1 **Functions of anionic lipids in plants**

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Abstract: (150 words max)

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

Keywords: (6 max)

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

Main text

1. INTRODUCTION

Biological membranes delimit cellular compartments, as well as the inner and outer of the cell. Each compartment has its proper chemical environment allowing numerous and specialized chemical reactions in the same cell. To enable the proper localization of proteins, each membrane has its own biochemical and biophysical identity. Membranes are composed of lipids forming a bilayer and transmembrane or membrane-associated proteins (63). Glycerophospholipids associated with sphingolipids and sterols are the main lipid components within the eukaryotic **endomembrane system**. Among the glycerophospholipids some present negative charges and, as such, are anionic phospholipids. Anionic phospholipids include phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol and phosphatidylinositol phosphates (PIPs also referred to as phosphoinositides), the phosphorylated forms of phosphatidylinositol (Figure 1.a). Phosphatidylinositol display an inositol ring that can be phosphorylated in three different positions - 3, 4 and 5 - creating a total of seven possible phosphoinositide species: 3 phosphoinositide monophosphates (PI3P, PI4P, PI5P), 3 phosphoinositide biphosphates (PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂) and one phosphoinositide triphosphate (PI(3,4,5)P₃).

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

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

It should be noted that anionic lipids also accumulate in mitochondria and plastids where they play a role in division, lipid synthesis and photosynthesis. Anionic lipids, such as PA, PI4P and PI(4,5)P₂ are also found in the nucleoplasm of the nucleus where they may regulate transcription factors, chromatin status and epigenetic landmarks (125) but this will not be further discussed, as we decided to focus this review on the endomembrane system.

2. ANIONIC LIPIDS ARE DYNAMIC LANDMARKS OF BIOLOGICAL MEMBRANES

2.1. Anionic phospholipids as landmarks for organelle identity

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

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

The combined study of the localization of anionic lipid generating enzymes and genetically encoded lipid sensors suggest that in Arabidopsis epidermal root cells, the PM accumulates PI4P, PI(4,5)P₂, PA and PS (Figure 3) (93, 112, 113, 127, 128). TGN/EE are enriched in PI4P, PS and to a lesser extent PI3P (93, 113, 114, 128). LE/MVBs accumulate both PI3P and PI(3,5)P₂, although both lipids localize in distinct compartments (37, 38, 112, 129). Finally, the tonoplast and autophagosomes carry PI3P (112, 129). However, this subcellular accumulation pattern may vary according to the cell type or the stress status of the cell (Figure 3) (34, 41, 44, 48, 57 in revision, 61, 110, 115). For example, PI(3,5)P₂ is found in LE/MVBs in most cell type but accumulates at the PM in elongating root hairs (34) (Figure 3). PI(4,5)P₂ and PA are constitutively produced at the PM at low or moderate levels, respectively, in resting condignition but upon stimulation, such as salt stress, they can be massively produced, which triggers specific stress responses (82, 121, 127). Alternatively, PI(4,5)P₂ production can be inhibited by external stimuli such as pathogen-associated molecular patterns (80).

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

2.2.1. PA production and stress responses

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

2.2.2. PIPs and rapid inter-conversion

The phosphorylation of the inositol ring of phosphatidylinositol is mediated by PI-kinases that are specific of the phosphate position (Figure 1.d). In plants, the genome does not encode for type-I and type-II PI3-kinases (PI3Ks), able to phosphorylate PI4P or PI(4,5)P₂ (84). As a consequence, PI(3,4)P₂ and PI(3,4,5)P₃ likely do not exist and have not been detected in plants. The dephosphorylation of PIPs is mediated by PI-phosphatases that belong in plants to three main families, the SUPPRESSOR OF ACTIN (SAC) domain-containing proteins (SAC1 to SAC9), the 5PTase family (5PTase1 to 15) and the PHOPHATASE AND TENSIN Homolog (PTEN) family (PTEN1 to 3) (24) (Figure 1.d). PI-kinases and phosphatases are highly processive enzymes *in*

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

In vitro experiments testing PI-kinases activity often require the use of detergents, which destroy liposomal structures and free lipid substrates in order to increase kinase activity (52). These results suggest that PI-kinases have difficulties to recognize their substrate embedded into the membrane and might get “help” *in planta*. The main candidates to fulfil this function are the SEC14 lipid transfer proteins. The yeast Sec14 protein is able to transfer phosphatidylcholine and phosphatidylinositol from distinct membranes *in vitro*, possibly by-passing the classical vesicular transport of lipids (52, 103). However, the current model, based on crystallographic data, advocates the idea that Sec14 dislodges the phosphatidylinositol head from the TGN membrane making it accessible for PI4-kinases (Figure 4.a). Based on sequence homology, Arabidopsis contains 32 Sec Fourteen Homologs (AtSFHs), each containing a highly conserved sec14 domain. The sec14 domain of several AtSFHs displays the same *in vitro* activity than the yeast sec14 and can rescue the sec14 yeast mutant (40, 130). So far, the best-characterized AtSFH protein is AtSFH1. AtSFH1 belongs to a subclass of 13 members that contains a Nlj16 domain in C-terminal. This domain has two functions. It contains a polybasic patch that localizes at the PM in a PI(4,5)P₂-dependent manner and a coiled-coiled domain that mediates homo-oligomerization (25). The model built with the combination of yeast and Arabidopsis data is that AtSFH1 is targeted at the root hair tip PM by PI(4,5)P₂ where it stimulates the activity of PI-kinases (PI4K or PIP5K), thereby enhancing the production of PIPs (directly PI(4,5)P₂ or PI4P that will then be converted into PI(4,5)P₂). PI(4,5)P₂ recruit new AtSFH1 which oligomerizes and further creates PI(4,5)P₂ clusters at the root hair tip PM (Figure 3).

2.3. PS synthesis and trafficking

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

2.3.1. Flippases and vesicular trafficking

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

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 brought 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.

3. ANIONIC LIPIDS AS DETERMINANT OF MEMBRANE PHYSICOCHEMICAL PROPERTIES

3.1. Surface charges

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)P₂. 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 contains 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.

3.2. Curvature

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

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

3.3. Clustering

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

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

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

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

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

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

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

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

models are not mutually exclusive.

4.1. V-EPCS

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

4.2. S-EPCS

The mammalian E-SYT1 is able *in vitro* to trigger glycerolipids exchanges between liposomes (105). E-SYTs contain C2 domains that bind anionic phospholipids from the PM in a Ca²⁺ depend manner (144). In favour of the tethering arrangement model, E-SYTs Ca²⁺ binding leads to clustering of E-SYTs proteins and an increase of the interactions between E-SYTs and PM anionic lipids (26). This results in stabilization and tightening of S-EPCS (26, 138, 144). In Arabidopsis, ionic stress triggers the expansion of S-EPCS in association with accumulation of PI(4,5)P₂ at the PM supporting the tethering arrangement model (62). Furthermore, the C2 domains of Arabidopsis SYT1 bind PS in a Ca²⁺ dependent-manner *in vitro* (90) (Figure 6). The relevance of this Ca²⁺-dependent PS binding *in vivo* is currently unknown but also argues in favour of the tethering arrangement model.

4.3. PD-EPCS

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

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

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

5. TRAFFICKING BETWEEN THE PM AND THE TGN

5.1. Clathrin mediated endocytosis

In animal cells, it is well documented that anionic lipids play a fundamental role in endocytosis. Clathrin-mediated endocytosis mainly involves PI(4,5)P₂ produced from PI4P by PIP5P kinases (PI4P-5Ks) (81). PI(4,5)P₂ allows the initiation and stabilisation of the **clathrin-coated pits** by the recruitment of several components of the endocytic machinery including the adaptor protein AP2 (51, 95). Moreover, PIP5Ks are directly recruited at the site of endocytosis by AP2, creating a local pool of PI(4,5)P₂, which induces a high rate of

clathrin-coated pit nucleation (81). Then, both PI(4,5)P₂ and PI(3,4)P₂ are involved in pit maturation (81).

5.1.1. PI(4,5)P₂ a main actor in clathrin-mediated endocytosis

In plant cells, the recruitment of the endocytic machinery to the PM is far less understood. However, the role of PI(4,5)P₂ in endocytosis has been demonstrated indirectly using PIP5Ks over-expression and loss-of-function approaches (43, 146). Notably, a *pip5k1pip5k2* (hereafter designed as *pip5k1k2*) mutant has abnormal **clathrin-coated pits**, which are less dense than in the wild type but bigger (43). This mutant, as well as PIP5K1 and PIP5K2 overexpression line, have defects in PIN endocytic trafficking, which ultimately impact both their accumulation at the PM, their polar targeting and hence auxin distribution, leading to various degrees of auxin-related developmental defects (43, 79, 120). Over-expression of PIP5K also induces a phloem differentiation phenotype (30). PIP5K overexpression redirects the trafficking toward the vacuole leading to protein degradation and vacuole biogenesis, which in protophloem file induces vascular discontinuities (30). However, it is unclear if this increase in vacuolar trafficking is caused by an overall heightened endocytic rate at the PM or by regulation of downstream trafficking steps. In any case, PI(4,5)P₂ levels are constantly kept in check in the root phloem by two PM-localized PI(4,5)P₂ 5-phosphatases, COTYLEDON VASCULAR PATTERN 2 (CVP2) and CVP2-LIKE 1, whose double mutant shows similar phloem discontinuity as PIP5K overexpression lines (101).

In growing pollen tube, the exocytosis at the tip is balanced by endocytosis in the subapical regions (Figure 3). These regions are marked by PI(4,5)P₂ locally produced by PIP5Ks and restricted by degradation into diacylglycerol (DAG) by Phosphoinositide Phospholipase C (PI-PLC) (17, 32) (Figure 3). Loss-of function mutants of PIP5Ks show reduced endocytosis, which lead to low pollen germination rate and slow tube elongation (41, 115). Conversely,

pollens overexpressing PIP5K6 show PM invagination and the formation of several tip branches due to a higher rate of endocytosis at the tip of pollen tube (146). The expression of a clathrin dominant negative can rescue the PIP5K6-OE phenotype indicating that PI(4,5)P₂ synthesis enhances clathrin-mediated endocytosis (146). A similar mechanism was proposed in responses to salt treatment which increases the level of PI(4,5)P₂ and induces the formation of **clathrin-coated vesicles** in a PIP5K1 and PI5PK2-dependent manner (43, 56).

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

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

To conclude, a role for PI(4,5)P₂ in clathrin-mediated endocytosis is established in different cell types and developmental contexts. Nevertheless, the proteins regulated by PI(4,5)P₂ during endocytosis are still largely unknown. In addition, the role of other anionic lipids such as PI4P and PA in the regulation of the endocytic machinery needs to be clarified.

5.2. Polar secretion and exocytosis: the targeting of the exocyst

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

5.2.1. PI(4,5)P₂ targets the Exocyst to the PM

The exocyst complex is targeted to the PM via two subunits, SEC3 and EXO70 known to bind anionic phospholipids, in particular PI(4,5)P₂. In Arabidopsis, SEC3 contains a PH domain that binds to PI(4,5)P₂ *in vitro* (8). The extensive use of biosensors and the localization of lipid metabolic enzymes in plants allowed the cartography of anionic lipids in cells exhibiting polar secretion highlighting specific lipid accumulation patterns, notably for PI(4,5)P₂ (Figure 3) (41, 42, 57, 60, 61, 115, 117, 146). Interestingly, the localizations of SEC3 and/or EXO70 isoforms often correlate with the PI(4,5)P₂ subcellular accumulation pattern, suggesting that PI(4,5)P₂ contribute to their localization (8, 48, 108). In pollen tube, SEC3 and EXO70A1 partially colocalize with PI(4,5)P₂ biosensors (8, 108). Since PIP5K mutants display slower tube elongation and reduced secretion of cell wall materials (41, 115), it is likely that PI(4,5)P₂ participates in targeting exocyst complexes to specific PM domains.

5.2.2. PI(4,5)P₂ and PA: working together to confine exocytosis

EXO70A1 and EXO70B1 are expressed in growing pollen tube and partially colocalize with both PI(4,5)P₂ and PA biosensors (108). Therefore, it is possible that the coincidence detection of PI(4,5)P₂ and PA in pollen tube restricts the site of exocytosis to the narrow PM strip that contains both lipids (Figure 3). These EXO70 subunits may directly recognize each

lipid. Alternatively, they may be sensitive to the overall membrane surface charges. One may speculate that in pollen tube, the PM membrane domain that contains a combination of both PI(4,5)P₂ and PA is highly electronegative and thereby could recruit specific EXO70 isoforms. Additional experiments are required to distinguish between these two possibilities.

5.3. Proper secretion requires PI4P production in the TGN

Exocytosis not only requires specific anionic lipids at the PM but also a regulation of the lipid composition in the TGN for proper vesicle formation. Indeed, root hair studies revealed the importance of the pool of PI4P in the TGN for polar secretion (49, 99, 122, 143). PI4Kinases, PI4Kβ1 and PI4Kβ2, as well as the PI4P phosphatase ROOT HAIR DEFECTIVE 4, RHD4/SAC7, localize to the TGN. *pi4kβ1β2* and *rh4* mutants display short or bulged root hairs (49, 99, 122). Ultrastructural studies by electron tomography showed the presence of enlarged, likely deficient, **secretory vesicles** in the TGN of *pi4kβ1β2* double mutant, suggesting that PI4Kβs are indeed involved in secretion (49). PI4Kβs and RHD4/SAC7 have opposite catalytic activities, yet apparently, locate to the same compartment. It is possible that PI4Kβs and RHD4/SAC7 localize in different TGN subdomains. Consistent with this scenario, PI4Kβ1 localizes specifically in the secretory vesicle subdomain of the TGN (49). According to this hypothesis, PI4Kinases could be necessary for the production of the pool of PI4P at the TGN which confers the proper physical properties (mild electrostatics, high curvature) necessary for secretory vesicle formation. By contrast, RHD4/SAC7 could localize in a different TGN subdomain, perhaps directly connected to endocytosis. In such model, RHD4/SAC7 would degrade incoming PI4P from the PM to maintain the PI4P gradient along the endocytic pathway (113).

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

The cell plate is an organelle formed from the secretion and fusion of vesicles from the TGN and will form the new PM of the two daughter cells (13). A combination of clathrin-mediated endocytosis and exocytosis occurs at the cell plate. During this process, the lipid composition of the cell plate varies from the bulk PM. Lipid binding biosensors suggest that PI4P, PA and PS accumulate and confer a highly electronegative field at the cell plate to a similar extent, or even higher, than at the PM (16, 93, 113) (Figure 3). However, PI(4,5)P₂ is excluded from the cell plate during its creation and extension and appears at the edges of the cell plate just before its full attachment to the PM (16, 113, 127). Knowing that PI(4,5)P₂ is a major regulator of both exocytosis and endocytosis, it is therefore likely that specific trafficking mechanisms occur at the cell plate compared to the bulk PM, which have yet to be uncovered. This difference of membrane composition between the bulk PM and the cell plate is crucial for proper cell division. Indeed, several mutants for enzymes involved in anionic lipid homeostasis show cell plate and/or cytokinesis phenotypes. Those include mutants for PI4P production (*pi4kβ1β2*), PI4P/PI(4,5)P₂ interconversion (*sac9*, *pi5k1k2*), and PI3P/PI(3,5)P₂ interconversion (*fab1afab1b*, *fra7/sac1*) (49, 120, 131, 137, 148). Some of these enzymes directly act at the cell plate. For instance, PI4Kβ1 localizes at the cell plate and the absence of PI4Kβ1 and PI4Kβ2 directly affect the vesicular trafficking along phragmoplast microtubules (68). However, others might act indirectly on cell division, such as FORMATION OF APLOID AND BINUCLEATE CELLS 1, FAB1A and FAB1B proteins, that do not localize at the cell plate (36, 137). It is therefore likely that the *fab1afab1b* cell plate phenotype results from a broad alteration of membrane trafficking. We refer the reader to a recent review on this subject, which details the potential involvement of each anionic lipid during cytokinesis (13).

5.6. Anionic phospholipid remodelling at the Plants-Fungi interface

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

5.6.1. Periarbuscular membrane

During the endosymbiotic association of roots with arbuscular mycorrhizal (AM) fungi in *M. truncatula*, the fungus develops intracellular branched hyphae called arbuscules (44). In response, the root cortical cells form a new membrane in continuity with the PM called periarbuscular membrane (PAM) (31). The PAM envelops the hyphal branches and is thought to be generated by polar exocytosis. Indeed, subunit of the exocyst complex and SNARE proteins known to be involved in exocytosis and membrane fusion are required for the PAM formation (31, 145). By analogy to tip growing cells, anionic lipids might regulate this particular case of polarized secretion. So far, only the pattern of localization of PI4P, PI(4,5)P₂ and PA were documented during AM interaction (44) (Figure 3). Both PI4P and PA are found in the PAM to similar extent than the PM. However, in the trunk part of hyphal branches, discrete regions where PI(4,5)P₂, PI4P and PA reporters co-accumulate can be observed (44). This accumulation could correspond to an accumulation of membranes before formation of small bulges. Furthermore, anionic lipids accumulation could influence membrane curvature and thereby physically contribute to the establishment of the PAM.

5.6.2. Extra invasive hyphal membrane

Using another model of fungi-plant interactions, it was recently shown in *Arabidopsis* infected by *Colletotrichum* that extra invasive hyphal membrane (EIHM), which is formed in continuity with the PM, strongly accumulates PI(4,5)P₂ and PIP5K3 all along the membrane (Figure 3), as well as a subunit of the exocyst complex and SNARE proteins (39, 109, 110). This accumulation of PI(4,5)P₂ seems to be specific to *Colletotrichum* (110). PI(4,5)P₂

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

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

6. ANIONIC LIPIDS IN VACUOLAR TRAFFICKING

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

6.1. From PI4P to PI3P and PI(3,5)P₂: PIP cascade leading to protein sorting

One of the main characteristics of PIPs is to be quickly interconverted by kinases and phosphatases (Figure 1.c). This is well illustrated along the road leading to protein degradation into the vacuole in which the different maturation steps correlate with a PIP interconversion (Figure 7). PI(4,5)P₂ from the PM is converted into PI4P by 5-phosphatases either in TGN/EEs or during the endocytic process. At the surface of TGN/EEs, PI4P is gradually replaced by PI3P through the combined action of 4-phosphatases (e.g. RHD4/SAC7 in root hair) and the PI3K, VPS34. Some MVBs exhibit PI(3,5)P₂ due to the action of the

PI3P5K from the FAB1 family (36, 38, 112) (Figure 7). The acquisition of PI3P is essential for **protein sorting**. Indeed, FYVE1/FREE1, a component of the ESCRT complex that binds ubiquitinated cargo, localizes in the MVB by interacting with PI3P (22, 55). Similarly, SORTING NEXIN 1 (SNX1), a component of the retromer complex also localizes at the MVB through interactions with PI3P and/or PI(3,5)P₂ (98). In addition, PI3P and/or PI(3,5)P₂ also mediate the association of endosomes with the cytoskeleton, since the loss of FAB1 releases MVBs from cortical microtubules (35, 36) and SNX1 interact with the microtubule regulator CYTOPLASMIC LINKER ASSOCIATED PROTEIN, CLASP (1).

6.2. Autophagosome and protein degradation

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

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

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

6.3. PIPs and vacuole morphology

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

vacuoles (147). The homotypic fusion and vacuolar protein sorting (HOPS) tethering complex is required for vacuole morphology (12). VACUOLAR PROTEIN SORTING33 (VPS33) and VPS45, two HOPS complex members, binds phosphoinositide in vitro and localize to the vacuole in a PI3P-dependent manner (12). SAC2, SAC3, SAC4 and SAC5 are likely PI(3,5)P₂ 5-phosphatases that localize at the tonoplast and could hydrolyse the incoming PI(3,5)P₂ from MVB into PI3P (87) (Figure 7). Loss-of-function and overexpression of these SAC proteins lead to abnormal vacuolar morphology consistent with deficit in vacuole fusion due to lack of PI3P (87). PI4P is also involved in the morphological changes of the vacuole that are regulated by auxin in the root (69, 106). However, the molecular mechanism connecting PI4P to vacuole morphology are so far unknown.

7. CONCLUSIONS, PERSPECTIVES AND FUTURE CHALLENGES

Many aspects of the role and operation of anionic lipids along the endomembrane system are conserved among eukaryotes, including many of the enzymes. However, the anionic lipid distribution of plant cells present several specificities compare to yeast or animal cells, starting with the high and low abundance of PI4P and PI(4,5)P₂ at the PM, respectively. Thus, the establishment of the anionic lipid distribution in plant is still not fully understood. Moreover, the mechanisms by which this distribution is maintained despite the constant exchanges of membrane between compartments due to vesicular trafficking must be explored. This includes the coupling between the enzymes that produce anionic lipids and the ones that convert or degrade them. Additionally, links or compensatory effects could exist between the different lipid pathways at the level of enzymatic activity, membrane recruitment or 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.

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 combinaison 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...) status.
670

671

- **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.

Acknowledgement: Research on phospholipids in Y.J. lab is supported by the French National Research Agency: ANR caLIPSO (ANR-18-CE13-0025-02) and ANR STAYING-TIGHT (ANR-18-CE13-0016-02). L.N. is funded by a PhD fellowships from the French Ministry of Higher Education.

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1151 • **Reference Annotations:** *brief (15 words maximum) explanation of citations’*
1152 *importance (as many as 10); insert below the Literature Cited section*
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- 1154 1. **Brault et al., 2019:** Proposes MCTP proteins, a class of putative PS/PI4P binding
1155 proteins, as tethering factors at PD-EPCS.
- 1156 2. **Gronnier et al., 2017:** Describes how anionic lipids and sterol influence REMORIN
1157 conformation, localization and activity by forming nanoclusters.
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1159 lipids (i.e. PS) in live plant cells.
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1161 cytokinesis and phragmoplast dynamics.
- 1162 5. **Kang et al., 2011:** Uses electron tomography to reveal distinct TGN subdomains
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1167 plants, which corresponds to post-Golgi membranes.
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1169 anionic phospholipids and regulate endocytic trafficking.
- 1170 9. **Simon et al., 2016:** Establishes the importance of membrane electrostatics and PI4P
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1173 helper protein mediating PI(4,5)P₂ polar localization.

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

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

- 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 specify.

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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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

are composed of a diacylglycerol backbone attached to a phosphate group. PS and PI display a serine (star) and an inositol ring (hexagon), respectively, linked to the glycerol by a phosphodiester linkage. PI can be phosphorylated in position 3, 4 and 5 to form 7 different PIPs: Phosphatidylinositol 3-phosphate (PtdIns3P or PI(3)P), Phosphatidylinositol 4-phosphate (PtdIns4P or PI(4)P), Phosphatidylinositol 5-phosphate (PtdIns5P or PI(5)P), Phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂ or PI(3,4)P₂), Phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂ or PI(3,5)P₂), Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂, PI(4,5)P₂ or PIP₂), Phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃ or PI(3,4,5)P₃). Pathways for PA (**b**), PS (**c**), PI and PIP (**d**) production and degradation. PC, phosphatidylcholine; PE, phosphatidylethanolamine; DAG, diacylglycerol; DGPP, Diacylglycerol pyrophosphate; Gly3P, Glycerol 3-phosphate; PLC, Phospholipase C; PLD, Phospholipase D; DGK, Diacylglycerol kinase; PAP, Phosphatidate phosphatase; PAK, PA Kinase; LPP, lipid-phosphate phosphatase; LPAAT, lyso-phosphatidic acid acyltransferase; GPAT, Glycerol-3-phosphate acyltransferase; PSS1, PS Synthase1; CMP-PA, Cytidine Monophosphoryl-Phosphatidic Acid; Ins, Inositol; SAC, SUPPRESSOR OF ACTIN; PTEN, PHOPHATASE AND TENSIN Homolog; FAB1, FORMATION OF APLOID AND BINUCLEATE CELLS 1; RHD4, ROOT HAIR DEFECTIVE 4; CVP2, COTYLEDON VASCULAR PATTERN 2; CVL1, CVP2-LIKE1; PI4K, PI 4-Kinases.

Figure 2: Physicochemical properties of membranes and membrane targeting by anionic lipids.

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. **(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. **(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). Note that for clarity purpose only the cytoplasmic membrane leaflet was drawn. BK11, BRI1 KINASE INHIBITOR 1; BAR, Bib-Amphyphisin-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.

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)P₂, 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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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 β -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 β -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.

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.

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)P₂, 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.

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)P₂, Phosphatidylinositol 4,5-bisphosphate; PI(3,5)P₂, Phosphatidylinositol 3,5-bisphosphate; PI4K, PI4P Kinases; RHD4, ROOT HAIR DEFECTIVE 4; FAB1, FORMATION OF APLOID AND BINUCLEATE CELLS 1; SAC, SUPPRESSOR OF ACTIN.