intracellular dotted
160 structures (Figure 4A and 4C). Thus, SAC9 is required for PI(4,5)P₂ restriction at the plasma membrane.
161 We also observed an increase of the number of intracellular compartments labeled by the PI4P
162 biosensors (Simon et al. 2014; Simon et al. 2016) mCIT-PH^{FAPP1} and mCIT-P4M^{SidM}, but not for mCIT-
163 PH^{OSBP} (Figure 4B and 4C). These results are consistent with a diminution of the PI4P pool at the plasma
164 membrane and therefore the relocalization of the PI4P biosensor at intracellular compartments, as
165 previously reported (Simon et al. 2016). The subcellular localization of PI3P sensors (mCIT-FYVE^{HRS}
166 and mCIT-PHOX^{p40}) was identical between Col-0 and *sac9^{-/-}* cells (Figure 4C, Supplemental Figure
167 2A). We also detected a slight but significant decrease in the density of intracellular compartments
168 decorated by mCIT-C2^{Lact} phosphatidylserine biosensor in *sac9-3^{-/-}* (Figure 4C; Supplemental Figure
169 2B). Taken together, these results indicate that SAC9-depletion leads to a massive change in PI(4,5)P₂
170 subcellular patterning, which is present on intracellular cortical structures instead of only being present
171 at the plasma membrane.

172 Previous investigation of the fine structure in *SAC9* mutant allele reported a massive accumulation
173 of vesicles, presumably containing cell wall, at the close vicinity to the plasma membrane (Vollmer et
174 al. 2011). When co-imaging 2xmCH-2xPH^{FAPP1} together with mCIT-TUBBYc in *sac9-3^{-/-}* (Figure 4D), the
175 dotted structures decorated by the PI(4,5)P₂ biosensor—but not with the PI4P biosensor—were observed
176 at the cortex of the cell, at the close vicinity with the plasma membrane (Figure 4D upper panel and
177 4E), whereas those structures were rarely observed in the internal part of the cell (Figure 4D lower panel
178 and 4E). Therefore dotted structures containing PI(4,5)P₂ were associated with PI4P labelled
179 complements but they did not strictly overlap. Confocal imaging of *sac9-3^{-/-}* mutant co-expressing
180 mCIT-TUBBYc with endosomal markers revealed that the dotted structures containing PI(4,5)P₂ were
181 found associated with FM4-64 endocytic tracer as well as TGN markers (Supplemental Figure 3A-B).
182 Furthermore, BFA treatment efficiently induced 2xmCH-2xPH^{FAPP1} positive BFA bodies in *sac9-3^{-/-}*
183 but did not affect the distribution of mCIT-TUBBYc compartments in the same cells (Supplemental
184 Figure 3C). Therefore, PI(4,5)P₂ intracellular compartments in *sac9-3^{-/-}* are devoid of any ARF GTPase
185 activated by BFA sensitive ARF-GEF. *In vivo* time-lapse imaging of PI(4,5)P₂ biosensor mCIT-
186 TUBBYc and mCIT-2xPH^{PLC} in *sac9-3^{-/-}* mutant revealed that those intracellular structures were mobile

187 in the cortex of root epidermal cells, hence, behaving like intracellular compartments (Supplemental
188 Figure 3D, Supplemental movie 2).

189 We next addressed the turnover and the origin of the cortical intracellular PI(4,5)P₂ compartment
190 observed in *sac9-3^{-/-}*. We previously showed that short-term treatment (15-30 min) with phenyl arsine
191 oxide (PAO), a pan PI 4-kinases inhibitor, significantly depletes PI4P (Figure 5E) but not PI(4,5)P₂
192 pools at the plasma membrane of plant cells, whereas longer treatment (> 60 min) affects the synthesis
193 of both lipids (Figure 5A, Platre et al., 2018; Simon et al., 2016). We used this pharmacological
194 approach to test the effect of the inhibition of either PI4P, or both PI4P and PI(4,5)P₂ synthesis, on *sac9-3^{-/-}*
195 anomalous PI(4,5)P₂ intracellular compartments (Figure 5B to 5E). Solubilization of mCIT-PH^{FAPP1-}
196 ^{E50A} PI4P biosensor in *sac9-3^{-/-}* cells treated for either 30 or 120 min with PAO confirmed the efficient
197 PI4P depletion in both conditions (Figure 5E). Solubilization of mCIT-TUBBYc PI(4,5)P₂ biosensor in
198 WT after 120 min of PAO exposure, but not after 30 min, confirmed that an efficient PI(4,5)P₂ depletion
199 occurred only for the longest treatment (Figure 5B and 5C). 30 min PAO treatment did not affect
200 anomalous *sac9-3^{-/-}* PI(4,5)P₂ compartments, but 120 min PAO treatment significantly reduced the
201 number of anomalous PI(4,5)P₂ compartments compared to both 120 min mock treatments or 30 min
202 short treatment (Figure 5B and 5C), showing that intracellular PI(4,5)P₂ compartments in *sac9-3^{-/-}* are
203 dependent on the PI4P synthesis, itself substrate for PI(4,5)P₂ production.

204

205 **SAC9 is required for efficient endocytic trafficking**

206 Because of the specific localization of SAC9 at the cortex of the cell and its colocalization with early
207 TGN/EE markers, we wondered whether PI(4,5)P₂ defective patterning in *sac9-3^{-/-}* correlated with
208 endocytic defects. We counted the number of labeled endosomes following FM4-64 endocytic tracer
209 treatment in cells from WT and *sac9-3^{-/-}* seedlings (Rigal et al. 2015). We observed a significant near
210 two-fold decrease in the number of FM4-64-labelled endosomes per cells in *sac9-3^{-/-}* compared to WT
211 (Figure 6A and B), that was not caused by smaller cells in *sac9-3^{-/-}* as the density of FM4-64-labelled
212 endosomes was also strongly and significantly decreased compared to WT (Supplemental Figure 4). We
213 inhibited recycling with BFA, and used FM4-64 tracer to monitor the endocytosis by measuring the
214 number of BFA bodies labeled by FM4-64 in Col-0 and *sac9-3^{-/-}*. We observed significantly less FM4-
215 64-labelled BFA bodies per cells in *sac9-3^{-/-}* compared to wild-type (Figure 6C and D), confirming the
216 lower rate of endocytosis in this mutant. We then assessed whether SAC9 depletion affected the
217 trafficking of cargo proteins. We, therefore, performed another BFA assay, but using the integral
218 membrane protein PIN-FORMED2 fused with GFP (PIN2-GFP) which localizes at the plasma
219 membrane and in intracellular organelles as it continuously recycles (Armengot et al. 2016). We
220 observed significantly less PIN2-GFP-labelled BFA bodies per cell in *sac9-3^{-/-}* compared to WT (Figure

221 6E and F). PIN2-GFP being partially located on intracellular organelles before BFA treatment, the effect
222 observed may, therefore, indicate an endocytic defect as supported by the others experiments, , and/or
223 a more general defect in trafficking in *sac9-3^{-/-}*.

224 To gain further insights into the function of SAC9 in bulk endocytosis, we investigated the sensitivity
225 of the *sac9* mutant to pharmacological inhibition of endocytosis. To this end, we used the recently
226 described ES9-17, which is a specific inhibitor of clathrin-mediated endocytosis (Dejonghe et al. 2019).
227 We treated wild-type and *sac9-3^{-/-}* seedlings with ES9-17 for 180 min and labeled the plasma membrane
228 and endosomes with FM4-64. Cells from both mock-treated wild-type and *sac9-3^{-/-}* seedlings did not
229 display any obvious defect (Figure 6G). On the contrary, we observed dome-shaped plasma membrane
230 invaginations in ES9-17 long-term treatment of Col-0 seedlings, almost exclusively in elongating or
231 differentiated cells (epidermal or root cap cells), substantiating the possibility that these invaginations
232 constitute read-outs of long term disturb endocytosis (Figure 6G and H). Strikingly, we observed a
233 much higher number of dome-shaped plasma membrane invagination in cells from ES9-17 treated *sac9-*
234 *3^{-/-}* (Figure 6G and H), showing that SAC9 depletion causes over-sensitivity to inhibition of endocytosis.
235 Hypersensitivity to endocytosis inhibition, decreased internalization of the bulk endocytic tracer FM4-
236 64 and defects in PIN2 protein trafficking together indicate that endocytic trafficking is impacted in the
237 absence of SAC9.

238

239 **SAC9 is required for SH3P2 localization at the plasma membrane.**

240 To gain some insights into SAC9 function, we next screened for SAC9 interactors using a yeast
241 two hybrid assay against a universal Arabidopsis normalized dT library (ULTimate Y2H SCREEN,
242 hybrigenics). As a bait, we used a portion of SAC9 (AA 499 to 966), which includes the WW domain,
243 a putative protein-protein interaction platform. Out of 59.6 million screened interaction, we recovered
244 and sequenced 260 independent clones corresponding to 107 different proteins. However, among these
245 proteins, most were classified as proven artifact or low confidence interaction and only three candidates
246 (2.8 %) were ranked as very high confidence in the interaction (Supplemental Table 2). Among the high
247 confident interaction candidate, we identified 11 clones corresponding to SH3P2 (5 independent
248 clones). In the yeast-two-hybrid screen, the selected interaction domain identified for SH3P2,
249 corresponds to the C-terminal part of the proteins (aa 213-368), which includes the SH3 domain (Figure
250 7A). We decided to focus on this candidate because, like SAC9, SH3P2 is linked to both clathrin-
251 mediated endocytosis and membrane phosphoinositides. Indeed, SH3P2 i) colocalizes with clathrin
252 light chain, ii) cofractionates with clathrin-coated vesicles (Nagel et al. 2017), and iii) co-
253 immunoprecipitates in planta with clathrin heavy chain (Nagel et al. 2017), clathrin light chain
254 (Adamowski et al. 2018) and the dynamin DRP1A (Ahn et al. 2017). Furthermore, SH3P2 binds to

255 various phosphoinositides in vitro (Zhuang et al. 2013; Ahn et al. 2017), and its plasma membrane
256 localization is dependent on PI(4,5)P₂ in vivo (Doumane et al. 2021).

257 Using live cell imaging we observed that fluorescently tagged SAC9^{C459A} and SH3P2 colocalized
258 in a subcortical population of endomembrane compartments (Figure 7BC), probably TGN/EEs (Figure
259 3E and F). Because SH3P2 was described to play a role at different steps of the trafficking, from the
260 endocytosis at the plasma membrane to autophagy, we next addressed more precisely which pool of
261 SH3P2 was affected in absence of SAC9. Using confocal imaging, we observed a diminution of the
262 signal corresponding to SH3P2-sGFP at the plasma membrane compared to the cytoplasm in *sac9*
263 (Figure 7D-E). Thus, consistently with the idea that SAC9 regulates SH3P2 localization at the plasma
264 membrane through its interaction, these results point toward a role of SAC9 in endocytosis.

265

266 **The endocytic protein T-PLATE has an altered dynamic in the absence of SAC9**

267 SAC9 is observed at the close vicinity to the plasma membrane and its absence causes a mislocalization
268 of its protein partner SH3P2, as well as a reduction of the overall endocytosis process. We then assessed
269 if the clathrin-mediated endocytosis at the plasma membrane was affected in absence of SAC9. Using
270 Total Internal Reflection Fluorescence (TIRF) microscopy, we determined the density and dynamic
271 behavior of the endocytic protein from the T-PLATE complex, T-PLATE-GFP, in the *sac9* mutant
272 compared with wild-type plants. Quantitative analysis revealed that the density of T-PLATE-GFP was
273 reduced in *sac9-3^{-/-}* compared with the WT (Figure 8A, B; supplemental movie 3). Analysis of the
274 dynamics of T-PLATE-GFP at the plasma membrane of etiolated hypocotyl revealed a decrease of the
275 dwell time of T-PLATE-GFP at the plasma membrane in the *sac9* mutant compared to the WT. This
276 result is in line with a reduction of the endocytic flow in the absence of SAC9, and suggests that
277 maintaining a strict plasma membrane accumulation of PI(4,5)P₂ is critical for clathrin-mediated
278 endocytosis.

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284 DISCUSSION

285 Here, we showed that the phosphatase activity of SAC9 is required for its function, and that
286 fluorescent SAC9 protein fusions colocalize with TGN/EE markers in a subpopulation of endosomes
287 close to the plasma membrane. We found that the subcellular patterning of PI(4,5)P₂ is defective in *sac9*
288 mutants, consistent with the idea that SAC9 is a PI(4,5)P₂ 5-phosphatase. In planta, SAC9 interacts and
289 colocalizes with the endocytosis component SH3P2. In the absence of SAC9, and therefore when the
290 patterning of the PI(4,5)P₂ at the plasma membrane and at its close vicinity is affected, SH3P2 no longer
291 localizes at the plasma membrane and the rate of clathrin-mediated endocytosis is significantly reduced.
292 Together, these findings underlie the importance of strictly restricting PI(4,5)P₂ to the plasma membrane
293 during the endocytic process.

294

295 SAC9 and phosphoinositide interconversion along the endocytic pathway

296 In animal cells, a phosphoinositide conversion cascade has been described through the successive
297 action of phosphoinositide kinases and phosphatases. This cascade starts with PI(4,5)P₂ at the plasma
298 membrane and ends-up with PI3P in the membrane of early endosomes (Noack and Jaillais 2017;
299 Schmid and Mettlen 2013; Abad et al. 2017; Posor et al. 2015; Posor et al. 2013; Shin et al. 2005).
300 Briefly, PI(4,5)P₂ is dephosphorylated at the plasma membrane or during the endocytic process by 5-
301 phosphatase enzymes such as OCRL and synaptojanin (De Matteis et al. 2017) (Cauvin et al. 2016;
302 Nandez et al. 2014; Ben El Kadhi et al. 2011; Posor et al. 2013). PI4P is then phosphorylated into
303 PI(3,4)P₂ inside clathrin-coated pits by a PI3-Kinase (PI3K C2α), before being converted into PI3P
304 through the action of a PI4P phosphatase on clathrin-coated vesicles (Schmid and Mettlen 2013; Posor
305 et al. 2013). Because both PI(4,5)P₂ and PI3P are essential regulators of endocytic trafficking, this
306 conversion cascade, and in particular the precise recruitment of dedicated enzymes at the right place
307 and at the right time during endocytosis, is of critical importance of clathrin-mediated endocytosis to
308 proceed normally (Noack and Jaillais 2017; Schmid and Mettlen 2013).

309 Plants have a much simpler endosomal system, where the TGN functions as an early endosome.
310 This hybrid compartment collects endocytic vesicles and redirects cargo proteins either to the plasma
311 membrane for recycling or to late endosomes/vacuole for degradation (Dettmer et al. 2006; Narasimhan
312 et al. 2020; Rodriguez-Furlan et al. 2019). The plant TGN/EE is enriched in PI4P, not PI3P, which
313 instead accumulates in late endosomes (Noack and Jaillais 2020; Simon et al. 2014). Interestingly, we
314 found that i) SAC9 localizes to clathrin-coated vesicles close to the plasma membrane, and ii) the *sac9*
315 mutant accumulates PI(4,5)P₂ in vesicular structures at the cortex of the cells. Together, we propose
316 that SAC9 represent the long sought-after enzyme which performs the PI(4,5)P₂-to-PI4P conversion

317 during the plant endocytic process (Figure 9). As such, SAC9 is required to erase PI(4,5)P₂ in endosomal
318 membranes and thereby maintains this lipid strictly at the plasma membrane.

319

320 **Accumulation of PI(4,5)P₂-containing vesicles in the *sac9* mutant**

321 In the absence of SAC9, PI(4,5)P₂ accumulates inside the cell in what appears to be abnormal
322 compartments that are associated with but independent from TGN/EEs. We can speculate that the
323 prolonged accumulation of PI(4,5)P₂ on clathrin-coated vesicles, and perhaps the lack of PI4P
324 production on these structures, impairs the function of these vesicles. Ultrastructural analyses
325 previously revealed that *sac9* accumulates extra vesicles close to the cell surface (Vollmer et al. 2011).
326 The finding of these unknown subcortical vesicles in *sac9* resonates with our confocal microscopy
327 observations of ectopic PI(4,5)P₂-containing vesicles in this mutant. Importantly, we found that these
328 vesicles are not extensively labeled after a short-term FM4-64 treatment. This suggests that these
329 vesicles are accumulating slowly over time in the *sac9* mutants and are not actively exchanging
330 membrane materials with the TGN/EEs or the plasma membrane, at least not in the 30-minute time
331 frame of the FM4-64 pulse labeling experiment. Together, we propose that PI(4,5)P₂ accumulation on
332 endocytic vesicles after scission could lead to an altered dynamics of these vesicles, which may be
333 unable to fuse correctly with TGN/EEs, ultimately leading to their accumulation in the cell cortex
334 (Figure 9).

335 We observed that the endocytic pathway is partially impaired in absence of SAC9, and that *sac9* was
336 oversensitive to ES9-17-mediated inhibition of endocytosis. In animal cells, membrane protuberances
337 have been reported in cultured cells knocked-out or knock-down for enzymes of PI(4,5)P₂ metabolism
338 (Gurung et al. 2003; Terebiznik et al. 2002; Mochizuki and Takenawa 1999). These protuberances could
339 therefore be a conserved process affected by disturbed PI(4,5)P₂ homeostasis in both plant and animal
340 cells leading to such plasma membrane distortion. It is not the first observation of plasma membrane
341 protuberances in cells where PI(4,5)P₂ homeostasis is affected: upon PIP5K6 over-expression in pollen
342 tubes, which increases PI(4,5)P₂ levels, plasma membrane invaginations also occurs. The authors
343 elegantly demonstrated that these plasma membrane distortions are due to clathrin-mediated
344 endocytosis defects (Zhao et al. 2010). These structures were also reported when inducible depletion of
345 PI(4,5)P₂ at the plasma membrane of root cells was performed using the iDePP system (Doumane et al.
346 2021), suggesting that the tight regulation of the PI(4,5)P₂ metabolism is important for the integrity of
347 the plasma membrane. Previous study on the cellular organization of the primary roots of *sac9-1*
348 seedlings at ultrastructural level reported the excessive membrane material either in direct contacts or
349 close to the wall forming the protuberances (Vollmer et al. 2011). The irregular cell wall deposition in
350 the *sac9* mutant leads to cell wall ingrowths, coined “whorl-like structures” (Vollmer et al. 2011). We

351 propose that such protuberances could be a readout of long-term endocytic defects, for instance caused
352 by an excess of plasma membrane due to unbalanced exocytosis. If so, the appearance of plasma
353 membrane protuberances upon PI(4,5)P₂ metabolism perturbation could be an additional indication of
354 disturbed endocytosis.

355

356 **How could SAC9 regulate endocytosis?**

357 We observed that the endocytic pathway is partially impaired in the absence of SAC9. We envision
358 several scenarios to explain these endocytic defects. They are not mutually exclusive and ultimately, it
359 would not be surprising if the *sac9* endocytic phenotype results from a combination of altered cellular
360 pathways relying, directly or indirectly, on the precise spatio-temporal regulation of anionic lipid
361 homeostasis. First, as mentioned in the paragraph above, the *sac9* mutant likely accumulates ectopic
362 vesicle following endocytosis. These vesicles trap membrane components inside the cell, which may
363 overall impact the endocytic process. In addition, it is possible that the localization or dynamics of
364 PI(4,5)P₂ binding proteins, which regulate clathrin-mediated endocytosis, could be impacted, as we
365 observed for SH3P2 and T-PLATE (Zhuang et al. 2013; Yperman et al. 2021b; Yperman et al. 2021a).
366 Other proteins from the T-PLATE complex also interact with anionic lipids, including PI(4,5)P₂, and
367 could be affected in *sac9* (Yperman et al. 2021b; Yperman et al. 2021a). Furthermore, the dynamics of
368 dynamin-related proteins, which contain a lipid-binding plekstrin homology domain, and the AP2
369 adaptor complex, which relies on PI(4,5)P₂ for localization in planta (Doumane et al. 2021), could also
370 be altered in *sac9*. It was also recently reported that perturbation of clathrin-containing vesicles at the
371 TGN/EEs also impacts clathrin-mediated endocytosis (and conversely) (Yan et al. 2021). It is thus
372 possible that perturbing phosphoinositides metabolism after the scission of clathrin-coated vesicles
373 from the plasma membrane impacts the dynamics of clathrin-mediated endocytosis at the cell surface
374 in an indirect way, for example because of slowed recycling of the clathrin pool or other endocytic
375 components at the surface of TGN/EEs.

376 It is also important to note that bulk endocytosis in general and clathrin-mediated endocytosis in
377 particular are still going-on in the *sac9* mutant, albeit at a reduced rate. Thus, while PI(4,5)P₂ strict
378 exclusion from intracellular membranes is important for the endocytic process, the plant membrane
379 trafficking system appears to be extremely resilient, as it manages to operate despite the accumulation
380 of these membrane of mixed identity inside the cell. Here, we can speculate that SAC9 may not be the
381 only 5-phosphatase enzyme that can control PI(4,5)P₂ homeostasis during endocytosis. In addition,
382 while PI(4,5)P₂ is the main phosphoinositide regulating the recruitment of endocytic regulators in
383 animals, it is likely not the case in plants. Indeed, PI4P, not PI(4,5)P₂, is the major anionic lipids that
384 powers the plasma membrane electrostatic field (Simon et al. 2016), and is very likely key to recruit

385 endocytic proteins in plants (Yperman et al. 2021b; Yperman et al. 2021a). In addition, phosphatidic
386 acid (PA) is also important to drive the electrostatic field of the plasma membrane (Platre et al. 2018)
387 and PA could be involved in the localization of AtEH1/Pan1, a component of the T-PLATE complex
388 (Yperman et al. 2021a).

389

390 **Limitation of the study**

391 SAC9 was previously proposed to act as a PI(4,5)P₂ 5-phosphatase in planta, mainly because the
392 *sac9* mutant accumulates PI(4,5)P₂ (Williams et al. 2005). We found an accumulation of PI(4,5)P₂
393 sensors inside the cells in *sac9*, which is fully consistent for a 5-phosphatase enzyme. However, we
394 could not confirm this activity in vitro. This is mainly due to problems in purifying SAC9 catalytic
395 domain in sufficient quantity. Yet, it represents a weakness of our study that we should keep in mind.
396 Indeed, although this is not the most parsimonious explanation, it is possible that SAC9 is not a 5-
397 phosphatase and that instead it controls the localization or activity of another enzyme bearing this
398 catalytic activity. Nonetheless, the fact that catalytically inactive mCIT-SAC9^{C459A} fusion protein does
399 not rescue *sac9* macroscopic phenotype suggests that SAC9 phosphatase activity is required for its
400 function.

401 To explain the endocytic defects observed in the *sac9* mutant, we mainly focused on PI(4,5)P₂ mis-
402 patterning. However, biochemical measurements also showed that the *sac9* mutant accumulates less
403 PI4P than its wild-type counterpart. Given the importance of PI4P for plant cell function (Noack et al.
404 2020; Simon et al. 2016), it is possible that PI4P rather than (or in combination with) PI(4,5)P₂ defects
405 are involved in the *sac9* phenotypes. Given that phosphoinositide metabolism is highly intricate, we
406 fully recognize that it is difficult to fully untangle the specific involvement of each lipid in the observed
407 phenotypes. In addition, the SAC9 protein may carry specific functions outside of its catalytic activity.
408 For example, SAC9 directly interacts with SH3P2, yet the physiological relevance of this interaction
409 for both SAC9 and SH3P2 function remains unclear. Structure-function analyses aiming at dissecting
410 this interaction will be instrumental to clarify this point. In addition, the exact function of SH3P2 in
411 endocytosis is also elusive and it will be important to analyze it in details in the future. Finally, one of
412 the clear limitations in interpreting the *sac9* phenotype, which is common to most genetic approaches,
413 is the accumulation of defects over a long-time period in the mutant. As such, it is impossible to pin-
414 point toward direct or indirect effects of PI(4,5)P₂ mis-patterning on the cellular and developmental
415 phenotypes of this mutant. Future studies, aiming at rapidly manipulating SAC9 function and
416 localization in an inducible manner will be instrumental in disentangling the direct and indirect effects
417 of SAC9 on lipid dynamics and endocytosis regulation.

418 **MATERIALS AND METHODS**

419 **Growth condition and plant materials**

420 *Arabidopsis thaliana* Columbia-0 (Col-0) accession was used as wild-type (WT) reference genomic
421 background throughout this study. *Arabidopsis* seedlings *in vitro* on half Murashige and Skoog (½ MS)
422 basal medium supplemented with 0.8% plant agar (pH 5.7) in continuous light conditions at 21 °C.
423 Plants were grown in soil under long-day conditions at 21 °C and 70% humidity 16 h daylight.

424

425 **Cloning**

426 *SAC9* promoter (*SAC9pro*; 800 bp) was amplified from Col-0 genomic DNA using *SAC9prom_fwd_gb*
427 TTGTATAGAAAAGTTGCTATTGAAAAAAGATAGAGGCGCGTG and *SAC9prom_rev_gb*
428 TTTTTTGTACAAACTTGCCTGAGCTCAGGACCAAGCGG primers and the corresponding PCR
429 product was recombined into *pDONR-PIRP4* (life technologies www.lifetechnologies.com/) vector by
430 BP reaction to give *SAC9pro/pDONR-PIRP4*. The *mCIT* and *TdTOM* containing vectors
431 *cYFPnoSTOP/pDONR221* and *TdTOMnoSTOP/pDONR221* were described before (Simon et al., 2014;
432 Jaillais et al., 2011).

433 The genomic sequence of *SAC9* (At3g59770) was amplified by PCR using 7-day old *Arabidopsis*
434 seedlings gDNA as template and the *SAC9-B2R*
435 GGGGACAGCTTTCTTGTACAAAGTGGCTATGGATCTGCATCCACCAGGTTAGT and *SAC9-*
436 *B3wSTOP* GGGGACAACCTTTGTATAATAAAGTTGCTCAGACACTTGAAAGGCTAGTCCAT
437 primers. The corresponding PCR product was recombined into *pDONR-P2RP3* vector by BP reaction
438 to give *SAC9g/pDONR-P2RP3*.

439 *SAC9*^{C459A} mutation was obtained by site-directed mutagenesis using the partially overlapping *SAC9-*
440 *C459A-F* TACGTTTTAACGCTGCTGATTCCCTTGGATCGAACAAATGC and *SAC9-C459A-R*
441 AGGAATCAGCAGCGTTAAAACGTATCACCCATTTTGATGTG primers on *SAC9g/pDONR-*
442 *P2RP3*.

443 Final destination vectors were obtained using three fragments LR recombination system (life
444 technologies, www.lifetechnologies.com/) using pB7m34GW destination vector (Karimi et al. 2007).
445 The following reactions were set-up to generate the corresponding destination vectors:
446 UBQ10prom:tdTOM-SAC9g/pH, *SAC9*prom:mCIT-SAC9g/pB, pAt*SAC9*:mCIT-SAC9g^{DEAD}/pB,
447 *SAC9*prom:TdTOM-SAC9g^{DEAD}/pH (Supplemental Table 1).

448

449 **FM4-64 staining and drug treatments**

450 Seedlings were incubated in wells containing 1 μ M FM4-64 (Life Technologies T-3166; from a stock
451 solution of 1.645 mM = 1 mg/ml in DMSO) in half Murashige and Skoog ($\frac{1}{2}$ MS) liquid basal medium
452 without shaking for 30 min and in dark. Seedlings were then mounted in the same medium and imaged
453 within a 10 min time frame window (1 h \pm 5 min).

454 Seedlings were incubated in wells containing Brefeldin A (BFA; Sigma B7651) applied at 50 μ M (from
455 a stock solution of 30 mM in DMSO), or a corresponding volume of DMSO as ‘mock’ treatment,
456 dissolved in liquid $\frac{1}{2}$ MS for one hour in dark without shaking before mounting in the same medium
457 and imaging. For co-treatment with 50 μ M BFA and 1 μ M FM4-64, FM4-64 and BFA were added at
458 the same time. Imaging was performed within a 14 min time frame window (1 h \pm 7 min).

459 For PAO treatment, seedlings were incubated in wells containing 30 μ M PAO (Sigma P3075,
460 www.sigmaaldrich.com, PAO stock solution at 60 mM in DMSO), or a volume of DMSO as mock
461 treatment, during the indicated time. Roots were imaged within a 10 min time frame window around
462 the indicated time. The PAO effects on the localization of our biosensors were analyzed by counting
463 manually the number of labelled compartments per cell.

464 ES9-17 was stored at -20°C as a 30 mM stock solution in DMSO. Seedlings were incubated in dark
465 without shaking for the indicated duration (\pm 7 min) in liquid $\frac{1}{4}$ MS (pH 5.7) in wells containing 1 %
466 DMSO to help solubilization of ES9-17, and either 1 μ M ES9-17 or the corresponding additional
467 volume of DMSO (mock treatment). For 1 μ M ES9-17 and 1 μ M FM4-64, FM4-64 was added 30 min
468 after the ES9-17 (the indicated time corresponds to ES9-17 exposure).

469

470 **Western-Blot**

471 To verify that SAC fusion proteins were present in the plant cells, leaves from 1-month-old plants
472 were vacuum infiltrated three times with 100 μ M MG132 (inhibitor of proteasome). Treated leaves
473 were grind in liquid nitrogen, and ground material resuspended in 4 ml of extraction buffer (100 mM
474 Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 10 mM DTT, 0.5% Triton X-100, 1%
475 Igepal and 1% protease inhibitors (Sigma) into $\frac{1}{2}$ MS 100 μ M MG132) and centrifuged at 17896 g for
476 40 min at 4°C to obtain the total protein extract in the supernatant. 1 ml of the total protein extract was
477 incubated 20 min with 50 μ L of magnetic protein G Dynabeads (Millipore) fused to a GFP monoclonal
478 antibody (1/500 in PBS 0.002 % tween). Beads, antibodies and antibodies bund proteins were

479 magnetically precipitated on columns, eluted and denatured in 40 μ L of Lamelli buffer 2X at 95°C for
480 5 min. 20 μ L of immunoprecipitated proteins were loaded onto 7.5 % polyacrylamide gel, run for 105
481 min at 125 V and blotted on nitrocellulose membranes over-night at 20 V on ice. Membranes were
482 blocked in 5 % milk dissolved in TBST buffer (10 mM Tris-HCL, 150 mM NaCL, 0.05 % Tween20)
483 for 2 h at 4°C. mCIT tagged proteins were revealed by using respectively GFP monoclonal antibody
484 (1/1000 in 5 % milk) as primary antibodies and anti-mouse IgG-HRP conjugated secondaries
485 antibodies (1/5000 in TBST). Finally tagged proteins were detected by chemiluminescence using ECL,
486 substrate to HRP enzyme, revelation.

487

488 **Live cell imaging**

489 Most images (see exceptions below) were acquired with the following spinning disk confocal
490 microscope set up: inverted Zeiss microscope (AxioObserver Z1, Carl Zeiss Group,
491 <http://www.zeiss.com/>) equipped with a spinning disk module (CSU-W1-T3, Yokogawa,
492 www.yokogawa.com) and a ProEM+ 1024B camera (Princeton Instrument,
493 <http://www.princetoninstruments.com/>) or Camera Prime 95B (www.photometrics.com) using a 63x
494 Plan-Apochromat objective (numerical aperture 1.4, oil immersion). GFP and mCITRINE were excited
495 with a 488 nm laser (150 mW) and fluorescence emission was filtered by a 525/50 nm BrightLine!
496 single-band bandpass filter (Semrock, <http://www.semrock.com/>), mCHERRY en TdTOM was excited
497 with a 561 nm laser (80 mW) and fluorescence emission was filtered by a 609/54 nm BrightLine! single-
498 band bandpass filter (Semrock, <http://www.semrock.com/>). For quantitative imaging, pictures of
499 epidermal root meristem cells were taken with detector settings optimized for low background and no
500 pixel saturation. Care was taken to use similar confocal settings when comparing fluorescence intensity
501 or for quantification.

502 Colocalization experiments were performed on an inverted Zeiss CLSM710 confocal microscope or an
503 inverted Zeiss CLSM800 confocal microscope using a 63x Plan-apochromatic objective. Dual-color
504 images were acquired by sequential line switching, allowing the separation of channels by both
505 excitation and emission. In the case of colocalization, we also controlled for a complete absence of
506 channel crosstalk. GFP was excited with a 488 nm laser, mCIT was excited with a 515nm laser and
507 mCH/tdTOM were excited with a 561 nm laser. Imaging was performed in the root epidermis in cells
508 that are at the onset of elongation.

509

510 **Yeast two-hybrid screen**

511 The yeast two hybrid screen was performed by hybrigenics services ([https://www.hybrigenics-](https://www.hybrigenics-services.com/contents/our-services/interaction-discovery/ultimate-y2h-2)
512 [services.com/contents/our-services/interaction-discovery/ultimate-y2h-2](https://www.hybrigenics-services.com/contents/our-services/interaction-discovery/ultimate-y2h-2)), using the ULTIimate Y2H
513 screen against their Universal Arabidopsis Normalized library obtained using oligo_dT. A codon
514 optimized residue (aa 499-966) of SAC9
515 gttaataatcaggggggataaacgctcccctccaccgggatgggaaaaagagctgatgccgtaactggaaaatcatattatagatcacaata
516 caaagacaacaacatggagtcacatccatgtcctgataaacatggaagagacttgacatgaggttgaggaatttaagagatcaactatcttctcctg
517 tgcagaactgcccgatcttttctgcaacaaggtgatatccatgcaaccctctatactggctcgaagctatgcacagccaaattctcaacatcttcagt
518 gaagaatcaggagcattaaacagtttctgcagcacagaaaaacatgaagattacactacagagaagatataaaaatgctatggttgatagttcacg
519 gcaaaaacagctcgagatgttctgggaatgaggctttcaagcatctccatcaattcctgtccagcctttacatgtactttctcgaccatctggtttctt
520 ctgaaacctgtacctaacaatgtccgaaagtccaatgatgggtccagtctgctgagatcaagaggaaggacataaactggctatgtccacaagctgc
521 agatattgtgaattattatctatctcagtgagccttgccatgatgcaactctactgacatcacacgggtgcgggatgattgacatgtccatccact
522 gtggacgtgagaactggacgccacatagaggaccttaattagttggtgagtttagttcaactggattaccgattacctgtaattatgtttctggacagg
523 gtgcttcaataaccagctgtgcaaatggtacaatctctggtacccttaccagggccaattagttctgaggatagggctgttactggagctggtgcac
524 gtcttcatgaaaaagatacgtcaagcttttcaactgctatgatgtttgaagaactagaaggacagttggatttctaaccctgtagttgctgttacatttat
525 ccagctggtgctgttagaattcctatgactctgtgctagatagaagtccttgaatttcttccatggaaaggaatgttactgtgaactgactggagg
526 aagattagctgaactgcaaggaaaccagatgaagatggaagtccttttcatctgttctgactgaaatccggttctgcaacaacatcttaccagctg
527 aaactgtttccacaccagtagacaacagaaggatcccttccagtaactctgctgacctttgacaggagaggactcttcttctgacccttcccacaacc
528 agtggtggaatgtattgcaagtgaggcaatgacatgcttgatttcttagacgaagcagttgtgaaatcgcggctctgacactgttctgacgggtc
529 t was cloned in a pB66 vector (N-GAL4-bait-C fusion). The screen was performed on 0.5 mM 3AT. 59
530 million interactions were analyzed, and 260 positive clones were sequenced (ATNOR_dT_hgx4515v1
531 _pB66, Supplemental Table 2.

532

533 **TIRF imaging**

534 3 days old etiolated seedlings were used for hypocotyl epidermal cells observations. TIRF-VAEM
535 imaging were made using an ilas2 TIRF microscope (Roper Scientific) with 100x Apo NA 1.46 Oil
536 objective and a Prime 95B camera (Photometrics, <https://www.photometrics.com/>) and 1.5 coverslips
537 were used (VWR 61-0136). Time lapses were acquired during 300 time-points for 300s (acquisition
538 time 500ms). Spot density were measured using Spot_detector ImageJ macro (Bayle et al. 2017).
539 Particle identification and tracking was performed using ImageJ plugin Trackmate
540 (<https://research.pasteur.fr/fr/software/trackmate/>) using following parameters: Particle min size 0.5,
541 threshold 50, particles with contrast >0.04 were filtered. For tracks filtering 0 gap, min distance between
542 two spots 0.2.

543

544 **Dissociation indexes**

545 Dissociation indexes of membrane lipid fluorescent biosensors were measured and calculated as
546 previously described (Platre et al., 2018). Briefly, we calculated “indexNoDex”, defined as the ratio
547 between the fluorescence intensity (Mean Grey Value function of Fiji software) measured in two
548 elliptical regions of interest (ROIs) from the plasma membrane region (one at the apical/basal plasma
549 membrane region and one in the lateral PM region) and two elliptical ROIs in the cytosol in the mock
550 condition.

551

552 **Measures, counting and statistical analysis**

553 Primary root length and number of lateral roots were manually measured from pictures. For comparing
554 the primary root length and lateral root density between each genotype, we used a one-way ANOVA
555 and post hoc Tukey HSD pairwise tests (95 % confidence level).

556 For quantitative imaging, pictures of epidermal root meristem cells were taken with detector settings
557 optimized for low background and no pixel saturation. Care was taken to use similar confocal settings
558 when comparing fluorescence intensity. Pseudo-colored images were obtained using the “Green Fire
559 Blue” look-up-table (LUT) of Fiji software (<http://www.fiji.sc/>). The intracellular compartments
560 (“spots”) per cell were automatically counted.

561 We automatically measured the density of intracellular compartments labeled per root for each
562 biosensor, and we used two-sided non-parametric Wilcoxon rank-sum tests to compare Col-0 and *sac9*-
563 *3^{-/-}* genotypes. To account for multiple testing, we used a Bonferroni correction and lowered the
564 significance level of each test at $\alpha = 0.05/11 = 0.004$.

565 Because the signal of mCIT-SAC9 is mainly soluble, no automatic spot detection could be used for
566 colocalization analysis. Therefore, for comparing the number of intracellular compartments containing
567 mCIT-SAC9 or mCIT-SAC9^{C459A} protein-fusions per cell across conditions, we manually counted them
568 and used a generalized linear mixed-effect model (Poisson family) accounting for image ID (*id est* root)
569 as a random factor was used. Since the localization of the marker for the membrane compartment was
570 larger in z compared to the restricted localization of SAC9 (only present at the surface of the cell), we
571 counted the number of mCIT-SAC9 labeled structure which was also labelled by the compartment
572 markers. The same approach was used to deduce the localization of the mutated version of SAC9. We
573 then computed a two-sided Tukey Post hoc tests (function “glht” in multcomp) to specifically compare
574 each pair of conditions.

575 To assess the effect of the inhibitor PAO on anomalous mCIT-TUBBYc intracellular compartments,
576 we manually counted their number per cell. We tested PAO effect and the effect of treatment duration

577 (30 min and 2 h) on the number of marked intracellular compartments using a generalized linear mixed-
578 effect model (Poisson family) to account for image ID as a random factor. Two sided Post-hoc tests
579 were performed using the package *lsmeans*. We compared the number of FM4-64 positive
580 compartments in Col-0 and *sac9-3^{-/-}* using a generalized linear mixed model (Poisson family) to account
581 for image ID (*id est* root) as a random factor. The density of FM4-64 labeled compartments was also
582 compared in Col-0 and *sac9-3^{-/-}* using a linear mixed model accounting for image ID as a random factor;
583 Type II Wald χ^2 .

584 To compare the effects of BFA on FM4-64 treated Col-0 and *sac9-3^{-/-}*, we only compared the BFA
585 treated Col-0 and *sac9-3^{-/-}* seedlings using a generalized linear mixed model (Poisson family) with
586 image ID (*id est* root) as a random factor (Type II Wald χ^2 test : $\chi^2 = 33.8$, $p < 0.001$).

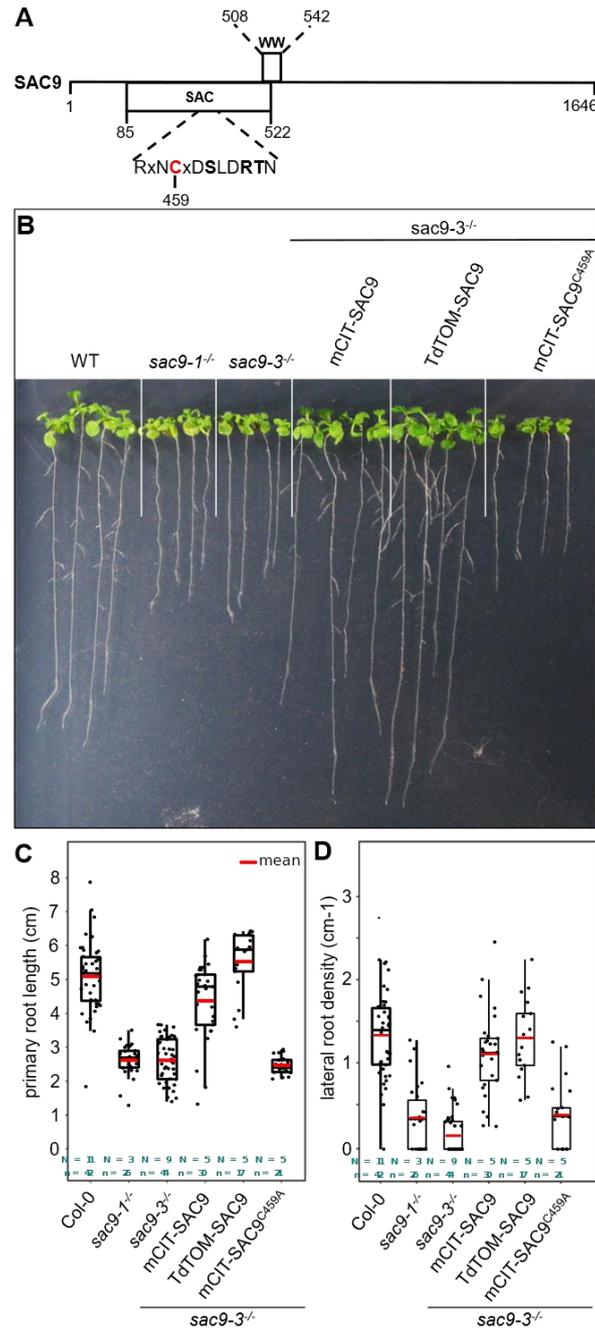
587 To compare the effects of BFA on Col-0 and *sac9-3^{-/-}* seedlings expressing PIN2-GFP, we only
588 compared the treatments BFA-Col-0 with BFA-Sac9 using a generalized linear mixed model (Poisson
589 family) with image ID (*id est* root) as a random factor. (Type II Wald χ^2 test : $\chi^2 = 42.1$, $p < 0.001$). For
590 dissociation index we performed all our statistical analyses in R (v. 3.6.1, (R Core Team, 2019), using
591 R studio interface and the packages ggplot2 (Wickham 2016), lme4 (Bates et al. 2014), car (Fox and
592 Weisberg 2011), multcomp (Hothorn et al. 2008) and lsmeans (Lenth and Lenth 2018). To compared
593 TPLATE-GFP density between Col-0 and *sac9-3^{-/-}* we used a two-sided non-parametric Kruskal Wallis
594 rank-sum tests for each replicate and obtained each time a statistical difference (p .value $< 0,05$ between
595 the two genotypes). Graphs were obtained with R and R-studio software, and customized with Inkscape
596 (<https://inkscape.org>).

597

598 ACKNOWLEDGEMENTS

599 We are grateful to the SiCE group (RDP, Lyon, France), Dr Yohann Boutté (LBM, Bordeaux, France),
600 Dr Fabrice Besnard and Dr Nicolas Doll (RDP, Lyon, France), Hugo Ducuing (INMG, Lyon, France),
601 Sébastien This (CIRI, Lyon, France) and Augustin Le Bouquin (IGFL, Lyon, France), Dr Sophie
602 Piquerez (IPS2, Paris-Saclay, France) for comments and discussions. We thank Justin Berger, Patrice
603 Bolland, and Alexis Lacroix from our plant facility, and Claire Lionnet from the imaging platform. We
604 are also grateful to Dr Daniël van Damme (VIB, Ghent, Belgium) for sharing with us T-PLATE-GFP
605 and for discussions. We would like to thanks Pr Dr Erika Isono (University of Konstanz, Konstanz,
606 Germany) for sharing SH3P2-sGFP transgenic line with us. We are also grateful to E. Russinova (VIB,
607 Ghent, Belgium) for kindly providing us ES9-17; S. Bednarek for sharing markers and for discussions.
608 This work was supported by two Seed Fund ENS LYON-2016 and LYON-2019 (to MCC), a Junior
609 Investigator grant ANR-16-CE13-0021 (to MCC), ERC no. 3363360-APPL under FP/2007–2013 (to
610 YJ). MD and AL were funded by Ph.D. fellowships from the French Ministry of Research and Higher
611 Education.

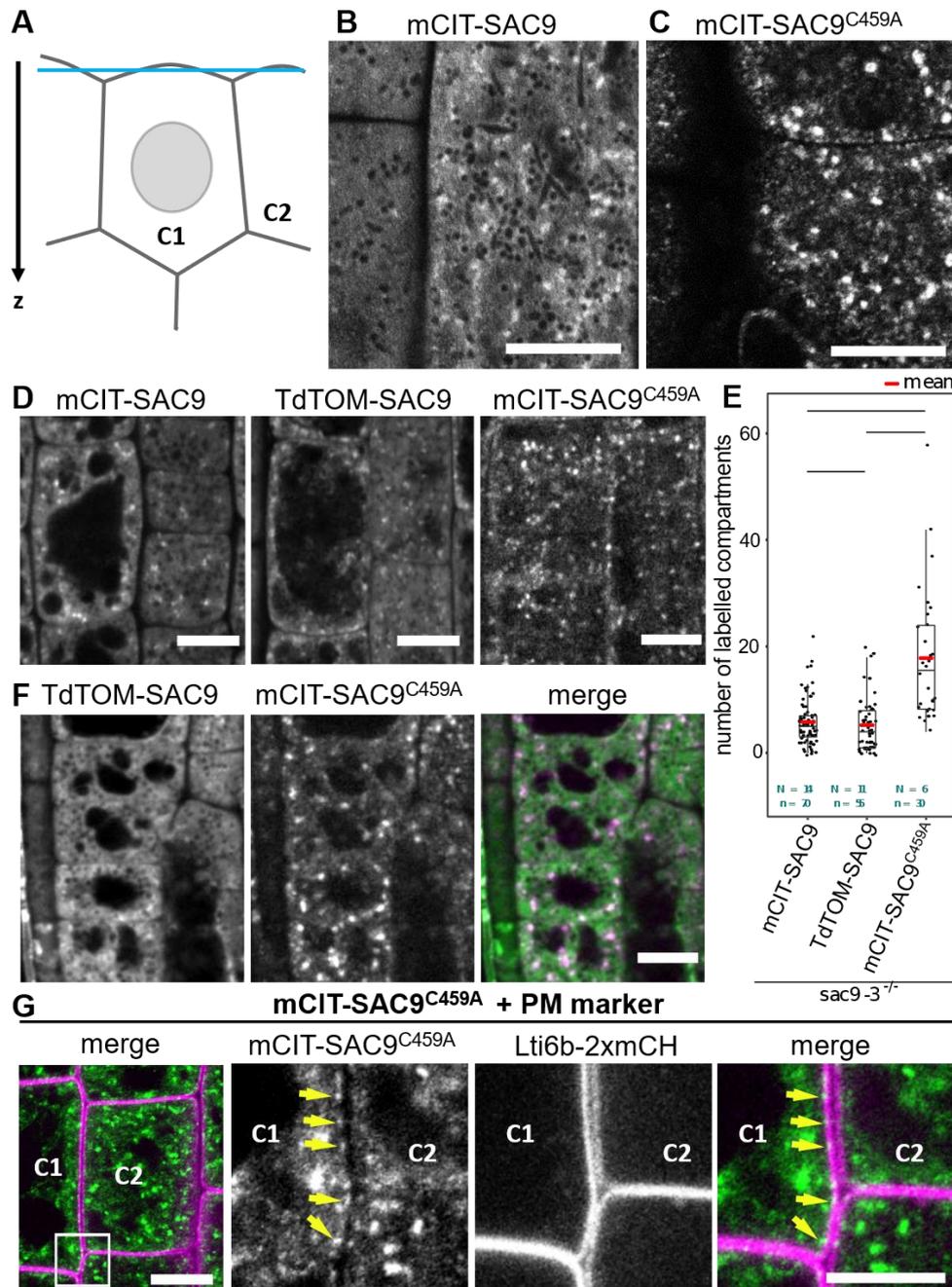
612 **FIGURES**



613

614 **Figure 1. Structure-function analysis of SAC9.** **A**, Schematic representation of SAC9 protein. The SAC catalytic domain, as well
 615 as the WW domain, are represented. **B**, Representative images of the macroscopic phenotype observed in (i) wild-type (Col-0), (ii)
 616 *sac9-1-/-* and *sac9-3-/-* loss of function mutants, (iii) *sac9-3-/-* complemented lines expressing full-length genomic DNA encoding SAC9
 617 fused to fluorescent protein (mCIT-SAC9 and TdTOM-SAC9) or a mutated version of the catalytic cysteine residue within the C-
 618 x(5)-R-[TS] catalytic motif in the SAC domain (mCIT-SAC9^{C459A}; right panel). Pictures are taken 12 days post germination (dpg).
 619 **C**, Quantification of primary root length in *sac9-3-/-* homozygous mutants expressing mCIT-SAC9 and TdTOM-SAC9, mCIT-
 620 SAC9^{C459A} under the control of the native promoter (*SAC9prom*). Wild-type (Col-0) seedlings and two independent mutant alleles
 621 of *SAC9*, *sac9-1-/-* and *sac9-3-/-*, are used as controls. **D**, Same as (C) but for the quantification of the lateral root density (ratio of
 622 the number of lateral roots to primary root length). In the plots, middle horizontal bars represent the median, while the bottom
 623 and top of each box represent the 25th and 75th percentiles, respectively. At most, the whiskers extend to 1.5 times the interquartile
 624 range, excluding data beyond. For range of value under 1,5 IQR, whiskers represent the range of maximum and minimum values.
 625 Details for statistical analysis can be found in the Methods section and supplementary Tables.

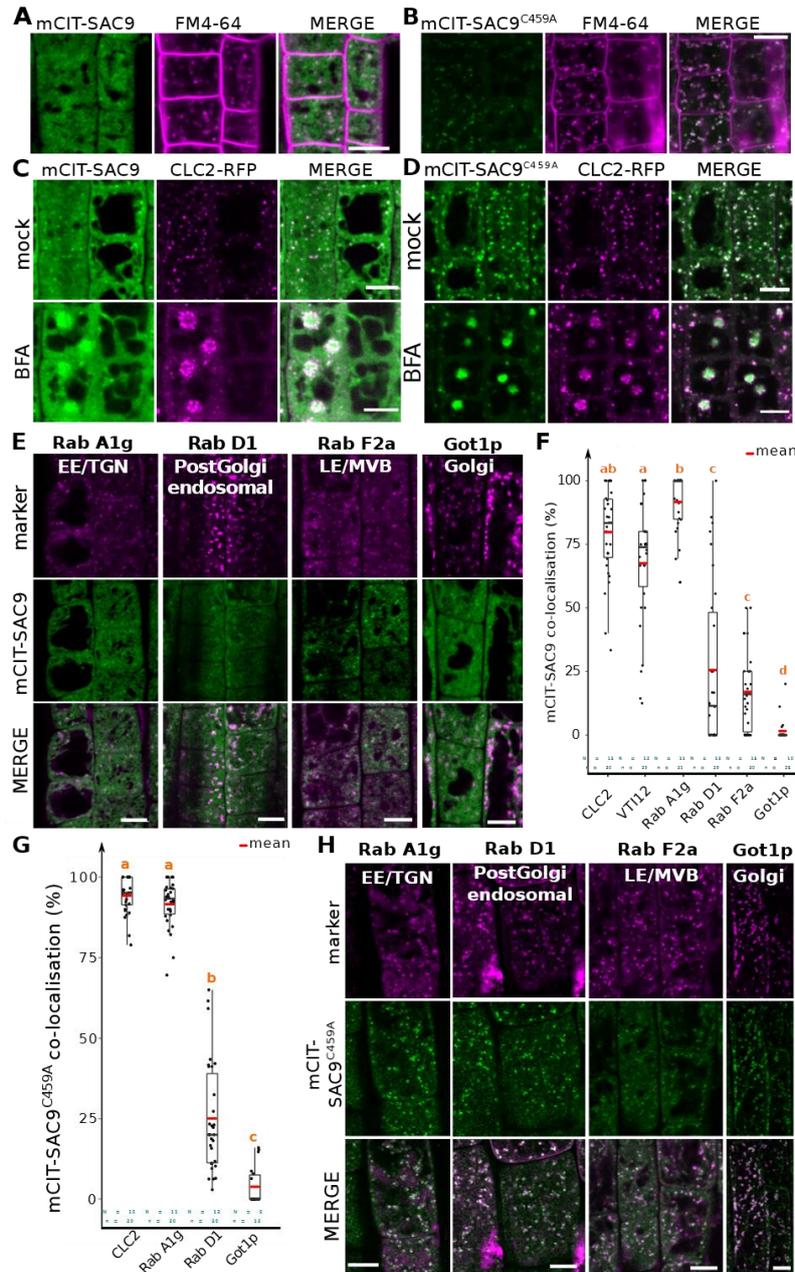
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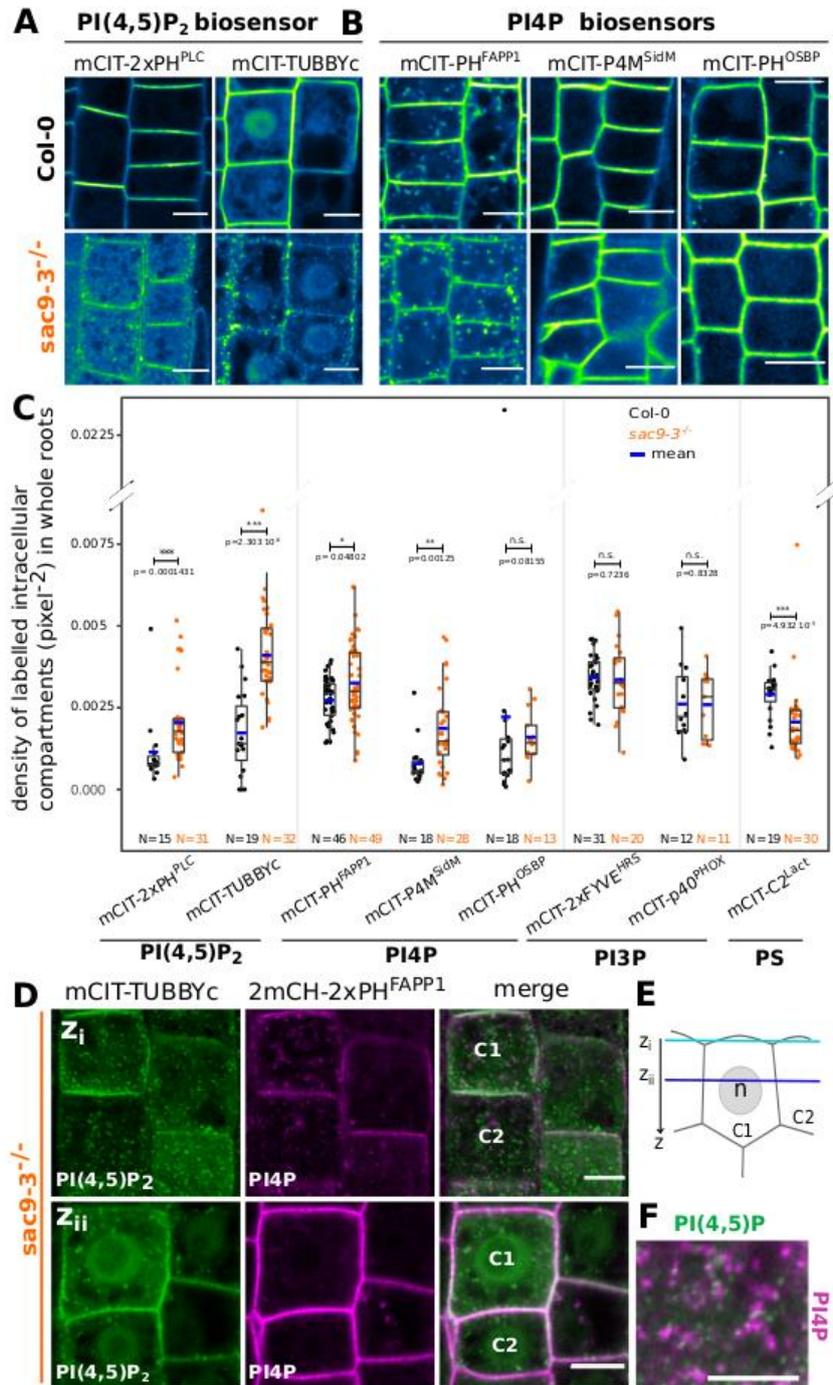
628 **Figure 2. A**, Schematic representation of two root epidermal cells (C1 and C2) imaged in this study. The blue bar represents the
 629 depth at which the imaging was performed. **B**, Confocal images of the subcortical part Arabidopsis root epidermal cell expressing
 630 mCIT-SAC9 under the control of *SAC9* native promoter (*SAC9prom*). **C**, Confocal images of the subcortical part Arabidopsis root
 631 epidermal cell expressing mCIT-SAC9^{C459A} under the control of *SAC9* native promoter (*SAC9prom*). **D**, Representative images of
 632 the fluorescent signal observe in *sac9-3^{-/-}* mutant expressing mCIT-SAC9, TdTOM-SAC9 and mCIT-SAC9^{C459A}. **E**, Comparison
 633 of the number of labeled intracellular compartments per cell in *sac9-3^{-/-}* root epidermis expressing mCIT-SAC9 and TdTOM-SAC9,
 634 mCIT-SAC9^{C459A}. In the plot, middle horizontal bars represent the median, while the bottom and top of each box represent the
 635 25th and 75th percentiles, respectively. At most, the whiskers extend to 1.5 times the interquartile range, excluding data beyond.
 636 For range of value under 1,5 IQR, whiskers represent the range of maximum and minimum values. **F**, Colocalization analysis on
 637 *Ub10pro:TdTom-SAC9* (magenta) and *SAC9pro:CIT-SAC9^{C459A}* (green). **G**, Co-visualization of *SAC9pro:CIT-SAC9^{C459A}* (green)
 638 together with the membrane localized reporter Lti6b-2xmCH (magenta) close to the basal plasma membrane of an epidermal root
 639 cell (C1). The yellow arrows indicate the intracellular compartment decorated by CIT-SAC9^{C459A} observed at the close vicinity to
 640 the labelled plasma membrane. Scale bar: 10 μm in A-F; 5 μm in G (left panel) and 2 μm in the close up of G (right panel).

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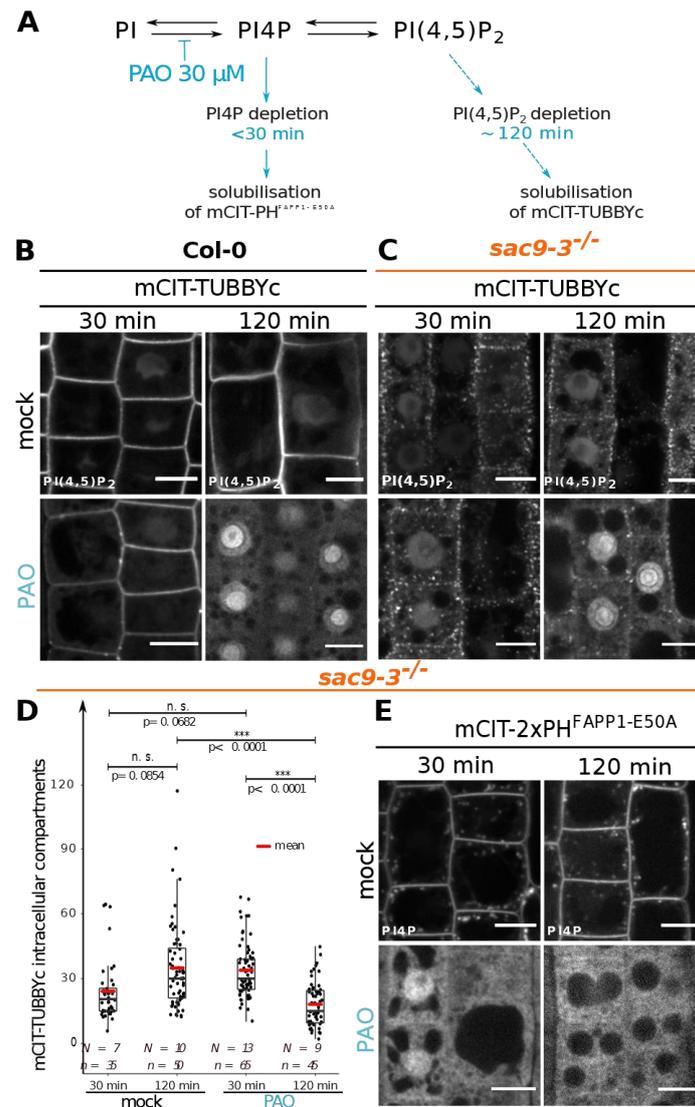
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643 **Figure 3. A, B** Confocal images of *SAC9pro:mCIT-SAC9* (A, green) or *SAC9pro:mCIT-SAC9^{C459A}* (B, green) in vivo in Arabidopsis
644 root epidermis together with the endocytic tracer FM4-64 (magenta). Scale bar: 10 μ m. **C, D** Confocal images of Arabidopsis root
645 epidermis co-expressing *SAC9pro:mCIT-SAC9* (C, green) or *SAC9pro:mCIT-SAC9^{C459A}* (D, green) together with and Clathrin Light
646 Chain 2 (CLC2) fused to RFP (*UBQ10pro:CLC2-RFP*; magenta). Upper panel: fluorescent signals observed in the mock treatment;
647 Lower panel: fluorescent signals observed after 50 μ M 60 min BFA treatment. Scale bars: 10 μ m. **E**, Confocal images of Arabidopsis
648 root epidermis co-expressing *SAC9pro:mCIT-SAC9* (green) and TGN markers *mCH-RabA1g*, *mCH-RabD1* (magenta), the late
649 endosome/pre-vacuolar compartment (LE/MVB) marker *mCH-RabF2a/Rba1* (magenta) and the Golgi marker *mCH-Got1p*
650 (magenta). Scale bars: 10 μ m. **F**, Quantification of the percentage of colocalization between mCIT-SAC9 and a given endosomal
651 compartment marker per cell. **G**, Quantification of the percentage of colocalization between mCIT-SAC9^{C459A} and a given
652 endosomal compartment marker. Similar letters correspond to markers with non-significantly different percentages of
653 colocalization with mCIT-SAC9^{C459A} (one-way ANOVA and post hoc Tukey HSD pairwise test with a 95 % confidence level). In
654 the plots, middle horizontal bars represent the median, while the bottom and top of each box represent the 25th and 75th
655 percentiles, respectively. At most, the whiskers extend to 1.5 times the interquartile range, excluding data beyond. For range of
656 value under 1,5 IQR, whiskers represent the range of maximum and minimum values. **H**, Confocal images of Arabidopsis root
657 epidermis co-expressing *SAC9pro:mCIT-SAC9^{C459A}* (green) and TGN markers *mCH-RabA1g*, *mCH-RabD1* (magenta), the late
658 endosome/pre-vacuolar compartment (LE/MVB) marker *mCH-RabF2a/Rba1* (magenta) and the Golgi marker *mCH-Got1p*
659 (magenta). Scale bars: 10 μ m.



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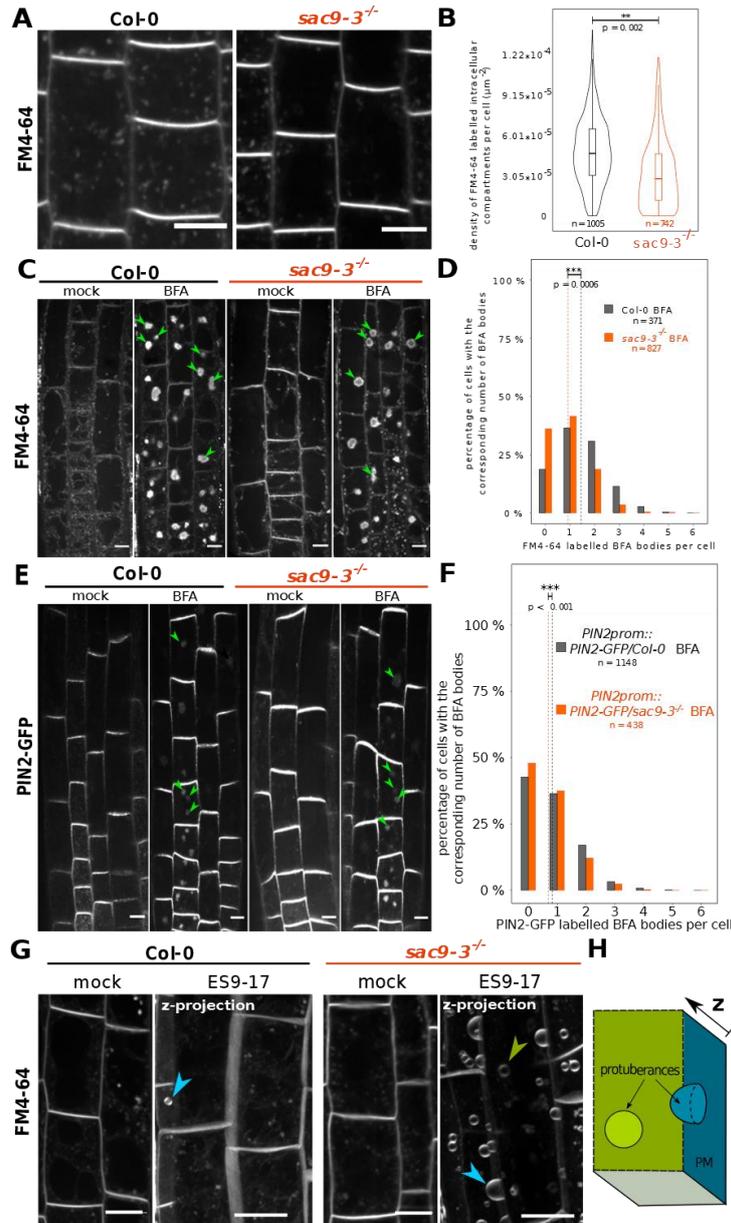
661 **Figure 4. A**, Confocal images of Arabidopsis root epidermis expressing mCIT-tagged PI(4,5)P₂ sensors in WT (Col-0) and *sac9-3*^{-/-}
 662 *sac9-3*^{-/-} genetic backgrounds. **B**, Confocal images of Arabidopsis root epidermis expressing mCIT-tagged PI4P sensors in Wild-type (Col-
 663 0) and *sac9-3*^{-/-} genetic backgrounds. Fluorescence intensity is color-coded (green fire blue scale). **C**, Quantification of density of
 664 labelled intracellular compartments (pixel⁻²) in whole roots of epidermis expressing mCIT-tagged lipid sensors in wild-type (Col-0)
 665 and *sac9-3*^{-/-}. Non-parametric Wilcoxon rank sum tests with Bonferroni correction. In the plots, middle horizontal bars represent
 666 the median, while the bottom and top of each box represent the 25th and 75th percentiles, respectively. At most, the whiskers
 667 extend to 1.5 times the interquartile range, excluding data beyond. For range of value under 1,5 IQR, whiskers represent the range
 668 of maximum and minimum values. Details for statistical analysis can be found in the Methods section and supplementary Tables.
 669 **D**, Confocal images of *sac9-3*^{-/-} Arabidopsis root epidermal cell (C1 and C2) expressing mCIT-TUBBYc (green) and 2xmCH-PH^{FAPP1}
 670 (magenta) at their cortex (upper panel, Z_i) and at their center (bottom panel, Z_{ii}). **E**, Schematic representation of two root epidermal
 671 cells (C1 and C2) imaged in D. The blue bars represent the depth at which the imaging was performed. **F**, close up (from a different
 672 cell) of the distribution of the intracellular compartments in *sac9-3*^{-/-} epidermal cell labelled by mCIT-TUBBYc (green) and 2xmCH-
 673 PH^{FAPP1} (magenta). Scale bar: 10 μm in A-D, 5 μm in F.



674

675 **Figure 5. A**, Schematic representation of the mode of action of the PAO on the PI4P and PI(4,5)P₂ metabolism based on
 676 previously published work (Doumane et al. 2020; Platre et al. 2018). **B**, Confocal images of WT root epidermal cells expressing
 677 mCIT-TUBBYc PI(4,5)P₂ biosensor that underwent mock, or 30 μM PAO treatments for 30- or 120- min. **C**, Confocal images of
 678 *sac9-3^{-/-}* root epidermal cells expressing mCIT-TUBBYc PI(4,5)P₂ biosensor that underwent mock, or 30 μM PAO treatments for
 679 30- or 120- min. **D**, Quantification of the number of mCIT-TUBBYc labeled intracellular compartments per cell in *sac9-3^{-/-}*
 680 following mock, or 30 μM PAO treatments for 30- or 120- min. Generalized linear mixed effect model (Poisson family) to account
 681 for root as a random factor, and two sided post hoc *lsmeans* pair wise test with a 95 % confidence level. In the plot, middle horizontal
 682 bars represent the median, while the bottom and top of each box represent the 25th and 75th percentiles, respectively. At most,
 683 the whiskers extend to 1.5 times the interquartile range, excluding data beyond. For range of value under 1,5 IQR, whiskers
 684 represent the range of maximum and minimum values. All statistical tests were two-sided. **E**, Confocal images of *sac9-3^{-/-}* root
 685 epidermal cells expressing mCIT-PHF^{FAPP1-E50A} PI4P biosensor that underwent mock or 30 μM PAO treatments for 30- or 120-
 686 min. Scale bar: 10 μm.

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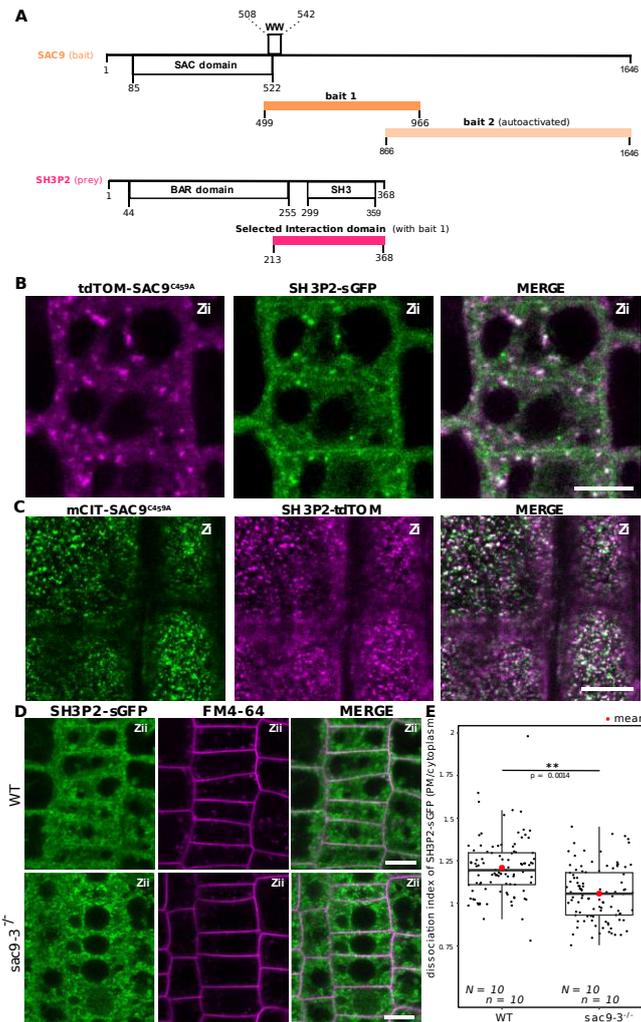
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689 **Figure 6. A**, Representative images of Col-0 and *sac9-3^{-/-}* seedlings treated for 30 min with FM4-64, which is endocytosed and
690 labels endocytic intracellular compartments. Scale bar: 10 μm . **B**, Quantification from the experiment showed in (A). Violin and
691 box plots quantifying the number of FM4-64 labeled intracellular compartments in Col-0 and *sac9-3^{-/-}*. **C**, Representative images of
692 root epidermis following BFA and FM4-64 treatment of Col-0 and *sac9-3^{-/-}* seedlings. Examples of FM4-64 labeled BFA bodies are
693 pointed out (green arrowheads). **D**, Quantification from the experiment showed in (C). For Col-0 and *sac9-3^{-/-}*, the proportion (%)
694 of cells containing from none to six BFA bodies is displayed. Dotted line: means. **E**, Representative images following BFA
695 treatment of PIN2-GFP/WT and PIN2-GFP/*sac9-3^{-/-}* seedlings. Examples of PIN2-GFP labeled BFA bodies are pointed out
696 (green arrowheads). Scale bars: 10 μm . **F**, Quantification from the experiment showed in (E). For PIN2-GFP/WT and PIN2-
697 GFP/*sac9-3^{-/-}*, the proportion (%) of cells containing from none to six BFA bodies is displayed. Dotted line: means. **G**, Over-
698 sensitivity of *sac9-3^{-/-}* to prolonged inhibition of endocytosis. Seedlings were treated 180 min with 30 μM ES9-17 or DMSO (mock),
699 FM4-64 being added after 30 min (150 min of exposure). The picture showed after the ES9-17 treatment are the results of the
700 projection of a z-stack. **H**, ES9-17 treatments led to dome-shaped plasma membrane invagination. blue arrowheads: invaginations
701 with an obvious connection to the plasma membrane; green arrowheads: invaginations without a clear connection to the plasma
702 membrane (often connected to medullar plasma membrane). Scale bars: 10 μm .

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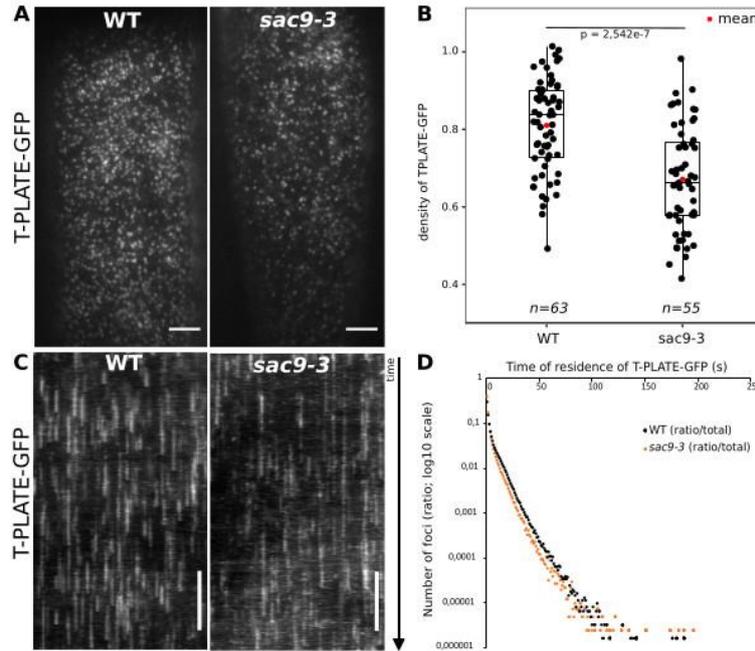
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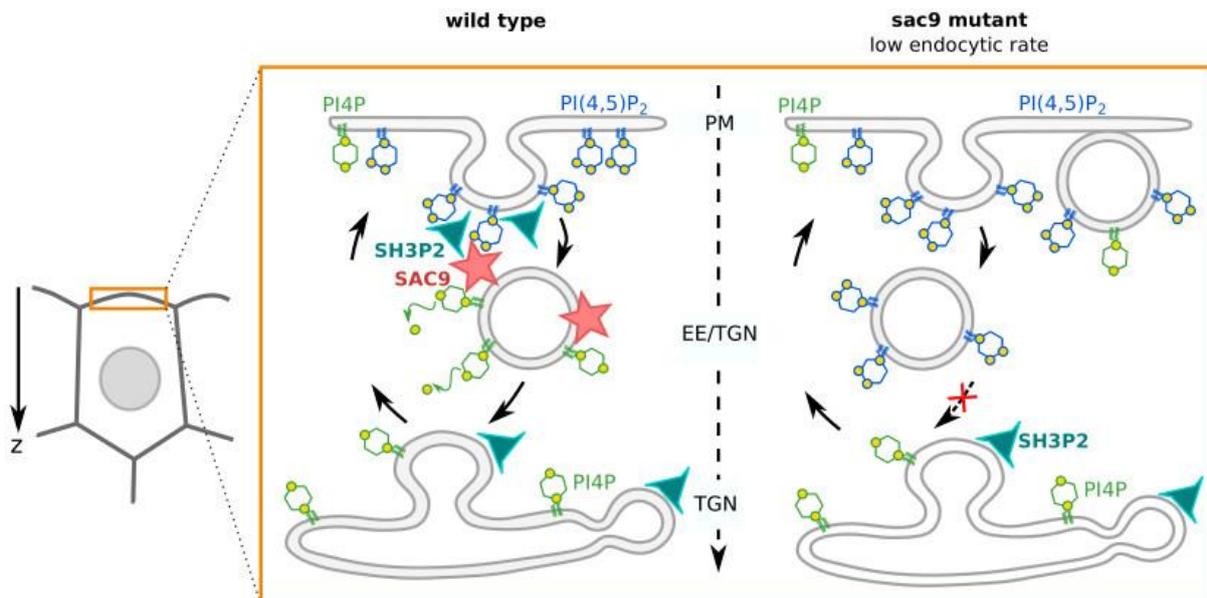
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Figure 7. A, Schematic representation of the yeast two-hybrid screen using SAC9 as a bait, where SH3P2 was found as a protein partner. The selected interaction domain (interacting with bait 1) corresponds to the amino acid sequence shared by all the eleven prey fragments matching the same reference protein. Note that one other fragment of SAC9 was attended to be screened for interacting proteins (bait 2), but the construct was autoactivate in yeast. For design purpose, the scale between SAC9 and SH3P2 was not respected. **B**, Representatives images of the in vivo colocalization analysis between tdTOM-SAC9^{C459A} (magenta) and SH3P2-sGFP (green) in a distal region from the plasma membrane. Scale = 5µm. **C**, Representatives images of the in vivo colocalization analysis between mCIT-SAC9^{C459A} (green) and SH3P2-tdTOM (magenta) near the plasma membrane. Scale = 5µm. The light blue line (zi) corresponds to the in vivo imaging near the plasma membrane observed by confocal imaging and the dark blue line (zii) corresponds to the in vivo imaging in a distal region from the plasma membrane. (See Figure 4E for details) **D**, Representatives images of the SH3P2-sGFP localization (green) in WT and *sac9-3* mutant in which the plasma membrane was labelled using FM4-64 (magenta). **E**, Double blinded quantification of the dissociation index of SH3P2-sGFP in WT and *sac9-3* mutant in which the plasma membrane was labelled using FM4-64. Linear mixed model (lmer in lme4) then type II Wald chisquare test: Chisq = 10.152, df=1, p = 0.001441. In the plot, middle horizontal bars represent the median, while the bottom and top of each box represent the 25th and 75th percentiles, respectively. At most, the whiskers extend to 1.5 times the interquartile range, excluding data beyond. For range of value under 1,5 IQR, whiskers represent the range of maximum and minimum values.



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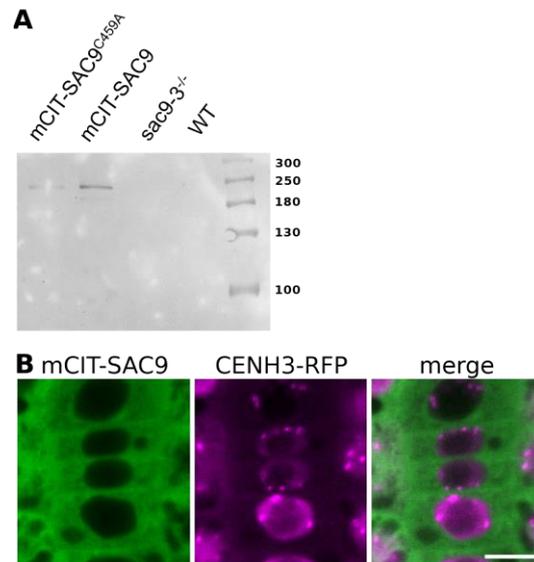
726 **Figure 8. A**, Representatives images of the TPLATE-GFP localization in WT and *sac9-3* mutant at the plasma membrane observed
 727 by TIRF microscopy in etiolated hypocotyl. Scale = 5 μ m. **B**, Quantification of the density at the plasma membrane of SH3P2-
 728 sGFP in WT and *sac9-3* mutant observed by TIRF microscopy. Only one of the three replicates is represented. In the plots, middle
 729 horizontal bars represent the median, while the bottom and top of each box represent the 25th and 75th percentiles, respectively.
 730 At most, the whiskers extend to 1.5 times the interquartile range, excluding data beyond. For range of value under 1,5 IQR, whiskers
 731 represent the range of maximum and minimum values. **C**, Representatives kymograph of the TPLATE-GFP dynamics at the
 732 plasma membrane in WT and *sac9-3* mutant observed by TIRF microscopy in etiolated hypocotyl over 5 min, scale =60s. **D**,
 733 Quantification of the time of residency at the plasma membrane of TPLATE-GFP in WT (black dots) and *sac9-3* mutant (orange
 734 dots) observed by TIRF microscopy in a time lapses acquired during 300 time-points for 300s (acquisition time 500ms). The data
 735 are represented in a log10 scale for the residency time at the PM, and the time scale is represented in sec.



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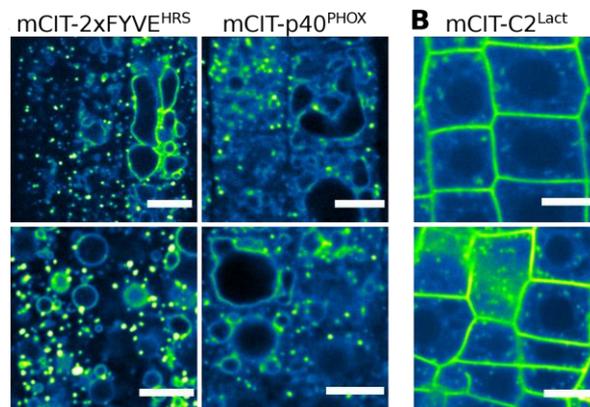
737 **Figure 9.** Model for the mode of action of SAC9 in regulating the PI(4,5)P₂ homeostasis at the close vicinity with the plasma
 738 membrane. In wild type plants, SAC9 restricts the localization of PI(4,5)P₂ at the plasma membrane allowing endocytic processes
 739 to occur. In the absence of SAC9, endocytosis PI(4,5)P₂ accumulates in atypical endomembrane compartments that are no longer
 740 able to fuse with the TGN because of their abnormal anionic lipid signature. SAC9 interacts with SH3P2 close to the plasma
 741 membrane. The defects in PI(4,5)P₂ patterning in absence of SAC9 leads to decreasing of the endocytic rate and the formation of
 742 membrane protuberances in contact with the plasma membrane.

743



744 **Supplemental Fig. 1. A**, Western blot analysis. Leaf lysates from Col-0, *sac9-3^{-/-}*, *SAC9pro:mCIT-SAC9/sac9-3^{-/-}*, *SAC9pro:mCIT-*
745 *SAC9^{C459A}/sac9-3^{-/-}* were immunoprecipitated with an anti-GFP antibody, run on an SDS-PAGE and immunoblotted with anti-
746 GFP primary antibody. **B**, Confocal imaging of mCIT-SAC9 (green) together with the nuclear marker CENH3-RFP (magenta).
747 Scale bars: 10 μ m.

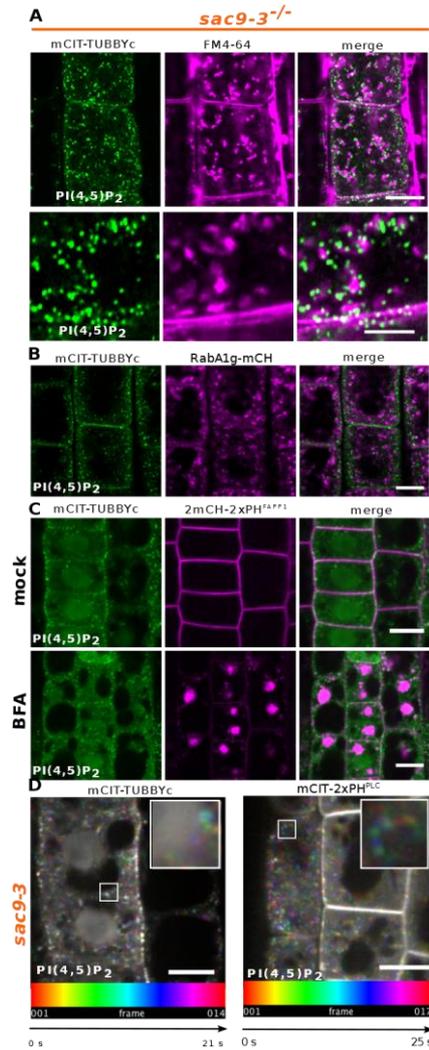
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750 **Supplemental Fig. 2. A**, Representative confocal images of *Arabidopsis* root epidermis expressing mCIT-tagged lipid sensors for
751 PI3P in wild-type (Col-0) and *sac9-3^{-/-}* genetic backgrounds. **B**, Confocal images of *Arabidopsis* root epidermis expressing mCIT-
752 tagged lipid sensors for phosphatidylinserine (PS) in wild-type (Col-0) and *sac9-3^{-/-}* genetic backgrounds. Fluorescence intensity is
753 color-coded (green fire blue scale). Scale bars: 10 μ m.

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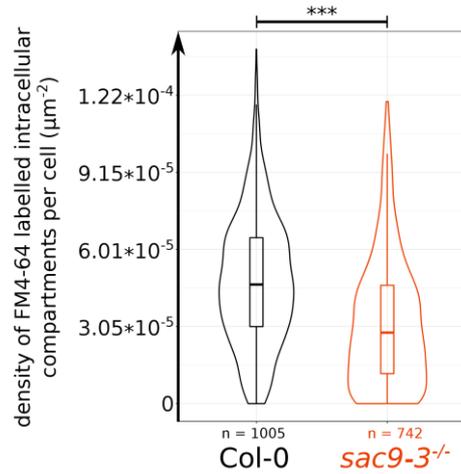


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756 **Supplemental Fig.3. A**, Confocal images of *sac9-3^{-/-}* Arabidopsis root epidermis expressing mCIT-TUBBYc (green) and *in vivo*
757 stained with FM4-64 endocytic tracer (magenta). **B**, Confocal images of *sac9-3^{-/-}* root epidermal cells co-expressing mCIT-TUBBYc
758 (green) and the TGN marker RabA1g-mCH (magenta). **C**, Confocal images of *sac9-3^{-/-}* root epidermal cells co-expressing mCIT-
759 TUBBYc (green) and 2mCH-PH^{FA}P1 (magenta). Upper panel: fluorescent signals observed in the mock treatment; lower panel:
760 fluorescent signal observed after 50 μ M 60 min BFA treatment. **D**, Temporal-color coded XY 2D image of mCIT-2xPH^{PLC} and
761 mCIT-TUBBYc in *sac9-3^{-/-}* root epidermis (Fiji Spectrum Temporal-color code). Scale bar: 10 μ m.

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765 **Supplemental Fig.4.** Quantification from the FM4-64 uptake assay. Violin and box plots quantifying the density of FM4-64
766 labeled intracellular compartments in Col-0 and *sac9-3*^{-/-}. N: number of cells. Linear mixed model (Poisson family) to account for
767 root as a random factor. *** p-value < 0.0005.

768

769 **Supplemental Movie 1.** Time-lapse imaging of mCIT-SAC9 using spinning disk confocal microscope

770 **Supplemental Movie 2.** Time-lapse imaging of mCIT-2xPH^{PLC} in *sac9* mutant (1 sec per frame) using spinning disk confocal
771 microscope.

772 **Supplemental Movie 3.** Time-lapse imaging of T-PLATE-GFP at the plasma membrane of WT and in *sac9-3* mutant using TIRF
773 microscopy. Time lapses were acquired during 300 time-points for 300s (acquisition time 500ms).

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