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Lise Noack, Yvon Jaillais

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1 **Functions of anionic lipids in plants**

2

3 Lise C. Noack and Yvon Jaillais

4

5 Laboratoire Reproduction et Développement des Plantes, Univ Lyon, ENS de Lyon, UCB

6 Lyon 1, CNRS, INRA, F-69342 Lyon, France

7 Corresponding author: Yvon Jaillais ([yvon.jaillais@ens-lyon.fr](mailto:yvon.jaillais@ens-lyon.fr))

8 Lise C. Noack ([lise.noack@ens-lyon.fr](mailto:lise.noack@ens-lyon.fr)) : ORCID# 0000-0001-5093-6374

9 Yvon Jaillais ([yvon.jaillais@ens-lyon.fr](mailto:yvon.jaillais@ens-lyon.fr)) : ORCID# 0000-0003-4923-883X

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12 **Abstract:** (150 words max)

13 Anionic phospholipids, which include phosphatidic acid, phosphatidylserine, and  
14 phosphoinositides, represent a low percentage of membrane lipids. They are able to modulate  
15 the physical properties of membranes, such as their surface charges, curvature or clustering of  
16 proteins. Moreover, by mediating interactions with numerous membrane-associated proteins,  
17 they are key components in the establishment of organelle identity and dynamics. Finally,  
18 anionic lipids also act as signalling molecules, as they are rapidly produced or interconverted  
19 by a set of dedicated enzymes. As such, anionic lipids are major regulators of many  
20 fundamental cellular processes, including cell signalling, cell division, membrane trafficking,  
21 cell growth or gene expression. In this review, we describe the functions of anionic lipids with  
22 a cellular perspective. Using the localization of each anionic lipids and their related metabolic  
23 enzymes as starting points, we summarize their roles within the different compartments of the  
24 endomembrane system and address their associated developmental and physiological  
25 consequences.

26

27 **Keywords:** (6 max)

28 membrane contact sites, endocytosis, exocytosis, autophagy, nanodomains, plant-microbe  
29 interphase

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32 **Main text**

33 **1. INTRODUCTION**

34 Biological membranes delimit cellular compartments, as well as the inner and outer of the cell.  
35 Each compartment has its proper chemical environment allowing numerous and specialized  
36 chemical reactions in the same cell. To enable the proper localization of proteins, each  
37 membrane has its own biochemical and biophysical identity. Membranes are composed of lipids  
38 forming a bilayer and transmembrane or membrane-associated proteins (63).  
39 Glycerophospholipids associated with sphingolipids and sterols are the main lipid components  
40 within the eukaryotic **endomembrane system**. Among the glycerophospholipids some present  
41 negative charges and, as such, are anionic phospholipids. Anionic phospholipids include  
42 phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol and phosphatidylinositol  
43 phosphates (PIPs also referred to as phosphoinositides), the phosphorylated forms of  
44 phosphatidylinositol (Figure 1.a). Phosphatidylinositol display an inositol ring that can be  
45 phosphorylated in three different positions - 3, 4 and 5 - creating a total of seven possible  
46 phosphoinositide species: 3 phosphoinositide monophosphates (PI3P, PI4P, PI5P), 3  
47 phosphoinositide biphosphates (PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, PI(4,5)P<sub>2</sub>) and one phosphoinositide  
48 triphosphate (PI(3,4,5)P<sub>3</sub>).

49

50 These lipids are present in low to very low abundance in eukaryotic membranes. In Arabidopsis  
51 leaf, phosphatidylinositol represents about 7% of total phospholipids, PA ~2% and PS ~1%. In  
52 addition, all phosphoinositides combined account for less than 1% of the total phospholipid  
53 content (72, 135). However, anionic lipids are key components of cell membranes. They are  
54 involved in the regulation of nearly all membrane-associated events including cell signalling,  
55 trafficking, cell division, and cell growth. Consequently, over-expression lines or loss-of-function  
56 mutants for lipid enzymes that perturb anionic lipid homeostasis lead to severe and pleiotropic  
57 phenotypes at the plant level, including defects in development, immunity, reproduction and  
58 stress adaptation (24, 94).

59 Anionic phospholipids are detected in most endomembrane compartments: endoplasmic  
60 reticulum (ER), Golgi, **trans-Golgi network/early endosomes** (TGN/EE), plasma membrane  
61 (PM), Late endosomes/**multi-vesicular bodies** (LE/MVB), vacuoles and autophagosomes. In  
62 this review, we first describe the key physicochemical properties of anionic lipids that mediate  
63 lipid-protein interactions and regulate protein dynamics in cell membranes. Then, we discuss  
64 the functions of anionic lipids with a cellular perspective, using as a starting point the  
65 localization of each anionic lipids and their related metabolic enzymes. We summarize the roles  
66 of anionic lipids within the different compartments of the endomembrane system and address  
67 their associated developmental and physiological consequences.

68 It should be noted that anionic lipids also accumulate in mitochondria and plastids where they  
69 play a role in division, lipid synthesis and photosynthesis. Anionic lipids, such as PA, PI4P and  
70 PI(4,5)P<sub>2</sub> are also found in the nucleoplasm of the nucleus where they may regulate  
71 transcription factors, chromatin status and epigenetic landmarks (125) but this will not be  
72 further discussed, as we decided to focus this review on the endomembrane system.

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## 75 **2. ANIONIC LIPIDS ARE DYNAMIC LANDMARKS OF BIOLOGICAL MEMBRANES**

### 76 **2.1. Anionic phospholipids as landmarks for organelle identity**

77 Cell compartments are enriched in different anionic lipids, which give specific properties to their  
78 membrane and thereby contribute to their identity. **Lipids binding domains** (LBDs) can  
79 mediate the interaction with the membrane (63, 126) (Figure 2.d). They allow the targeting of  
80 proteins to membranes by stereospecific interactions with a given phospholipid species. Those  
81 domains are, for example, Pleckstrin Homology (PH) domains, Fab1/YOTB/Vac1/EEA1 (FYVE)  
82 domains, Phox (PX) domains and C2 domains. The characterization of these domains and their  
83 specificity allowed the engineering of a set of anionic phospholipid biosensors by fusion of these  
84 domains with a fluorescent protein (92). These so called genetically encoded biosensors enable  
85 the study of the subcellular accumulation pattern and to some extent the dynamics of anionic

86 phospholipids *in vivo* (112, 127–129). However, in some cases, lipid-binding domains are not  
87 sufficient to target the protein to its proper compartment. Indeed, some proteins require the  
88 present of both, an anionic lipid and another protein (or lipid) to localize to the right  
89 compartment, in a mechanism called “**coincidence binding**” (Figure 2.d). For instance, the  
90 ubiquitin ligase PLANT-U-BOX13 (PUB13), which regulates the trafficking of the PM receptor  
91 FLAGELIN-INSENSITIVE 2 (FLS2), is recruited to the TGN via the concomitant interaction with  
92 RabA4b and PI4P (2). Furthermore, LBDs may also recognize additional membrane feature such  
93 as curvature or electrostatics. As such, results obtained with lipid biosensors should always  
94 been taken with care as their localization may not only reflect the accumulation of a single lipid  
95 species. This is exemplified by the fact that biosensors designed to bind to the same lipid species  
96 do not always have the exact same localization (112, 113). If possible, the potential  
97 accumulation of a lipid at a given membrane should be also supported by biochemical evidences  
98 and/or by the localization of their metabolic enzymes.

99

100 The combined study of the localization of anionic lipid generating enzymes and genetically  
101 encoded lipid sensors suggest that in Arabidopsis epidermal root cells, the PM accumulates PI4P,  
102 PI(4,5)P<sub>2</sub>, PA and PS (Figure 3) (93, 112, 113, 127, 128). TGN/EE are enriched in PI4P, PS and to  
103 a lesser extent PI3P (93, 113, 114, 128). LE/MVBs accumulate both PI3P and PI(3,5)P<sub>2</sub>, although  
104 both lipids localize in distinct compartments (37, 38, 112, 129). Finally, the tonoplast and  
105 autophagosomes carry PI3P (112, 129). However, this subcellular accumulation pattern may  
106 vary according to the cell type or the stress status of the cell (Figure 3) (34, 41, 44, 48, 57 in  
107 revision, 61, 110, 115). For example, PI(3,5)P<sub>2</sub> is found in LE/MVBs in most cell type but  
108 accumulates at the PM in elongating root hairs (34) (Figure 3). PI(4,5)P<sub>2</sub> and PA are  
109 constitutively produced at the PM at low or moderate levels, respectively, in resting condignition  
110 but upon stimulation, such as salt stress, they can be massively produced, which triggers specific  
111 stress responses (82, 121, 127). Alternatively, PI(4,5)P<sub>2</sub> production can be inhibited by external  
112 stimuli such as pathogen-associated molecular patterns (80).

113

114

115 **2.2. Dynamics of anionic lipids: rapid production and turnover thanks to a set of**  
116 **lipid-modifying enzymes**

117 **2.2.1. PA production and stress responses**

118 PA is structurally the simplest phospholipid. As such, it is a key intermediate for other  
119 phospholipid biosynthesis (94, 121). Multiple pathways involving different enzymes at various  
120 locations can lead to PA synthesis and are summarized in Figure 1.b. The rapid accumulation of  
121 PA is induced by numerous abiotic stresses including salt, cold, heat, or osmotic stress, but also  
122 by biotic stresses in case of pathogen infection (121). Moreover, PA synthesis is regulated in  
123 physiological and developmental context including hormonal responses such as SA, ABA, and  
124 auxin (121). In each case, PA accumulation leads to different adapted cellular responses raising  
125 the question: how can PA integrate different inputs to lead to specific outputs relevant to the  
126 physiological situation? One of the main hypothesis is that PA accumulation never occurs alone  
127 but rather that PA is one second messenger among others that together lead to concerted  
128 cellular responses (94). To know more about this topic, the different hypotheses to answer this  
129 question are discussed by Pokotylo *et al.* in a recent review.

130

131 **2.2.2. PIPs and rapid inter-conversion**

132 The phosphorylation of the inositol ring of phosphatidylinositol is mediated by PI-kinases that  
133 are specific of the phosphate position (Figure 1.d). In plants, the genome does not encode for  
134 type-I and type-II PI3-kinases (PI3Ks), able to phosphorylate PI4P or PI(4,5)P<sub>2</sub> (84). As a  
135 consequence, PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> likely do not exist and have not been detected in plants.  
136 The dephosphorylation of PIPs is mediated by PI-phosphatases that belong in plants to three  
137 main families, the SUPPRESSOR OF ACTIN (SAC) domain-containing proteins (SAC1 to SAC9),  
138 the 5PTase family (5PTase1 to 15) and the PHOPHATASE AND TENSIN Homolog (PTEN) family  
139 (PTEN1 to 3) (24) (Figure 1.d). PI-kinases and phosphatases are highly processive enzymes *in*

140 *vivo* and thereby PIPs can be quickly interconverted, cycling between two states or creating  
141 cascade regulations (86). Because anionic lipid pools can be enzymatically modified within  
142 second, they are able to respond to various stimuli and to act in fast cellular events such as  
143 membrane trafficking.

144

145 *In vitro* experiments testing PI-kinases activity often require the use of detergents, which  
146 destroy liposomal structures and free lipid substrates in order to increase kinase activity (52).  
147 These results suggest that PI-kinases have difficulties to recognize their substrate embedded  
148 into the membrane and might get “help” *in planta*. The main candidates to fulfil this function are  
149 the SEC14 lipid transfer proteins. The yeast Sec14 protein is able to transfer  
150 phosphatidylcholine and phosphatidylinositol from distinct membranes *in vitro*, possibly by-  
151 passing the classical vesicular transport of lipids (52, 103). However, the current model, based  
152 on crystallographic data, advocates the idea that Sec14 dislodges the phosphatidylinositol head  
153 from the TGN membrane making it accessible for PI4-kinases (Figure 4.a). Based on sequence  
154 homology, Arabidopsis contains 32 Sec Fourteen Homologs (AtSFHs), each containing a highly  
155 conserved sec14 domain. The sec14 domain of several AtSFHs displays the same *in vitro* activity  
156 than the yeast sec14 and can rescue the sec14 yeast mutant (40, 130). So far, the best-  
157 characterized AtSFH protein is AtSFH1. AtSFH1 belongs to a subclass of 13 members that  
158 contains a Nlj16 domain in C-terminal. This domain has two functions. It contains a polybasic  
159 patch that localizes at the PM in a PI(4,5)P<sub>2</sub>-dependent manner and a coiled-coiled domain that  
160 mediates homo-oligomerization (25). The model built with the combination of yeast and  
161 Arabidopsis data is that AtSFH1 is targeted at the root hair tip PM by PI(4,5)P<sub>2</sub> where it  
162 stimulates the activity of PI-kinases (PI4K or PIP5K), thereby enhancing the production of PIPs  
163 (directly PI(4,5)P<sub>2</sub> or PI4P that will then be converted into PI(4,5)P<sub>2</sub>). PI(4,5)P<sub>2</sub> recruit new  
164 AtSFH1 which oligomerizes and further creates PI(4,5)P<sub>2</sub> clusters at the root hair tip PM (Figure  
165 3).

166



167

### 168 **2.3. PS synthesis and trafficking**

169 Contrary to PA and PIPs that can be synthesized by several enzymes, a single enzyme produces  
170 PS in Arabidopsis: the Phosphatidylserine Synthase 1 (PSS1) (91, 93, 140) (Figure 1.b). PSS1 is  
171 an ER transmembrane protein which synthesizes PS in the luminal leaflet of this compartment  
172 (140). Therefore, by contrast to PIPs, PS is not locally produced directly at the membranes  
173 where it acts. This implies that PS must be transported from the luminal leaflet of the ER to the  
174 cytosolic leaflet of the endomembrane system. Two non-exclusive mechanisms may explain the  
175 localization of PS.

176

#### 177 **2.3.1. Flippases and vesicular trafficking**

178 As PS faces the lumen in the ER but the cytosol once at the PM or endosomes, it must switch  
179 membrane leaflet at some point. **Flippases** are proteins able to flip phospholipids from the  
180 luminal membrane leaflet to the cytosolic membrane leaflet (85) (Figure 4.b). The Arabidopsis  
181 genome encodes for 12 aminophospholipid flippases (ALAs) and at least ALA1, ALA2, ALA3 and  
182 ALA10 are able to flip PS in yeast (27, 70, 71, 85, 97). ALA proteins localize in the ER (ALA1, 2  
183 and 3) but also all along the endocytic pathway (ALA1 at the PM, ALA2 in LE/MVBs, ALA3 in the  
184 Golgi and TGN/EE) (9, 70, 71, 74, 96). Their localization, and function, depends on ALA  
185 interacting  $\beta$ -subunits, ALIS. These localization suggest that PS could be directly flipped in the  
186 ER and then be transported through vesicular trafficking to the PM and endosomes. Another  
187 hypothesis would be that PS is first transported by vesicular trafficking on the luminal side of  
188 organelle and then flipped in specific compartments, such as TGN/EE or PM. In yeast, the activity  
189 of the PS flippase Drs2p is promoted by PI4P (4, 123). As PI4P is present in the same  
190 compartments than PS along the endocytic pathway in plants, this could be an argument in  
191 favour of the later hypothesis. In any case, if the flippase activity occurs at the ER membrane, PS  
192 is then actively exported out of this compartment, since the ER cytosolic leaflet does not  
193 accumulate PS (93, 113).

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### 2.3.2. PS transfer at membrane contact sites

Another hypothesis suggests that PS bypasses classical vesicular trafficking and rather directly translocates from the ER to the PM, at **membrane contact sites** (MCSs). MCSs are static microdomains where membranes from two different organelles are bring together (at a distance lower than 30nm) and stabilize by **tethering proteins** (107). In animal and yeast, MCSs are major sites of lipid flow and are particularly important to control the localization of nearly all anionic lipids (138). **Lipid transfer proteins** present at MCS are able to transport lipids between two membranes extracting the lipid from the donor membrane, transferring it through the cytosol and inserting it in the acceptor membrane. Notably, proteins from the oxysterol-binding related protein (ORP) family localize at MCS via interactions with suppressor of choline sensitivity Scs2p (VAPS in animal cells) in the ER and PIPs at the PM (3, 138). Several ORPs transfer PS (among other lipids) from the ER to the PM and then counter transport PI4P from the PM back to the ER (Figure 4.c). The PI4P phosphatase SAC1, an ER resident protein, hydrolyses the incoming PI4P, thereby maintaining the PI4P gradient between the PM and ER membrane and fuelling PS export from the ER (3). Note that PS transfer at MCS has not been described so far in plants but that ORP proteins are conserved in plant genomes.

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## 3. ANIONIC LIPIDS AS DETERMINANT OF MEMBRANE PHYSICOCHEMICAL PROPERTIES

### 3.1. Surface charges

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As they carry negative charges, anionic phospholipids generate an **electrostatic field** on the cytosolic leaflet of membranes (76) (Figure 1.a and 2.b). The strength of this electrostatic

219 field depends upon the relative concentration in anionic phospholipids and their respective  
220 negative charges, which vary from -1 for PS to -5 for PI(4,5)P<sub>2</sub>. In plants, TGN/EEs are less  
221 electronegative than the PM but more electrostatics than MVB/LEs, whereas ER-derived  
222 compartments (ER, nuclear membrane, peroxisomes) are neutral (93, 113). Consequently, an  
223 electrostatic gradient is formed from the PM to the tonoplast, which follows the endocytic  
224 route and specifies an “**electrostatic membrane territory**” within the endomembrane system  
225 (93). The extensive use of biosensors locating anionic lipids and assessing membrane surface  
226 charge *in vivo* shows that the high electronegativity of the plant PM relies concomitantly on  
227 PI4P, PS and PA, with a major role of PI4P, whereas the surface charge of TGN/EE depends  
228 of both PI4P and PS (93, 113).

229 The surface charges of membrane compartments are a key determinant to control the  
230 localization of many membrane-associated proteins. For example, proteins which contain  
231 polybasic patches interact with the negative charges of the anionic phospholipids of the  
232 membrane by non-specific electrostatic interactions (Figure 2.d). Very often, such motifs also  
233 contain hydrophobic residues or are found adjacent to hydrophobic lipid anchors. For  
234 example, the plant AGC kinases PINOID and D6PK, which are well-characterized regulators  
235 of auxin efflux transporters from the PIN family, localize at the PM through this mechanism  
236 (5, 61, 113). Another prominent example of plant proteins, which rely, at least in part, on  
237 electrostatic interactions with anionic lipids for localization is the small GTPases from the  
238 Rho-Of-Plant (ROP) family, which have a polybasic stretch adjacent to a prenylation site (i.e.  
239 a geranylgeranylation lipid modification) (91).

240 There is no precise study on the number of proteins encoded in plant genomes that might  
241 contain such polybasic/hydrophobic stretches and localize to electrostatic membranes  
242 through this mechanism but it is likely a general mechanism that contributes to the  
243 localization of many proteins within the electrostatic membrane territory.

244

### 245 **3.2. Curvature**

246 Anionic phospholipids present different geometries (Figure 2.a). Indeed, PI and PIPs have a large  
247 head group that carries the phosphorylation, whereas PA has a very small one (10). The bulk of  
248 the lipid directly influences the curvature of the membrane. Thus, the conical shape of PIPs will  
249 enhance positive curvature while the inverted conical shape of PA and PS will favour negative  
250 curvature (Figure 2.a). Membrane curvature is of prime importance to form endocytic vesicles at  
251 the PM, **secretory vesicles** at the TGN/EE or to internalize proteins in MVB for instance (10,  
252 78). Similar than surface charges, the curvature of the membrane can also induce lipid-protein  
253 interactions, via curvature sensing domain such as Bib-Amphiphysin-Rsv (BAR) domains (7, 63)  
254 or the Amphipathic Lipid Packing Sensor (ALPS) motif, which recognizes packing defects found  
255 in curved membrane. This motif can be found in association with a small stretch of positive  
256 amino acids to form a so-called +ALPS motif, which allows the coincidence detection of  
257 membrane charge and curvature (139). In plants, the PI4-kinase PI4K $\beta$ 1 relies on such motif to  
258 localize to the curved and electrostatic membrane of TGN/EEs (93).

259

260 → Include the sidebar "**Lipid molecular species and lipid packing defects**" here

261

### 262 **3.3. Clustering**

263 Proteins and lipids can also laterally segregate in membrane domains, thereby creating sub-  
264 compartmentalization inside the membranes (Figure 2.c). Such clustering is often required for  
265 the protein to fulfil its biological function. While sterol has long been associated with membrane  
266 domain formation, it recently emerged that anionic lipids are also key determinant of protein  
267 clustering.

268 For example, Remorins (REM) are proteins that laterally segregate at the PM in domains of  
269 about 100nm in size (hence termed **nanodomains**) (46) (Figure 5.a). REMs are involved in  
270 biotic and abiotic responses, although their exact molecular function is still unknown (47). For

271 example, *Solanum tuberosum* REMORIN group 1 isoform three (StREM1.3) limits the spreading  
272 of Potato Virus X (PVX), and regulates **plasmodesmata** permeability (100). A combination of  
273 super-resolution microscopy and biophysical experiments showed that StREM1.3 targeting into  
274 nanodomains is dependent upon the presence of both sterol and PI4P and is required for  
275 function (29) (Figure 5.a). PI4P is not the only anionic lipid involved protein nanoclustering into  
276 membrane nanodomains. Indeed, PS stabilizes the small GTPase ROP6 into nanoclusters of  
277 about 50 to 70nm in diameter (91) (Figure 5.b). By contrast to REM proteins, which are  
278 constitutively localized in PM nanodomains, ROP6 clustering is triggered by its activation (i.e.  
279 GTP-loading) upon auxin treatment. ROP6 directly binds to PS (and other anionic lipids) via a  
280 polybasic region located next to its geranylgeranylation site (91). ROP6 localization into  
281 nanoclusters is essential for function since ROP6 signalling is impaired in mutant lines lacking  
282 PS biosynthesis or when the PS-binding site in ROP6 is mutated. Single molecule imaging and  
283 super resolution microscopy of PS biosensors at the plasma membrane suggest that PS itself, not  
284 only ROP6, is present in PM nanodomains (Figure 5.b). However, unlike ROP6, PS are present in  
285 PM nanodomains both in the absence or presence of auxin (91). Therefore, there are  
286 prepatterned nanoplatforms of PS at the PM, which recruit GTP-loaded ROP6 upon auxin  
287 treatment. Future studies are needed to uncover whether such PS nanoplatforms also contain  
288 additional lipids, such as sterol or PI4P and how they are formed. The cell wall and the  
289 cytoskeleton influence protein diffusion into membrane (73, 75). It will therefore be interesting  
290 to understand the interplay between anionic lipid-induced clustering, cell wall constraints and  
291 cytoskeleton dynamics, the latter being itself regulated by anionic lipids (24).

292

293 All the mechanisms described above converge on the idea that anionic lipids are key  
294 determinant of membrane identity, which ultimately allows the precise recruitment of proteins  
295 in space and time. There are countless examples of proteins that are targeted to specific  
296 membranes by anionic phospholipids either constitutively or upon certain stimuli. These  
297 examples will not be individually reviewed here. Instead, we will dedicate the rest of this

298 review to one of the key questions of cell biology: how compartments maintain a specific  
299 identity despite the constant flow and exchange of proteins and lipids between compartments.  
300 We will in particular highlight how anionic lipids may contribute to membrane contact sites  
301 establishment and analyse the interplay between membrane trafficking and anionic lipids.

302

#### 303 **4. ANIONIC LIPIDS AND ER/PM MEMBRANE CONTACT SITES IN PLANTS**

304 Several reports began to put EPCS (ER/PM MCS) at the centre of essential cellular processes  
305 including immune secretory pathway, viral movement at plasmodesmata, stabilization of the  
306 cortical ER network, endocytosis, membrane integrity, pollen, seed and root hair development  
307 (53, 64, 65, 90, 104, 134, 141). Lipid exchange at EPCSs is still an open question in plants but  
308 a clear role for anionic lipids in EPCS establishment is documented.

309

310 In Arabidopsis, three types of EPCSs have been described (Figure 6): the S-EPCSs, which are  
311 stabilized by synaptotagmins (SYTs, orthologs of the mammalian extended synaptotagmins  
312 (E-SYT)) (6, 90), the V-EPCSs that contain the vesicle-associated membrane-associated  
313 protein 27s (VAP27s) and VAP27-related proteins (orthologs of the mammalian VAPS and  
314 yeast Scs2) (102, 132, 134) and the PD-EPCSs, which are specifically enriched at  
315 plasmodesmata (PD) and contain MULTIPLE C2 AND TRANSMEMBRANE PROTEIN  
316 (MCTP) proteins (11). Importantly, all three classes of tethering molecules, VAP27s, SYTs,  
317 and MCTPs are directly interacting with anionic phospholipids and likely require these lipids  
318 to interact with the PM. Nonetheless, S-EPCS and V-EPCS are spatially separated within the  
319 same cell suggesting a tethering signature model where the abundance of one type of EPCS at  
320 the expense of the other could vary in response to environmental or developmental cues  
321 (111). Another model, the tethering arrangement model, proposes that the organization of  
322 EPCS could change in response to stimuli such as lipids or calcium fluxes (26). These two

323 models are not mutually exclusive.

324

#### 325 **4.1. V-EPCS**

326 VAP27 are ER transmembrane proteins that are conserved among eukaryotes (138). Ten  
327 homologs of VAP27 exist in Arabidopsis (134). Three of them localize at the ER-PM contact  
328 sites: VAP27-1, VAP27-3, and VAP27-4, together with the actin binding protein  
329 NETWORKED 3C, NET3C (133) (Figure 6). VAP27-1 and VAP27-3 interact with PIPs *in*  
330 *vitro* (116). Moreover, VAP27-3 is able to interact with a lipid transfer protein from the ORP  
331 family, ORP3a, that is able to bind sterol *in vitro*, suggesting possible lipid transfer similar to  
332 what has been described in yeast and animal cells (3, 102).

333

#### 334 **4.2. S-EPCS**

335 The mammalian E-SYT1 is able *in vitro* to trigger glycerolipids exchanges between  
336 liposomes (105). E-SYTs contain C2 domains that bind anionic phospholipids from the PM in  
337 a Ca<sup>2+</sup> depend manner (144). In favour of the tethering arrangement model, E-SYTs Ca<sup>2+</sup>  
338 binding leads to clustering of E-SYTs proteins and an increase of the interactions between E-  
339 SYTs and PM anionic lipids (26). This results in stabilization and tightening of S-EPCS (26,  
340 138, 144). In Arabidopsis, ionic stress triggers the expansion of S-EPCS in association with  
341 accumulation of PI(4,5)P<sub>2</sub> at the PM supporting the tethering arrangement model (62).  
342 Furthermore, the C2 domains of Arabidopsis SYT1 bind PS in a Ca<sup>2+</sup> dependent-manner *in*  
343 *vitro* (90) (Figure 6). The relevance of this Ca<sup>2+</sup>-dependent PS binding *in vivo* is currently  
344 unknown but also argues in favour of the tethering arrangement model.

345

#### 346 **4.3. PD-EPCS**

347 PD pores interconnect virtually all cells within the plant body, establishing direct PM and  
348 cytoplasmic continuity. A striking feature of PD is a strand of endoplasmic reticulum (ER)

349 running through the pores and tethered tightly to PM, making PD a unique case of EPCS in  
350 eukaryotes. While SYT1 localize to PD to some extent (64, 90), several MCTP proteins are  
351 specifically enriched at PD and likely contribute to ER-PM tethering at the PD pore (11)  
352 (Figure 6). Molecular dynamics suggest that MCTP4 C2 domains interact with anionic lipids  
353 at the PM, in particular PS and PI4P. Accordingly, pharmacological experiments support that  
354 PI4P is required for MCTP4 PD-specific localization. PDs have a specific composition in  
355 term of sterol and sphingolipid (28). By extension, an attractive hypothesis that remains to be  
356 tested, would be that the PM at PD has a unique anionic lipid signature that could contribute  
357 to MCTP PD-specific localization.

358

359 Communication between organelles and lipid exchanges occur not only at MCSs but also  
360 during vesicular trafficking. Vesicular trafficking implies exchange of membranes between  
361 compartments and anionic lipids are key actors of those exchanges. Therefore, compartments  
362 must maintain a specific lipid identity despite the constant flow and exchange of proteins and  
363 lipids that happen during vesicular trafficking. The interplay between membrane trafficking  
364 and anionic lipids will be detailed in the following section of this review.

365

## 366 **5. TRAFFICKING BETWEEN THE PM AND THE TGN**

### 367 **5.1. Clathrin mediated endocytosis**

368 In animal cells, it is well documented that anionic lipids play a fundamental role in  
369 endocytosis. Clathrin-mediated endocytosis mainly involves PI(4,5)P<sub>2</sub> produced from PI4P by  
370 PIP5P kinases (PI4P-5Ks) (81). PI(4,5)P<sub>2</sub> allows the initiation and stabilisation of the  
371 **clathrin-coated pits** by the recruitment of several components of the endocytic machinery  
372 including the adaptor protein AP2 (51, 95). Moreover, PIP5Ks are directly recruited at the site  
373 of endocytosis by AP2, creating a local pool of PI(4,5)P<sub>2</sub>, which induces a high rate of



374 clathrin-coated pit nucleation (81). Then, both PI(4,5)P<sub>2</sub> and PI(3,4)P<sub>2</sub> are involved in pit  
375 maturation (81).

376

### 377 **5.1.1. PI(4,5)P<sub>2</sub> a main actor in clathrin-mediated endocytosis**

378 In plant cells, the recruitment of the endocytic machinery to the PM is far less understood.  
379 However, the role of PI(4,5)P<sub>2</sub> in endocytosis has been demonstrated indirectly using PIP5Ks  
380 over-expression and loss-of-function approaches (43, 146). Notably, a *pip5k1pip5k2*  
381 (hereafter designed as *pip5k1k2*) mutant has abnormal **clathrin-coated pits**, which are less  
382 dense than in the wild type but bigger (43). This mutant, as well as PIP5K1 and PIP5K2  
383 overexpression line, have defects in PIN endocytic trafficking, which ultimately impact both  
384 their accumulation at the PM, their polar targeting and hence auxin distribution, leading to  
385 various degrees of auxin-related developmental defects (43, 79, 120). Over-expression of  
386 PIP5K also induces a phloem differentiation phenotype (30). PIP5K overexpression redirects  
387 the trafficking toward the vacuole leading to protein degradation and vacuole biogenesis,  
388 which in protophloem file induces vascular discontinuities (30). However, it is unclear if this  
389 increase in vacuolar trafficking is caused by an overall heightened endocytic rate at the PM or  
390 by regulation of downstream trafficking steps. In any case, PI(4,5)P<sub>2</sub> levels are constantly  
391 kept in check in the root phloem by two PM-localized PI(4,5)P<sub>2</sub> 5-phosphatases,  
392 COTYLEDON VASCULAR PATTERN 2 (CVP2) and CVP2-LIKE 1, whose double mutant  
393 shows similar phloem discontinuity as PIP5K overexpression lines (101).

394 In growing pollen tube, the exocytosis at the tip is balanced by endocytosis in the subapical  
395 regions (Figure 3). These regions are marked by PI(4,5)P<sub>2</sub> locally produced by PIP5Ks and  
396 restricted by degradation into diacylglycerol (DAG) by Phosphoinositide Phospholipase C  
397 (PI-PLC) (17, 32) (Figure 3). Loss-of function mutants of PIP5Ks show reduced endocytosis,  
398 which lead to low pollen germination rate and slow tube elongation (41, 115). Conversely,

399 pollens overexpressing PIP5K6 show PM invagination and the formation of several tip  
400 branches due to a higher rate of endocytosis at the tip of pollen tube (146). The expression of  
401 a clathrin dominant negative can rescue the PIP5K6-OE phenotype indicating that PI(4,5)P<sub>2</sub>  
402 synthesis enhances clathrin-mediated endocytosis (146). A similar mechanism was proposed  
403 in responses to salt treatment which increases the level of PI(4,5)P<sub>2</sub> and induces the formation  
404 of **clathrin-coated vesicles** in a PIP5K1 and PI5PK2-dependent manner (43, 56).

405

### 406 **5.1.2. PA as another regulator of clathrin-mediated endocytosis?**

407 Salt tolerance studies suggest a role for PA in endocytosis. Indeed, the level of PA is  
408 drastically increased under salt treatments (83). In addition of inducing membrane curvature,  
409 PA is able to bind clathrin heavy chain (CHC) and domain clathrin assembly proteins (77).  
410 Upon salt treatment, PA production by the phospholipase PLDζ<sub>2</sub> induces the internalisation  
411 of PIN2 in TGN/EE (21), whereas in normal condition PA and PLDζ<sub>2</sub> are necessary for the  
412 proper cycling of PIN2 in root cells (66). This mechanism decreases the amount of PIN2 at  
413 the PM on the salt-side and induces auxin redistribution that allows the plant to redirect its  
414 root growth away from highly salt concentrated soil (21). This data suggest that PA could act  
415 as a mediator of the auxin response by activating the endocytosis of PIN proteins upon high  
416 salinity conditions.

417

418 To conclude, a role for PI(4,5)P<sub>2</sub> in clathrin-mediated endocytosis is established in different  
419 cell types and developmental contexts. Nevertheless, the proteins regulated by PI(4,5)P<sub>2</sub>  
420 during endocytosis are still largely unknown. In addition, the role of other anionic lipids such  
421 as PI4P and PA in the regulation of the endocytic machinery needs to be clarified.

422

### 423 **5.2. Polar secretion and exocytosis: the targeting of the exocyst**

424 Exocytosis is led by a **tethering complex** called exocyst, which is conserved among  
425 eukaryotes and that targets exocytic vesicles to the PM (88). In many cases, exocytosis does  
426 not occur homogenously along the PM but it is polarized. Polar exocytosis has been reported  
427 in growing tip cells such as root hair and pollen tube (8, 14, 67, 108, 118, 136), for the polar  
428 localization of auxin efflux carriers from the PIN family (18, 89, 119), in the establishment of  
429 the casparian strip (48) or the Ortmannian ring in trichomes (57 in revision, 58–59).

430

### 431 **5.2.1. PI(4,5)P<sub>2</sub> targets the Exocyst to the PM**

432 The exocyst complex is targeted to the PM via two subunits, SEC3 and EXO70 known to  
433 bind anionic phospholipids, in particular PI(4,5)P<sub>2</sub>. In Arabidopsis, SEC3 contains a PH  
434 domain that binds to PI(4,5)P<sub>2</sub> *in vitro* (8). The extensive use of biosensors and the  
435 localization of lipid metabolic enzymes in plants allowed the cartography of anionic lipids in  
436 cells exhibiting polar secretion highlighting specific lipid accumulation patterns, notably for  
437 PI(4,5)P<sub>2</sub> (Figure 3) (41, 42, 57, 60, 61, 115, 117, 146). Interestingly, the localizations of  
438 SEC3 and/or EXO70 isoforms often correlate with the PI(4,5)P<sub>2</sub> subcellular accumulation  
439 pattern, suggesting that PI(4,5)P<sub>2</sub> contribute to their localization (8, 48, 108). In pollen tube,  
440 SEC3 and EXO70A1 partially colocalize with PI(4,5)P<sub>2</sub> biosensors (8, 108). Since PIP5K  
441 mutants display slower tube elongation and reduced secretion of cell wall materials (41, 115),  
442 it is likely that PI(4,5)P<sub>2</sub> participates in targeting exocyst complexes to specific PM domains.

443

### 444 **5.2.2. PI(4,5)P<sub>2</sub> and PA: working together to confine exocytosis**

445 EXO70A1 and EXO70B1 are expressed in growing pollen tube and partially colocalize with  
446 both PI(4,5)P<sub>2</sub> and PA biosensors (108). Therefore, it is possible that the coincidence  
447 detection of PI(4,5)P<sub>2</sub> and PA in pollen tube restricts the site of exocytosis to the narrow PM  
448 strip that contains both lipids (Figure 3). These EXO70 subunits may directly recognize each

449 lipid. Alternatively, they may be sensitive to the overall membrane surface charges. One may  
450 speculate that in pollen tube, the PM membrane domain that contains a combination of both  
451 PI(4,5)P<sub>2</sub> and PA is highly electronegative and thereby could recruit specific EXO70  
452 isoforms. Additional experiments are required to distinguish between these two possibilities.  
453

### 454 **5.3. Proper secretion requires PI4P production in the TGN**

455 Exocytosis not only requires specific anionic lipids at the PM but also a regulation of the lipid  
456 composition in the TGN for proper vesicle formation. Indeed, root hair studies revealed the  
457 importance of the pool of PI4P in the TGN for polar secretion (49, 99, 122, 143). PI4Kinases,  
458 PI4Kβ<sub>1</sub> and PI4Kβ<sub>2</sub>, as well as the PI4P phosphatase ROOT HAIR DEFECTIVE 4,  
459 RHD4/SAC7, localize to the TGN. *pi4kβ1β2* and *rhd4* mutants display short or bulged root  
460 hairs (49, 99, 122). Ultrastructural studies by electron tomography showed the presence of  
461 enlarged, likely deficient, **secretory vesicles** in the TGN of *pi4kβ1β2* double mutant,  
462 suggesting that PI4Kβs are indeed involved in secretion (49). PI4Kβs and RHD4/SAC7 have  
463 opposite catalytic activities, yet apparently, locate to the same compartment. It is possible that  
464 PI4Kβs and RHD4/SAC7 localize in different TGN subdomains. Consistent with this  
465 scenario, PI4Kβ<sub>1</sub> localizes specifically in the secretory vesicle subdomain of the TGN (49).  
466 According to this hypothesis, PI4Kinases could be necessary for the production of the pool of  
467 PI4P at the TGN which confers the proper physical properties (mild electrostatics, high  
468 curvature) necessary for secretory vesicle formation. By contrast, RHD4/SAC7 could localize  
469 in a different TGN subdomain, perhaps directly connected to endocytosis. In such model,  
470 RHD4/SAC7 would degrade incoming PI4P from the PM to maintain the PI4P gradient along  
471 the endocytic pathway (113).

472

### 473 **5.4. Cell plate establishment and the creation of a new PM**

474 The cell plate is an organelle formed from the secretion and fusion of vesicles from the TGN  
475 and will form the new PM of the two daughter cells (13). A combination of clathrin-mediated  
476 endocytosis and exocytosis occurs at the cell plate. During this process, the lipid composition  
477 of the cell plate varies from the bulk PM. Lipid binding biosensors suggest that PI4P, PA and  
478 PS accumulate and confer a highly electronegative field at the cell plate to a similar extent, or  
479 even higher, than at the PM (16, 93, 113) (Figure 3). However, PI(4,5)P<sub>2</sub> is excluded from the  
480 cell plate during its creation and extension and appears at the edges of the cell plate just  
481 before its full attachment to the PM (16, 113, 127). Knowing that PI(4,5)P<sub>2</sub> is a major  
482 regulator of both exocytosis and endocytosis, it is therefore likely that specific trafficking  
483 mechanisms occur at the cell plate compared to the bulk PM, which have yet to be uncovered.  
484 This difference of membrane composition between the bulk PM and the cell plate is crucial  
485 for proper cell division. Indeed, several mutants for enzymes involved in anionic lipid  
486 homeostasis show cell plate and/or cytokinesis phenotypes. Those include mutants for PI4P  
487 production (*pi4kβ1β2*), PI4P/PI(4,5)P<sub>2</sub> interconversion (*sac9*, *pi5k1k2*), and PI3P/PI(3,5)P<sub>2</sub>  
488 interconversion (*fab1afab1b*, *fra7/sac1*) (49, 120, 131, 137, 148). Some of these enzymes  
489 directly act at the cell plate. For instance, PI4Kβ1 localizes at the cell plate and the absence of  
490 PI4Kβ1 and PI4Kβ2 directly affect the vesicular trafficking along phragmoplast microtubules  
491 (68). However, others might act indirectly on cell division, such as FORMATION OF  
492 APLOID AND BINUCLEATE CELLS 1, FAB1A and FAB1B proteins, that do not localize  
493 at the cell plate (36, 137). It is therefore likely that the *fab1afab1b* cell plate phenotype results  
494 from a broad alteration of membrane trafficking. We refer the reader to a recent review on  
495 this subject, which details the potential involvement of each anionic lipid during cytokinesis  
496 (13).

497

## 498 **5.6. Anionic phospholipid remodelling at the Plants-Fungi interface**

499 Fungi infection and mycorrhizal symbiosis induce PM reorganization, which include lipid  
500 patterning at the PM with likely consequences on both endocytosis and exocytosis.

501

### 502 **5.6.1. Periarbuscular membrane**

503 During the endosymbiotic association of roots with arbuscular mycorrhizal (AM) fungi in *M.*  
504 *truncatulla*, the fungus develops intracellular branched hyphae called arbuscules (44). In  
505 response, the root cortical cells form a new membrane in continuity with the PM called  
506 periarbuscular membrane (PAM) (31). The PAM envelops the hyphal branches and is thought  
507 to be generated by polar exocytosis. Indeed, subunit of the exocyst complex and SNARE  
508 proteins known to be involved in exocytosis and membrane fusion are required for the PAM  
509 formation (31, 145). By analogy to tip growing cells, anionic lipids might regulate this  
510 particular case of polarized secretion. So far, only the pattern of localization of PI4P,  
511 PI(4,5)P<sub>2</sub> and PA were documented during AM interaction (44) (Figure 3). Both PI4P and PA  
512 are found in the PAM to similar extent than the PM. However, in the trunk part of hyphal  
513 branches, discrete regions where PI(4,5)P<sub>2</sub>, PI4P and PA reporters co-accumulate can be  
514 observed (44). This accumulation could correspond to an accumulation of membranes before  
515 formation of small bulges. Furthermore, anionic lipids accumulation could influence  
516 membrane curvature and thereby physically contribute to the establishment of the PAM.

517

### 518 **5.6.2. Extra invasive hyphal membrane**

519 Using another model of fungi-plant interactions, it was recently shown in *Arabidopsis*  
520 infected by *Colletotrichum* that extra invasive hyphal membrane (EIHM), which is formed in  
521 continuity with the PM, strongly accumulates PI(4,5)P<sub>2</sub> and PIP5K3 all along the membrane  
522 (Figure 3), as well as a subunit of the exocyst complex and SNARE proteins (39, 109, 110).  
523 This accumulation of PI(4,5)P<sub>2</sub> seems to be specific to *Colletotrichum* (110). PI(4,5)P<sub>2</sub>

524 production by PIP5K at the EIHM could increase polar exocytosis thereby allowing the  
525 formation of the EIHM and thus the successful infection by the fungi.

526

527 All together, these recent data suggest that anionic lipid patterns are modified in response to  
528 fungi and allow the symbiosis or infection to take place, possibly by induction of polar  
529 exocytosis to the membrane that surround the hypha.

530

## 531 **6. ANIONIC LIPIDS IN VACUOLAR TRAFFICKING**

532 Most membrane-associated proteins are degraded in the vacuole. To reach the vacuole, PM  
533 proteins are first polyubiquitinated, which induces endocytosis and targeting to TGN/EEs  
534 (19). Then TGN/EEs mature into LE/MVBs. During this maturation, endosomal-sorting-  
535 complex-required-for-transport (ESCRT) internalizes membrane proteins into intraluminal  
536 vesicles. Once the external membrane of MVB fuses with the tonoplast, the intraluminal  
537 vesicles containing the proteins are released into the vacuole and degraded. Non-ubiquitinated  
538 cargo can be retrieved in MVBs by the action of the retromer complex that antagonize the  
539 ESCRT complex (86).

540

### 541 **6.1. From PI4P to PI3P and PI(3,5)P<sub>2</sub>: PIP cascade leading to protein sorting**

542 One of the main characteristics of PIPs is to be quickly interconverted by kinases and  
543 phosphatases (Figure 1.c). This is well illustrated along the road leading to protein  
544 degradation into the vacuole in which the different maturation steps correlate with a PIP  
545 interconversion (Figure 7). PI(4,5)P<sub>2</sub> from the PM is converted into PI4P by 5-phosphatases  
546 either in TGN/EEs or during the endocytic process. At the surface of TGN/EEs, PI4P is  
547 gradually replaced by PI3P through the combined action of 4-phosphatases (e.g. RHD4/SAC7  
548 in root hair) and the PI3K, VPS34. Some MVBs exhibit PI(3,5)P<sub>2</sub> due to the action of the

549 PI3P5K from the FAB1 family (36, 38, 112) (Figure 7). The acquisition of PI3P is essential  
550 for **protein sorting**. Indeed, FYVE1/FREE1, a component of the ESCRT complex that binds  
551 ubiquitinated cargo, localizes in the MVB by interacting with PI3P (22, 55). Similarly,  
552 SORTING NEXIN 1 (SNX1), a component of the retromer complex also localizes at the  
553 MVB through interactions with PI3P and/or PI(3,5)P<sub>2</sub> (98). In addition, PI3P and/or PI(3,5)P<sub>2</sub>  
554 also mediate the association of endosomes with the cytoskeleton, since the loss of FAB1  
555 releases MVBs from cortical microtubules (35, 36) and SNX1 interact with the microtubule  
556 regulator CYTOPLASMIC LINKER ASSOCIATED PROTEIN, CLASP (1).

557

## 558 **6.2. Autophagosome and protein degradation**

559 Cytosolic cargoes can also be degraded by autophagy (20). In this process, cargoes are  
560 encapsulated by a double membrane to form autophagosomes. The outer membrane of the  
561 autophagosomes fuses with the tonoplast and the internal membrane, together with its luminal  
562 content, is subsequently degraded in the vacuole (20). The autophagosome is initiated by the  
563 formation of a **phagophore** that will expand, surround the cargoes and close. AuTophagy-  
564 related (ATG) proteins were first discovered by genetic screens in yeast but most of the  
565 actors of autophagy are conserved among eukaryotes (20). In all eukaryotes, PI3P is critical  
566 for autophagy. In animal and yeast, PI3P is produced at the initiation step of phagophore  
567 formation by a PI3K complex that contains the PI3K VPS34 (20). A member of the complex,  
568 ATG14, targets the PI3K complex to the phagophore (15). Furthermore, the production of  
569 PI3P is essential to recruit and stabilize autophagy-related proteins at the membrane of the  
570 phagophore. In addition, the geometry of the head group of PI3P could also directly help the  
571 curvature of the phagophore. In plants, the precise dynamics of PI3P during autophagy is not  
572 well understood. Furthermore, ATG14 does not exist, which raises the question of the  
573 recruitment of the PI3K complex at the phagophore (20). Yet, inhibitor of PI3Ks blocks



574 autophagy in plants (142), suggesting that, like in other eukaryotes, PI3P is important for this  
575 process.

576

577 In addition to stabilizing ATG-related protein at the membrane of the phagophore, several  
578 plant-specific proteins, involved in autophagy, directly interact with phosphoinositides. SRC-  
579 HOMOLOGY3-CONTAINING-PROTEINs SH3P2 and SH3P3 are two BAR-domain  
580 containing proteins that localize at the phagophore (149). As BAR domains can induce  
581 membrane curvature, they may participate in the membrane shaping of the phagophore.  
582 SH3P2 interacts with PI3P (and other phosphoinositides) and co-localize with ATG8 in the  
583 phagophore in a VPS34-dependent manner (149). SH3P2 interacts with ATG8 and the PI3K  
584 complex. *fyve1/free1* mutant presents autophagy-related phenotype with accumulation of  
585 autophagic structures (22, 149). FYVE1/FREE1 interacts with ATG8, the PI3K complex and  
586 SH3P2 (23). As such, FYVE1/FREE1 may play a role in phagophore maturation or closing.  
587 Another hypothesis argues for an indirect role of the ESCRT and retromer complexes in  
588 autophagy. Indeed, SNARE proteins, necessary for autophagosome fusion with the vacuole,  
589 need to be properly sorted to the tonoplast. It is therefore possible that the *fyve1/free1*  
590 phenotype results, at least in part, of a miss sorting of SNARE proteins (50).

591

### 592 **6.3. PIPs and vacuole morphology**

593 Mutant for the retromer and the ESCRT complexes present small unfused vacuoles (22, 23,  
594 45, 54, 55). This phenotype is also found with the loss-of- function of VTI11, a SNARE  
595 protein involved in the docking and fusion of MVB with the tonoplast (124). It is suggested  
596 that the PI3P-dependent protein sorting is important for the proper localization of SNARE  
597 proteins and thus vacuole fusion. Moreover, the vacuole phenotype of *vti11* mutant can be  
598 rescued by inhibition of PI3K activity suggesting a role of PI3P in fusion of homeotic

599 vacuoles (147). The homotypic fusion and vacuolar protein sorting (HOPS) tethering complex  
600 is required for vacuole morphology (12). VACUOLAR PROTEIN SORTING33 (VPS33) and  
601 VPS45, two HOPS complex members, binds phosphoinositide in vitro and localize to the  
602 vacuole in a PI3P-dependent manner (12). SAC2, SAC3, SAC4 and SAC5 are likely  
603 PI(3,5)P<sub>2</sub> 5-phosphatases that localize at the tonoplast and could hydrolyse the incoming  
604 PI(3,5)P<sub>2</sub> from MVB into PI3P (87) (Figure 7). Loss-of-function and overexpression of these  
605 SAC proteins lead to abnormal vacuolar morphology consistent with deficit in vacuole fusion  
606 due to lack of PI3P (87). PI4P is also involved in the morphological changes of the vacuole  
607 that are regulated by auxin in the root (69, 106). However, the molecular mechanism  
608 connecting PI4P to vacuole morphology are so far unknown.

609

610

## 611 **7. CONCLUSIONS, PERSPECTIVES AND FUTURE CHALLENGES**

612 Many aspects of the role and operation of anionic lipids along the endomembrane system are  
613 conserved among eukaryotes, including many of the enzymes. However, the anionic lipid  
614 distribution of plant cells present several specificities compare to yeast or animal cells,  
615 starting with the high and low abundance of PI4P and PI(4,5)P<sub>2</sub> at the PM, respectively. Thus,  
616 the establishment of the anionic lipid distribution in plant is still not fully understood.  
617 Moreover, the mechanisms by which this distribution is maintained despite the constant  
618 exchanges of membrane between compartments due to vesicular trafficking must be explored.  
619 This includes the coupling between the enzymes that produce anionic lipids and the ones that  
620 convert or degrade them. Additionally, links or compensatory effects could exist between the  
621 different lipid pathways at the level of enzymatic activity, membrane recruitment or  
622 expression. Recently, MCS appeared as a new path of lipids exchanges independent from

623 vesicular trafficking. The mechanisms, the nature of the exchanged lipids as well as relevance  
624 for the cell and plant life remains to be fully appreciated.

625 The role of anionic lipids in the regulation of membrane trafficking has been clearly  
626 established in all eukaryotes. The sorting of many of the proteins involved in membrane  
627 trafficking depends on anionic lipids. At the same time, many of the proteins involved in  
628 anionic lipids pathways are membrane-associated proteins. Thus, their sorting depends of  
629 vesicular trafficking. The constant flow of membrane trafficking and the interconnections  
630 between lipids and protein sorting make it difficult to sort the direct and indirect  
631 consequences of lipid perturbation using loss-of-function strategy only. The situation is  
632 further amplified by the fact that anionic phospholipids are highly interconnected, and  
633 perturbation of one lipid is likely to impact others. Similarly, although anionic lipids are of  
634 major importance for many developmental and physiological responses, including stress,  
635 cytokinesis or cell differentiation, the chain of events explaining the observed phenotype is  
636 often not solved. New live imaging and inducible perturbation technics to study lipid  
637 dynamics in its physiological context are therefore urgently needed in order to untangle direct  
638 from indirect effects of anionic phospholipids. Similarly, it will also be key to uncover and  
639 functionally characterized anionic lipid effectors (i.e. proteins that interact with anionic  
640 phospholipids for function and localization) as most of them are plant specific proteins.

641

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643

644 • **Summary Points list:** *highlight the central points of your review (as many as 8), in*  
645 *complete sentences; insert above the Acknowledgments and/or Literature Cited section*  
646

- 647 1. Anionic lipids influence the physicochemical properties of cell membranes, such as  
648 their surface charges, curvature, packing and nanoscale organization.
- 649 2. Anionic lipids define an “electrostatic membrane territory” in plants, which  
650 corresponds to post-Golgi membranes.
- 651 3. Enrichment of specific anionic lipid combination in different compartments  
652 participates to the establishment of organelle identity and the specific recruitment of  
653 proteins.
- 654 4. The distribution of anionic lipids inside a membrane is not homogeneous in term of  
655 lateral segregation and membrane leaflet accumulation. Anionic lipids, together  
656 with other lipids and proteins, organize in nanoclusters and are mostly oriented  
657 toward the cytosolic leaflet.
- 658 5. Most of the enzymes related to anionic lipids are conserved among eukaryotes,  
659 while anionic lipid effectors tend to be plant specific.
- 660 6. Anionic lipids can be transported either via vesicular trafficking or via lipid  
661 transport proteins at MCS. Conversely, anionic lipids are themselves master  
662 regulators of both vesicular trafficking and MCS.
- 663 7. Phosphoinositide kinases and phosphatases allow the fast production and  
664 interconversion of PIP, which ensure relatively stable patterns of phosphoinositide  
665 subcellular accumulation despite the constant flow of anionic lipids between  
666 organelles.
- 667 8. Anionic lipid repartition is a dynamic feature of the cell and can change according  
668 to its physiological (infection, symbiosis, stress...) or developmental (**polar**  
669 **growth**, cytokinesis, aperture establishment, casparian strip formation, trichome...)   
670 status.

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- □ **Future Issues list:** *note where research may be headed (as many as 8), in complete sentences; insert above the Acknowledgments and/or Literature Cited section*
  1. How the cell maintains the lipid homeostasis at both cellular and developmental levels? Addressing this question will require a deep understanding on how lipid production and degradation/consumption are coupled and regulated, and how each anionic lipid may work in functionally and physically independent pools within the same cells.
  2. Most enzymes involved in anionic lipid homeostasis have been described, but how their activity is regulated is still unknown. The role of post-translational modifications (e.g. phosphorylation) has been spear headed by Ingo Heilmann's group and this is an area of research that is likely to expand in the future (33, 80).
  3. It is difficult to determine the direct or indirect consequences of the perturbation of an anionic lipid in a given compartment. A key future research direction will be to address what is the chain of events linking defects in anionic lipids to the observed cellular or whole plant phenotypes? One of the key to unlock such question will be the development of fast inducible systems to perturb anionic phospholipids.
  4. What are the feedbacks and crosstalk between anionic lipids and the other membrane lipids, including phospholipids, sterol and sphingolipids? In particular, what is the importance of the membrane bilayer asymmetry in the way lipid pools contact and regulate each other, and what is the contribution of the cell wall/PM/cytoskeleton continuum in lipid interactions across the membrane bilayer?
  5. Compartment morphodynamics is a highly complex system. Understanding how anionic phospholipids contribute to membrane morphodynamics will require the development of minimal membrane systems in vitro and computer simulation together with continued efforts in molecular genetics and cellular biology.
  6. Anionic lipids are key molecules for membrane contact sites, yet the importance and mechanisms of MCS formation, regulation and function are still largely enigmatic in plants. It will be particularly interesting to investigate membrane contacts which include plant specific organelles, such as the chloroplast, tonoplast, plasmodesmata or TGN.
  7. The development of anionic lipid sensors compatible with live imaging and super-resolution microscopy techniques has allowed analysing the function of anionic lipids with unprecedented spatial and temporal resolution. However, given the current technical revolution in the field of super-resolution microscopy, we expect this line of research to rapidly grow in the future.
  8. A bottleneck in the field is our ability to biochemically detect the different anionic phospholipid molecular species with subcellular resolution. Efforts should be given to improve the current methods of anionic lipid identification by mass spectrometry and cellular fractionation.

713  
714

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- **Reference Annotations:** *brief (15 words maximum) explanation of citations' importance (as many as 10); insert below the Literature Cited section*
  1. **Brault et al., 2019:** Proposes MCTP proteins, a class of putative PS/PI4P binding proteins, as tethering factors at PD-EPCS.
  2. **Gronnier et al., 2017:** Describes how anionic lipids and sterol influence REMORIN conformation, localization and activity by forming nanoclusters.
  3. **Platre et al., 2019:** Visualizes and functionally characterizes nanoclusters of anionic lipids (i.e. PS) in live plant cells.
  4. **Lin et al., 2019:** Establishes that PI4P production at the cell plate contributes to cytokinesis and phragmoplast dynamics.
  5. **Kang et al., 2011:** Uses electron tomography to reveal distinct TGN subdomains with PI4KB1 highlighting the secretory vesicles subdomain.
  6. **Lee et al., 2019:** Proposes that S-EPCS dynamics is regulated by PI(4,5)P<sub>2</sub>-SYT interactions and is remodelled during osmotic stresses.
  7. **Platre et al., 2018:** Proposes the concept of an “electrostatic membrane territory” in plants, which corresponds to post-Golgi membranes.
  8. **Steffano et al., 2018:** Identifies that plant VAP27 proteins directly interact with anionic phospholipids and regulate endocytic trafficking.
  9. **Simon et al., 2016:** Establishes the importance of membrane electrostatics and PI4P in defining the plant PM identity.
  10. **Ghosh et al., 2015:** Proposes a model for Sec14-nodulin function as PI-kinase helper protein mediating PI(4,5)P<sub>2</sub> polar localization.

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- 1176 • **Sidebar** (50 words minimum, 200 words maximum) briefly discussing a fascinating  
1177 adjacent topic. Please give the sidebar a title and insert it below the Literature Cited  
1178 section, but be sure to call out the sidebar in text; it will be typeset near the section  
1179 containing the callout. The sidebar cannot contain figures or tables.

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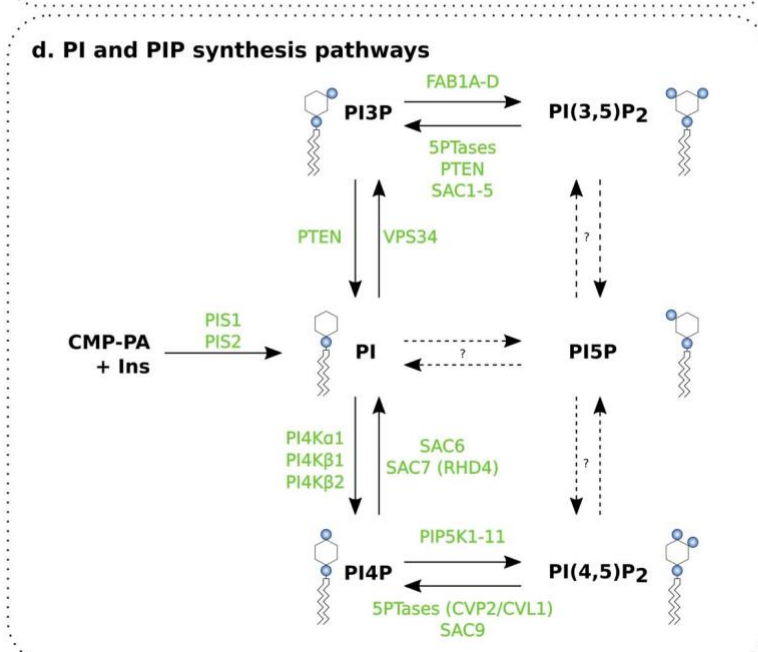
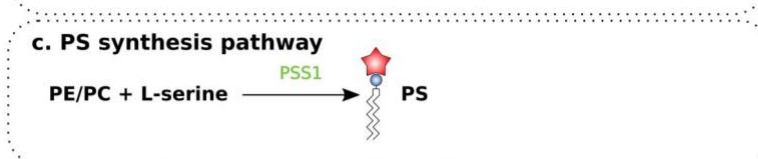
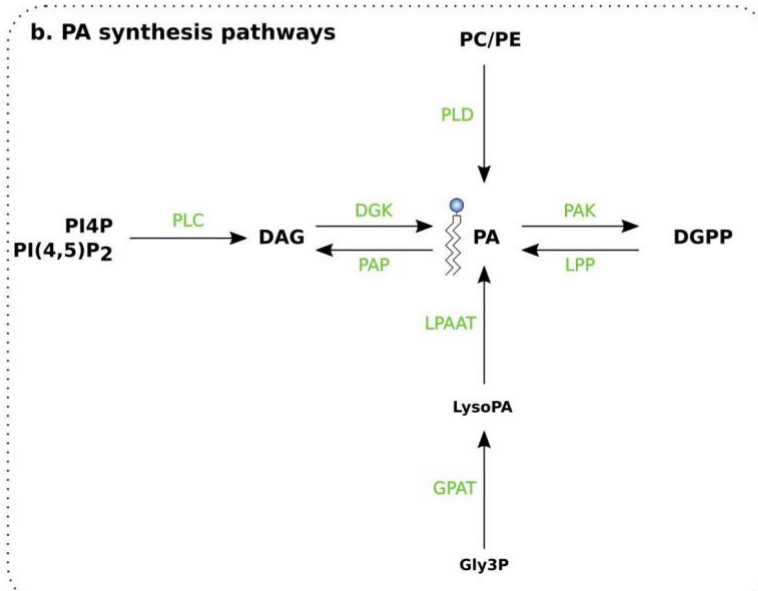
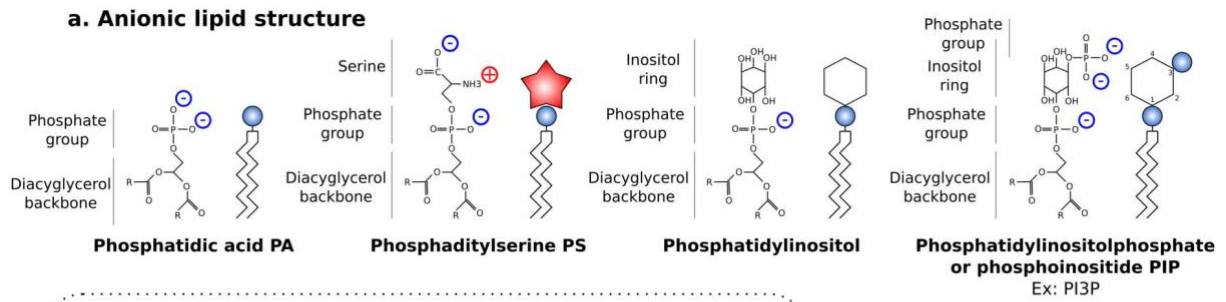
1181 **Lipid molecular species and lipid packing defects (7):** Anionic lipids are defined by their  
1182 head group. However, they are also constituted of two fatty acid chains that can vary in length  
1183 and unsaturation degree. These parameters influence membranes physical properties, notably  
1184 their fluidity and curvature, and thus, the recruitment and dynamics of membrane associated  
1185 proteins. In addition to membrane charge and curvature, another physical feature of the  
1186 membrane is of particular interest for protein targeting and is directly influenced by fatty acid  
1187 composition. Indeed, the density of lipids in the membrane is not homogeneous. The regions  
1188 with low density are called lipid-packing defects. Membrane curvature and conical shape  
1189 lipids (which can be linked to the degree of unsaturation) can induce packing defects. In this  
1190 region, the hydrophobic tails of the lipids are transiently exposed. Membrane associated  
1191 proteins can recognize and target specifically membrane with packing defects by inserting  
1192 hydrophobic residues into the membrane. In animal cells, high packing defects are found in  
1193 ER and cis-Golgi membrane while TGN and PM present low level of packing defects except  
1194 in highly curved-membranes. The relevance of lipid packing defects for protein recruitment in  
1195 plants is largely unexplored.

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- 1197 • **Terms and Definitions list:** *provide definitions for as many as 20 of the most important*  
 1198 *abbreviations or key terms, limited to 20 words maximum; insert below Literature Cited*  
 1199 *section*
- 1200 **Endomembrane system:** membranes system connected directly or through vesicular  
 1201 transport including nuclear envelop, ER, Golgi, PM, endosomes/vesicles,  
 1202 lysosomes/tonoplast, but not chloroplasts/mitochondria.
- 1203 **Lipid Binding Domain (LBD):** Protein domain that selectively recognize and target  
 1204 membranes via direct interactions with lipid(s) either via specific, non-specific and/or  
 1205 multivalent interactions.
- 1206 **Electrostatic field:** Field created by electric charges that exert forces able to attract or repel  
 1207 other charges entering the field.
- 1208 **Electrostatic membrane territory:** Within the endomembrane system, corresponds to  
 1209 negatively-charged membranes, whose cytosolic leaflet harbours anionic phospholipids (Post-  
 1210 Golgi compartments), as opposed to neutral membranes (ER-derived).
- 1211 **Nanodomain or nanocluster:** Small membrane zone below 100 nm, enriched in one or  
 1212 several lipid species or proteins.
- 1213 **Membrane Contact site (MCS):** Close (below 30nm) and stable apposition via tethering  
 1214 elements of two membranes allowing inter-organelle communication, without membrane  
 1215 fusion.
- 1216 **Protein sorting:** Delivery process of proteins to their appropriate cellular destination, based  
 1217 on information contained in the protein sequence and/or structure.
- 1218 **Polar secretion:** Delivery of proteins or material steered in one or several specific directions  
 1219 within the cell.
- 1220 **Cell plate:** Organelle formed by delivery of cell wall and membranes by Golgi-derived  
 1221 vesicles at the plane of cell division in plants.
- 1222 **trans-Golgi Network/Early endosome (TGN/EE):** Golgi-derived but Golgi-independent  
 1223 compartment involved in secretion, which also serves as early endosome in plants and  
 1224 involved in endocytic trafficking.
- 1225 **Secretory vesicles (SV):** specific subdomain of the TGN/EE made of uncoated vesicles  
 1226 involved in secretion.
- 1227 **Multivesicular bodies (MVB):** late endosome characterized by their spherical morphology,  
 1228 and containing intraluminal vesicles destined for vacuolar degradation.
- 1229 **Plasmodesmata (PD):** pores that connects plant cells by passing through their cell wall  
 1230 thereby allowing cytoplasmic, PM and ER continuity from cell-to-cell.
- 1231 **Phagophore:** a double membrane that encloses and isolates cytoplasmic components during  
 1232 autophagy.
- 1233 **Coincidence binding:** Recruitment of a protein at a specific membrane by interaction with

- 1234 several membrane landmarks, which together provides high targeting specificity.
- 1235 **Lipid transfer protein (LTP):** Proteins responsible for shuttling lipids between different  
1236 membranes via non-vesicular trafficking.
- 1237 **Flippase:** membrane transporters which translocates phospholipids from the luminal to the  
1238 cytosolic membrane leaflet, thereby maintaining lipid asymmetry across the bilayer.
- 1239 **Clathrin-coated vesicle (CCV):** vesicles decorated by a membrane-deforming Clathrin coat,  
1240 involved in many vesicular-mediated trafficking pathway within the cell.
- 1241 **Clathrin-coated pit (CCP):** clathrin-coated vesicles at the PM, corresponding to the initial  
1242 step of membrane deformation during endocytosis.
- 1243 **Tethering complex:** protein complex which brings together two membranes, either  
1244 transiently before fusion or in a more sustained manner at contact sites.
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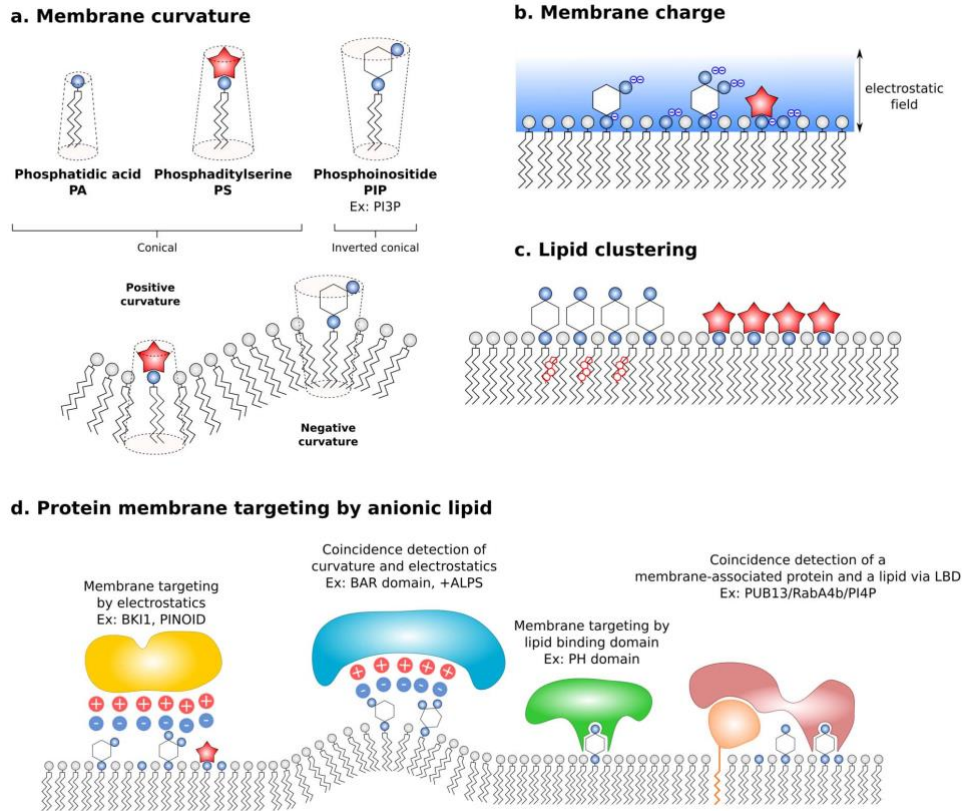


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**Figure 1: Anionic lipids.**

(a) Skeletal formula and schematic representation of anionic lipids: phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphoinositide (PIP). Anionic lipids

1253 are composed of a diacylglycerol backbone attached to a phosphate group. PS and PI display a  
1254 serine (star) and an inositol ring (hexagon), respectively, linked to the glycerol by a  
1255 phosphodiester linkage. PI can be phosphorylated in position 3, 4 and 5 to form 7 different  
1256 PIPs: Phosphatidylinositol 3-phosphate (PtdIns3P or PI(3)P), Phosphatidylinositol 4-  
1257 phosphate (PtdIns4P or PI(4)P), Phosphatidylinositol 5-phosphate (PtdIns5P or PI(5)P),  
1258 Phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub> or PI(3,4)P<sub>2</sub>), Phosphatidylinositol 3,5-  
1259 bisphosphate (PtdIns(3,5)P<sub>2</sub> or PI(3,5)P<sub>2</sub>), Phosphatidylinositol 4,5-bisphosphate  
1260 (PtdIns(4,5)P<sub>2</sub>, PI(4,5)P<sub>2</sub> or PIP<sub>2</sub>), Phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>  
1261 or PI(3,4,5)P<sub>3</sub>). Pathways for PA (**b**), PS (**c**), PI and PIP (**d**) production and degradation. PC,  
1262 phosphatidylcholine; PE, phosphatidylethanolamine; DAG, diacylglycerol; DGPP,  
1263 Diacylglycerol pyrophosphate; Gly3P, Glycerol 3-phosphate; PLC, Phospholipase C; PLD,  
1264 Phospholipase D; DGK, Diacylglycerol kinase; PAP, Phosphatidate phosphatase; PAK,  
1265 PA Kinase; LPP, lipid-phosphate phosphatase; LPAAT, lyso-phosphatidic acid  
1266 acyltransferase; GPAT, Glycerol-3-phosphate acyltransferase; PSS1, PS Synthase1; CMP-  
1267 PA, Cytidine Monophosphoryl-Phosphatidic Acid; Ins, Inositol; SAC, SUPPRESSOR OF  
1268 ACTIN; PTEN, PHOPHATASE AND TENSIN Homolog; FAB1, FORMATION OF  
1269 APLOID AND BINUCLEATE CELLS 1; RHD4, ROOT HAIR DEFECTIVE 4; CVP2,  
1270 COTYLEDON VASCULAR PATTERN 2; CVL1, CVP2-LIKE1; PI4K, PI 4-Kinases.  
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**Figure 2: Physicochemical properties of membranes and membrane targeting by anionic lipids.**

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Anionic lipids can confer several properties to the membranes. **(a) Curvature:** The geometry of the head group of anionic lipids gives a conic shape to the lipid which influences positively (in the case of PIP) or negatively (in the case of PA and PS) the curvature of the membrane.

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**(b) Electrostatics or membrane surface charge:** Accumulation of anionic lipids confers a negative charge to the membrane leading to an electrostatic field. **(c) Clustering:** Anionic lipids can cluster together to form nanodomains in which one or several lipid species accumulate.

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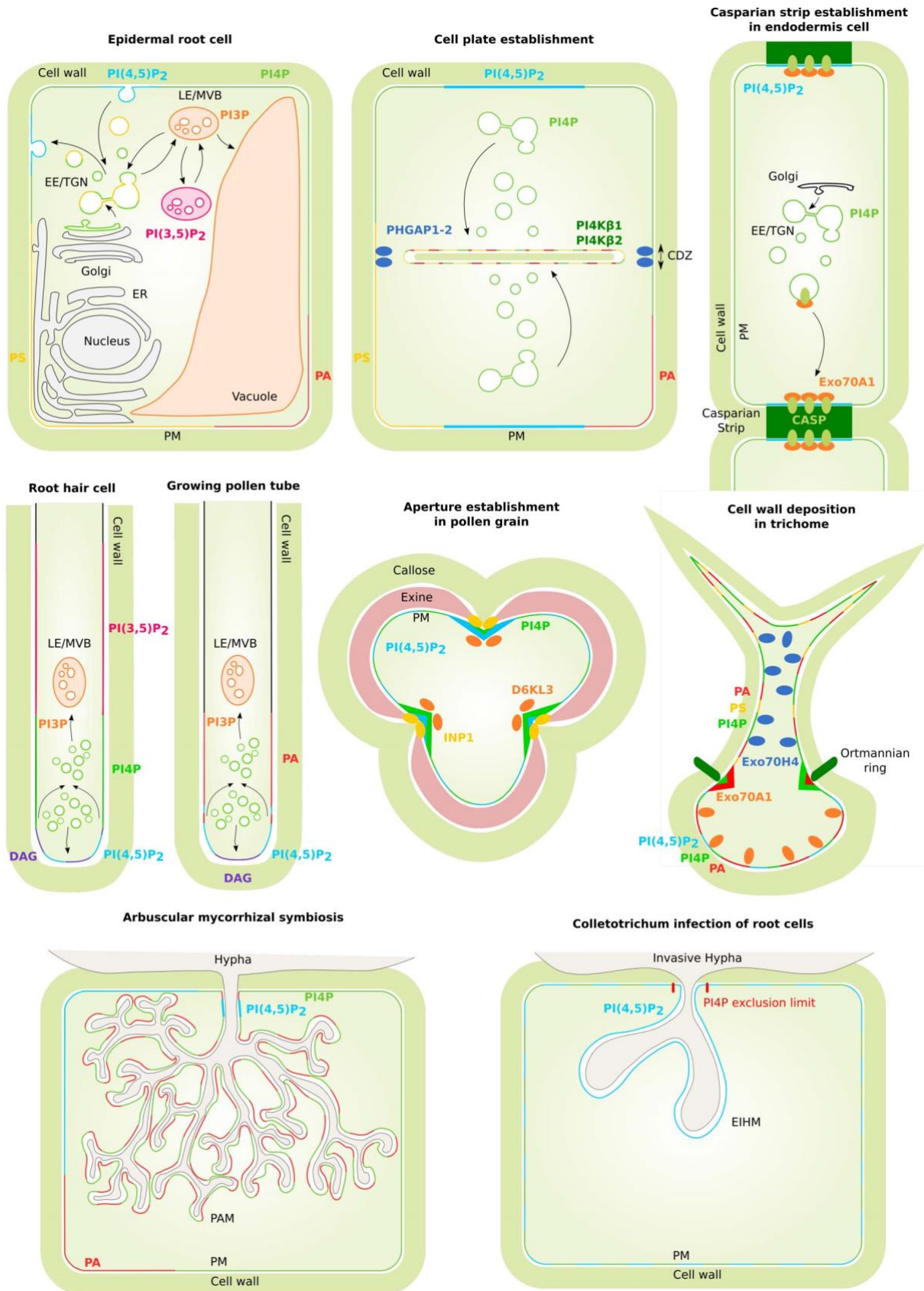
**(d) These properties can directly participate in the recruitment of proteins to the membrane through electrostatic interactions between the negative charges of the lipids and the positive charged amino acids, curvature recognition or lipid-binding domain that targets a specific lipid. In some cases, proteins can be recruited to the membrane by coincidence detection of several membrane features (electrostatics and curvature, or an anionic lipid and a membrane-associated protein for instance).**

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Note that for clarity purpose only the cytoplasmic membrane leaflet was drawn. BKI1, BKI1 KINASE INHIBITOR 1; BAR, Bib-Amphiphysin-Rsv; +ALPS, Amphipathic Lipid Packing Sensor; PH, Pleckstrin Homology; PUB13, PLANT-U-BOX13; PA, Phosphatidic Acid; PS, Phosphatidylserine; PIP, Phosphoinositide; PI3P, Phosphatidylinositol 3-phosphate; PI4P, Phosphatidylinositol 4-phosphate.

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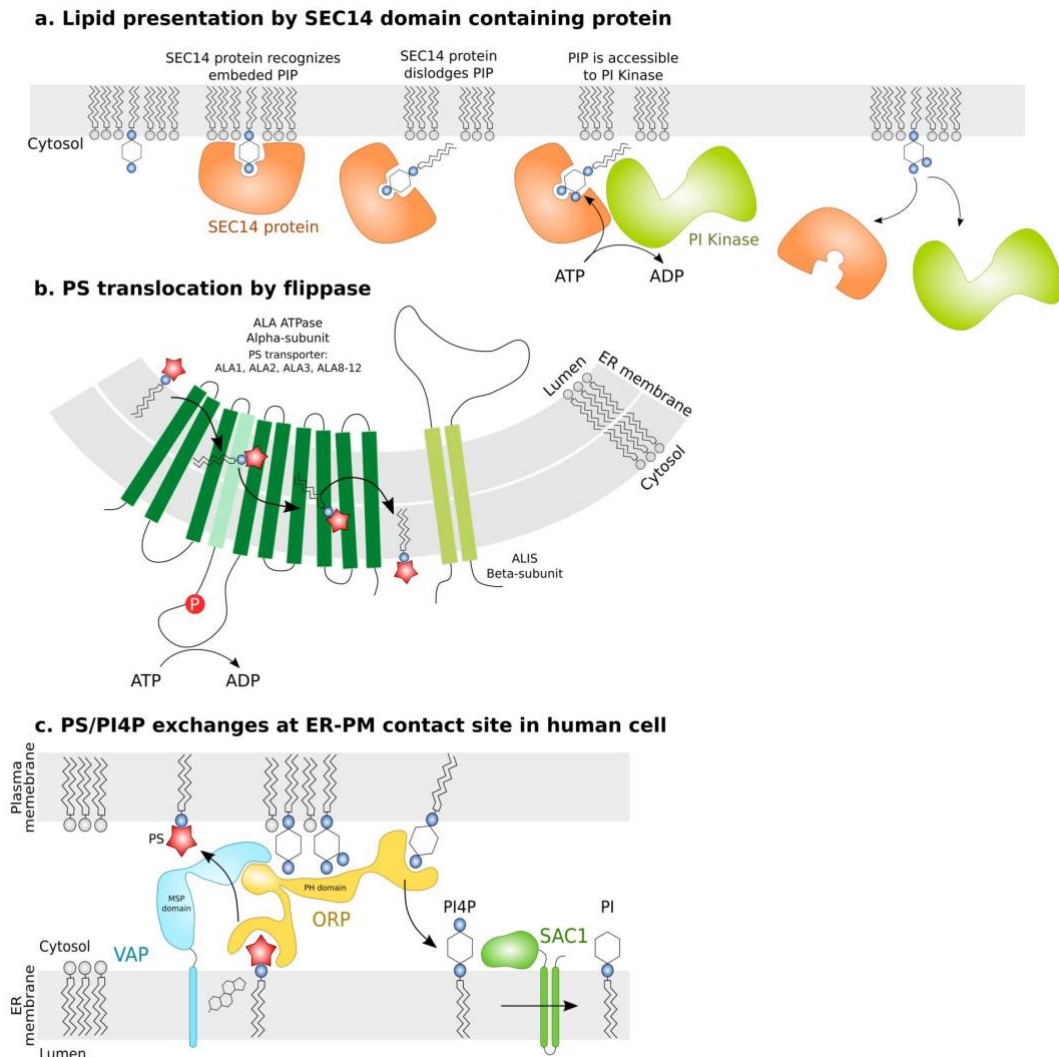
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**Figure 3: Anionic lipid distribution in plant cells.**

The localization of anionic lipids in between the different cellular compartment can change depending of the developmental and physiological context. Here, the distribution of anionic lipids in epidermal root cells is first represented. Anionic lipids can accumulate in specific

1298 zone of the PM during polarized cell growth (pollen tube, root hair cell for instance), when  
1299 interacting with another organism (symbiosis, infection), to determine the position of  
1300 developmental structure (aperture of the pollen grains, casparian strip, trichomes). PM,  
1301 plasma Membrane; EE/TGN, Early Endosome/Trans-Golgi Network; LE/MVB, Late  
1302 Endosome/Multi Vesicular Bodies; ER, Endoplasmic Reticulum; PAM, PeriArbuscular  
1303 Membrane; EIHM, Extra Invasive Hyphal Membrane; PA, Phosphatidic Acid; PS,  
1304 Phosphatidylserine; PI3P, Phosphatidylinositol 3-phosphate; PI4P, Phosphatidylinositol 4-  
1305 phosphate; PI(4,5)P<sub>2</sub>, Phosphatidylinositol 4,5-bisphosphate; DAG, Diacylglycerol; PI4K,;  
1306 PI4P Kinases; PHGAP, Pleckstrin Homology GTPase Proteins; D6KL3, D6 PROTEIN  
1307 KINASE-LIKE3; INE1, INAPERTURATE POLLEN1; CASP, CASPARIAN STRIP  
1308 MEMBRANE DOMAIN PROTEIN; Exo70, Exocyst Complex Component 70.  
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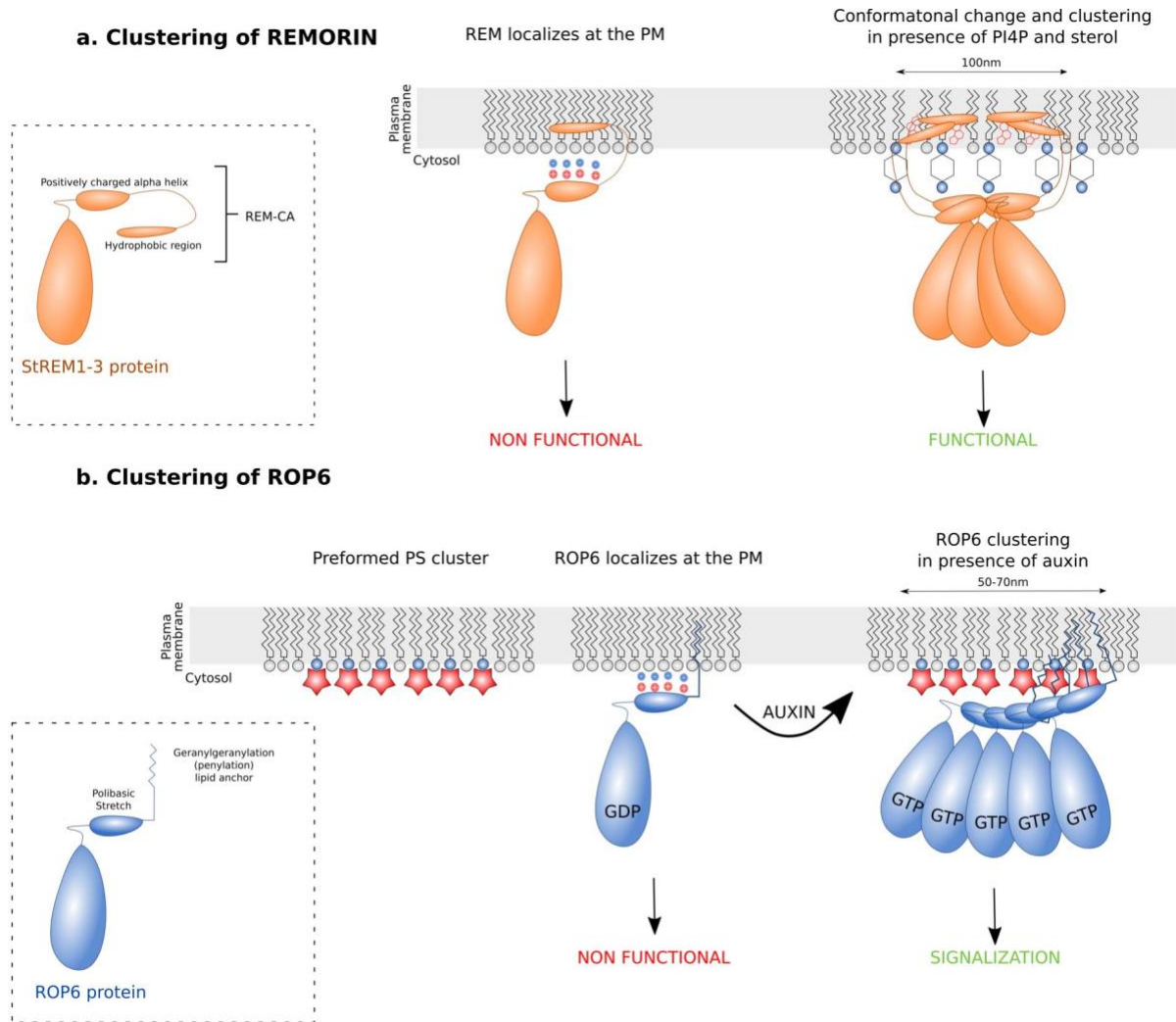
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**Figure 4: Mechanisms of lipid presentation and transport.**

(a) A mechanism of PIP presentation has been proposed to explain the high processive of PI-kinases. In this model, SEC14 protein digs up PI or PIP from the membrane and presents it to the PI-kinase for phosphorylation. (b) The translocation of PS from the luminal leaflet to the cytosolic leaflet of the ER membrane depends on flippase proteins composed of a alpha subunit called ALA which hydrolyses ATP and translocate PS and a  $\beta$ -subunit called ALIS which specify the localization of the flippase at different membrane compartments and is essential for activity. (c) Lipid exchanges take place at membrane contact sites. In human, ORP5 and 8 tether the ER and the PM by direct interaction with the ER-resident protein VAP and the PM-localized lipid PI4P. ORP5/8 then transfer PS from the ER membrane to the PM via its lipid transfer domain and then counter transport PI4P to the ER. PI4P is then dephosphorylated by SAC1 in the ER membrane maintaining the PI4P gradient between the two membranes. Note that for clarity purpose in panel a and c only the cytoplasmic membrane leaflet was drawn. ER, Endoplasmic Reticulum; EPCS; ER Plasma membrane Contact Site; PS, Phosphatidylserine; PI, Phosphatidylinositol; PIP, Phosphoinositide; SEC14; ALA, aminophospholipid flippase; ALIS, ALA interacting  $\beta$ -subunit; VAP, VAMP Associated Protein; SAC1, SUPPRESSOR OF ACTIN 1; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; ORP, OSBP-Related Protein; VAP, vesicle-associated membrane protein-associated proteins.



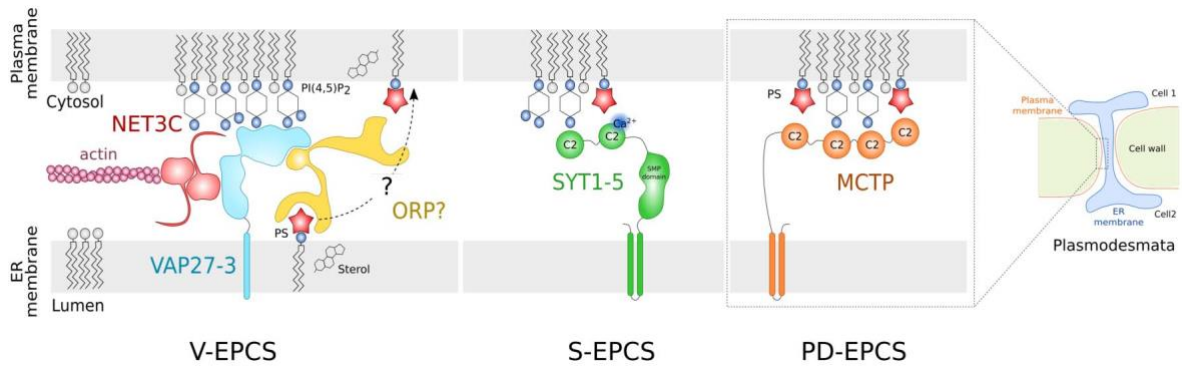
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**Figure 5: Protein/lipid clustering into nanodomains**

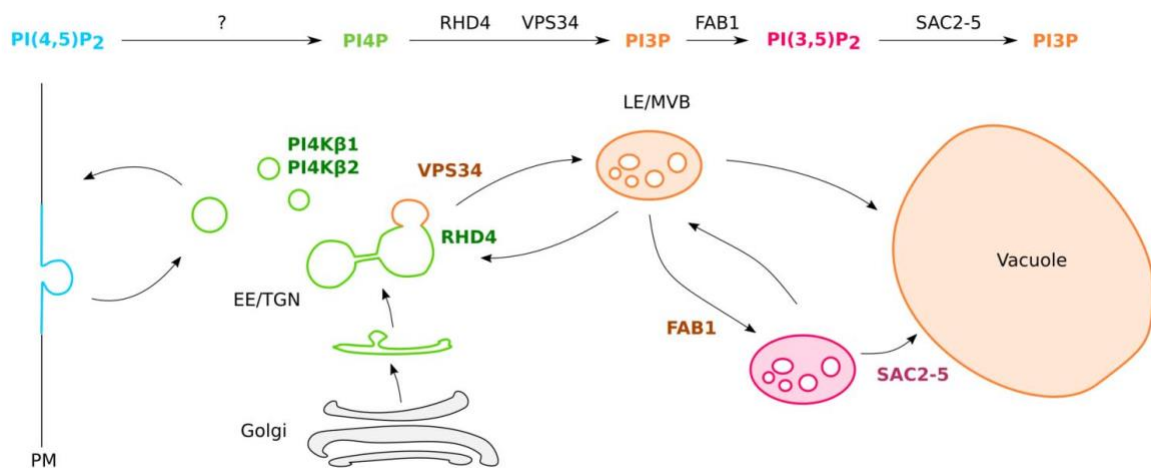
Lipids can accumulate in small regions of the membrane to form nanoclusters in which proteins are constrained. (a) StREM1.3 localizes at the PM through electrostatic interactions thanks to a positively charged alpha helix and hydrophobic interactions via a hydrophobic region that is embedded in the membrane. In presence of PI4P and cholesterol, StREM1.3 undergoes a conformational change that leads to its clustering. The distribution of StREM1.3 in cluster appears necessary to fulfill its function. (b) ROP6 localizes at the PM thanks to a polybasic patch and a lipid anchor. Upon auxin signaling, GTP-bound ROP6 relocates in pre-existing PS clusters. The clustering of ROP6 into PS nanodomains is necessary for ROP6 signaling. PM, Plasma Membrane; REM, REMORIN; ROP6, Rho-of-Plant 6; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; PS, Phosphatidylserine; PI4P, Phosphatidylinositol 4-phosphate.



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**Figure 6: ER-PM Membrane Contact Sites in plant**

Schematic representation of the three different classes of EPCS in plant and their relation with anionic lipids. Note that for clarity purpose only the cytoplasmic membrane leaflet was drawn. ER, Endoplasmic Reticulum; EPCS; ER Plasma membrane Contact Site; PD, Plasmodesmata; PS, Phosphatidylserine; PI(4,5)P<sub>2</sub>, Phosphatidylinositol 4,5-bisphosphate; VAP27, VAMP Associated Protein 27; ORP, OSBP-Related Protein; NET3C, NETWORKED 3C; VAP27, vesicle-associated membrane protein-associated proteins; SYT, SYNAPTOTAGMINE; MCTP, MULTIPLE C2 AND TRANSMEMBRANE PROTEIN, SMP, Synaptotagmin-like mitochondrial-lipid-binding domain.



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**Figure 7: Phosphoinositide cascade during endocytic trafficking to the vacuole**

Kinases and phosphatases create a phosphoinositide cascade along the endocytic route. PM, plasma Membrane; EE/TGN, Early Endosome/Trans-Golgi Network; LE/MVB, Late Endosome/Multi Vesicular Bodies; PI3P, Phosphatidylinositol 3-phosphate; PI4P, Phosphatidylinositol 4-phosphate; PI(4,5)P<sub>2</sub>, Phosphatidylinositol 4,5-bisphosphate; PI(3,5)P<sub>2</sub>, Phosphatidylinositol 3,5-bisphosphate; PI4K., PI4P Kinases; RHD4, ROOT HAIR DEFECTIVE 4; FAB1, FORMATION OF APLOID AND BINUCLEATE CELLS 1; SAC, SUPPRESSOR OF ACTIN.