Annual Review of Plant Biology

Functions of Anionic Lipids in Plants

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Annu. Rev. Plant Biol. 2020. 71:71–102

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

https://doi.org/10.1146/annurev-arplant-081519-035910

Keywords

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

Abstract

Anionic phospholipids, which include phosphatidic acid, phosphatidylserine, and phosphoinositides, represent a small 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 signaling 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 signaling, cell division, membrane trafficking, cell growth, and gene expression. In this review, we describe the functions of anionic lipids from a cellular perspective. Using the localization of each anionic lipid and its 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.

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1. INTRODUCTION

Biological membranes delimit cellular compartments as well as the inside and outside of the cell. Each compartment has its own 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 that form a bilayer and transmembrane or membrane-associated proteins (65). Glycerophospholipids associated with sphingolipids and sterols are the main lipid components within the eukaryotic endomembrane system. Some glycerophospholipids 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 displays an inositol ring that can be phosphorylated in three different positions—3, 4, and 5—creating a total of seven possible phosphoinositide species: three phosphoinositide monophosphates [phosphatidylinositol 3-, 4-, and 5-phosphate (PI3P, PI4P, and PI5P)], three phosphoinositide biphosphates {phosphatidylinositol 3,4-, 3,5-, and 4,5-bisphosphate [$PI(3,4)P_2$, $PI(3,5)P_2$, and $PI(4,5)P_2$]}, and one phosphoinositide triphosphate {phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$]}.

These lipids are present in low to very low abundance in eukaryotic membranes. In *Arabidopsis* leaf, phosphatidylinositol represents about 7% of total phospholipids, PA about 2%, and PS about 1%. In addition, all phosphoinositides combined account for less than 1% of the total phospholipid content (15, 75, 140). However, anionic lipids are key components of cell membranes. They are involved in the regulation of nearly all membrane-associated events, including cell signaling, trafficking, cell division, and cell growth. Consequently, overexpression 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 (26, 99).

Anionic phospholipids are detected in most endomembrane compartments: endoplasmic reticulum (ER), Golgi, *trans*-Golgi network/early endosomes (TGN/EEs), plasma membrane (PM), multivesicular bodies/late endosomes (MVBs/LEs), 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 from a cellular perspective, using as a starting point the localization of each anionic lipid and its 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 $PI(4,5)P_2$ are also found in the nucleoplasm of the nucleus, where they may regulate transcription factors, chromatin status, and epigenetic landmarks (130), but this is not further discussed, as we focus this review on the endomembrane system.

Endomembrane system: membrane compartments, connected directly or through vesicular transport (e.g., nuclear envelope, endoplasmic reticulum, Golgi, plasma membrane, endosomes, and tonoplast), excluding chloroplasts and mitochondria

***trans*-Golgi network/early endosome (TGN/EE):** Golgi-derived but Golgi-independent compartment involved in secretion that also serves as early endosome in plants and is involved in endocytic trafficking

Multivesicular body/late endosome (MVB/LE): late endosome characterized by its spherical morphology and containing intraluminal vesicles destined for vacuolar degradation

Lipid-binding domain (LBD): protein domain that selectively recognizes and targets membranes via direct interactions with lipid(s) via specific, nonspecific, and/or multivalent interactions

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. Lipid-binding domains (LBDs) can mediate the interaction with the membrane (65, 131) (**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 domains, Fab1/YOTB/Vac1/EEA1 (FYVE) domains, Phox 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 (97). 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 (117, 132–134). However, in some cases, LBDs are not sufficient to target the protein to its proper compartment. Indeed, some proteins require the presence of both an anionic lipid and

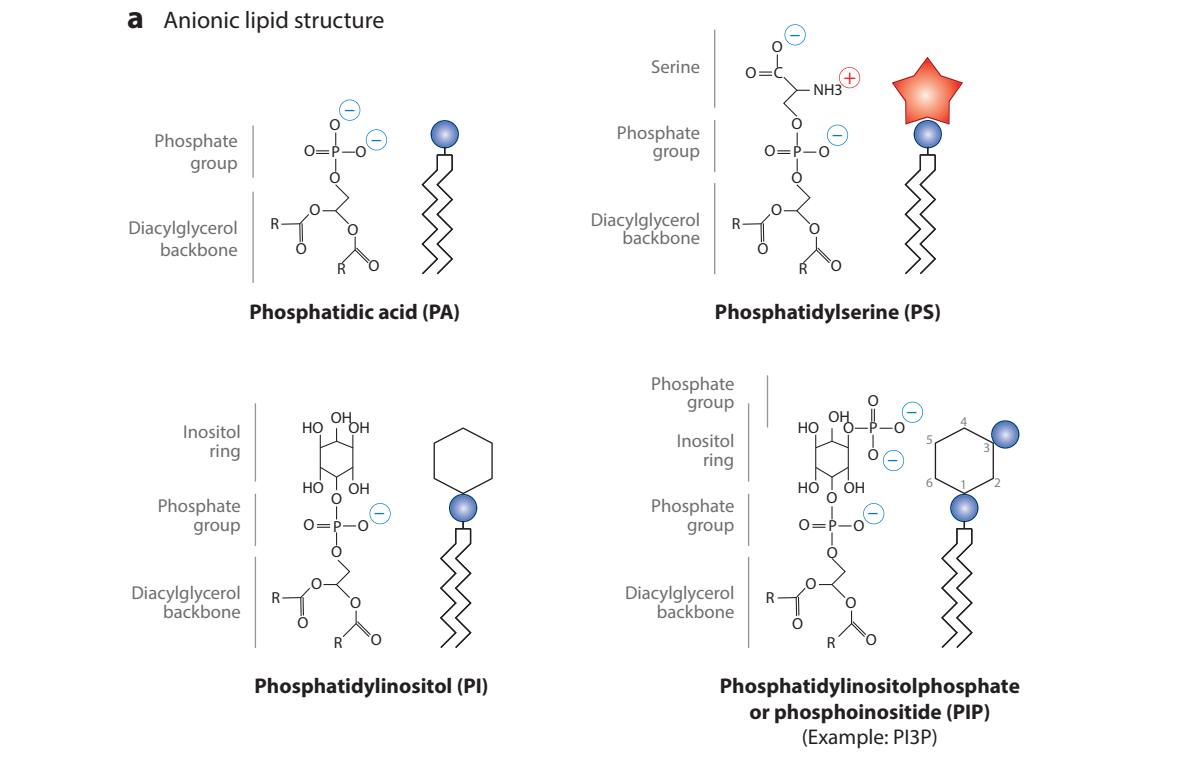

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Figure 1 (*Figure appears on preceding page*)

Anionic lipids. (*a*) Skeletal formula and schematic representation of PA, PS, PI, and 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 seven different PIPs: phosphatidylinositol 3-phosphate (PtdIns3P or PI3P), phosphatidylinositol 4-phosphate (PtdIns4P or PI4P), phosphatidylinositol 5-phosphate (PtdIns5P or PI5P), phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P2 or PI(3,4)P_2], phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P2 or PI(3,5)P_2], phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2, PI(4,5)P_2, or PIP2], and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3 or PI(3,4,5)P_3]. Pathways for PA (*b*), PS (*c*), and PI and PIP (*d*) synthesis and degradation are shown. Enzymes are in green, while substrates or products are in black. Abbreviations: 5PTase, 5-phosphatase; CMP-PA, cytidine monophosphoryl-phosphatidic acid; CVL1, CVP2-LIKE1; CVP2, COTYLEDON VASCULAR PATTERN2; DAG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; FAB1, FORMATION OF APLOID AND BINUCLEATE CELLS1; Gly3P, glycerol 3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; Ins, inositol; LPP, lipid-phosphate phosphatase; LPAAT, lysophosphatidic acid acyltransferase; LysoPA, lysophosphatidic acid; PA, phosphatidic acid; PAK, PA kinase; PAP, phosphatidate phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI4K, PI 4-kinase; PIP, phosphoinositide; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PIS, phosphatidylinositol synthase; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; PSD, PS decarboxylase; PSS1, PS synthase1; PTEN, PHOPHATASE AND TENSIN homolog; RHD4, ROOT HAIR DEFECTIVE4 (also called SAC7); SAC, SUPPRESSOR OF ACTIN; VPS34, VACUOLAR PROTEIN SORTING34.

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 FLAGELLIN-SENSING2 (FLS2), is recruited to the TGN via concomitant interaction with RabA4b and PI4P (2). Furthermore, LBDs may also recognize additional membrane features such as curvature or electrostatics. As such, results obtained with lipid biosensors should always be considered with care, as their localization may not 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 (117, 118). If possible, the potential accumulation of a lipid at a given membrane should be also supported by biochemical evidence, the localization of their metabolic enzymes, or both.

The combined study of the localization of anionic lipid–generating enzymes and genetically encoded lipid sensors suggests that, in *Arabidopsis* epidermal root cells, the PM accumulates PI4P, PI(4,5)P_2, PA, and PS (98, 117, 118, 132, 133) (**Figure 3**). TGN/EEs are enriched in PI4P, PS, and, to a lesser extent, PI3P (98, 118, 119, 133). MVBs/LEs accumulate both PI3P and PI(3,5)P_2, although both lipids localize in distinct compartments (39, 40, 117, 134). Finally, the tonoplast and autophagosomes carry PI3P (117, 134). However, this subcellular accumulation pattern may vary according to the cell type or the stress status of the cell (36, 43, 46, 51, 59, 63, 115, 120) (**Figure 3**). For example, PI(3,5)P_2 is found in MVBs/LEs in most cell types but accumulates at the PM in elongating root hairs (36) (**Figure 3**). PI(4,5)P_2 and PA are constitutively produced at the PM at low and moderate levels, respectively, but upon stimulation, such as salt stress, they can be massively produced, which triggers specific stress responses (86, 126, 132). Alternatively, PI(4,5)P_2 production can be inhibited in response to external stimuli such as pathogen-associated molecular patterns (84).

Coincidence binding: recruitment of a protein at a specific membrane by interaction with several membrane landmarks, which together provide high targeting specificity

2.2. Dynamics of Anionic Lipids: Rapid Production and Turnover Thanks to a Set of Lipid-Modifying Enzymes

Phosphatidic acid, phosphoinositides, and phosphatidylserine have distinct biosynthetic pathways, which we explain in detail below.

2.2.1. Phosphatidic acid production and stress responses. PA is structurally the simplest phospholipid. As such, it is a key intermediate for the biosynthesis of other phospholipids

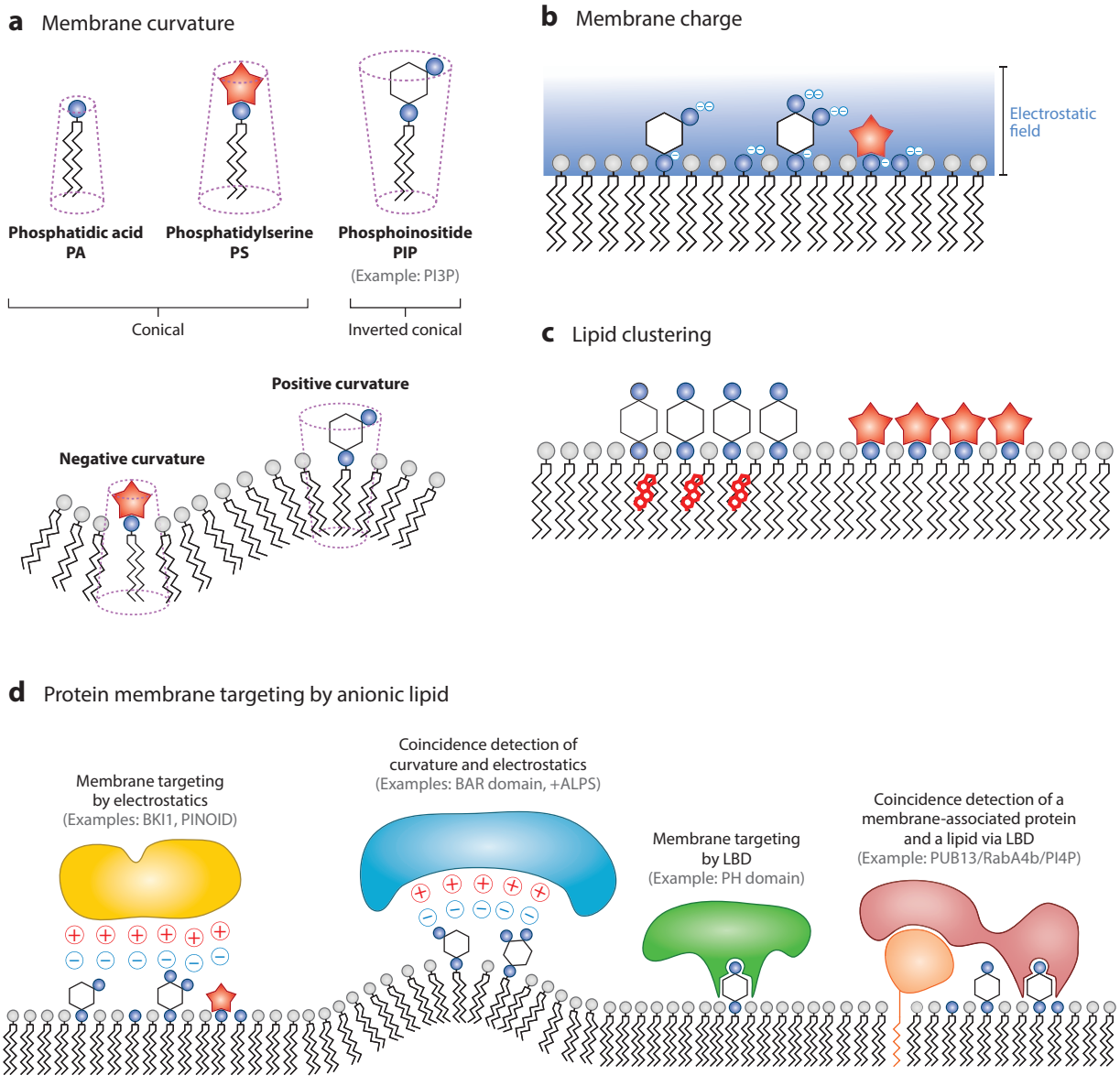

Figure 2

Physicochemical properties of membranes and membrane targeting by anionic lipids. Anionic lipids can confer several properties to the membranes. (*a*) 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*) Accumulation of anionic lipids confers a negative charge to the membrane, leading to an electrostatic field. (*c*) 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 positively charged amino acids, curvature recognition, or an LBD that targets a specific lipid. In some cases, proteins can be recruited to the membrane by coincidence detection of several membrane features (e.g., electrostatics and curvature, or an anionic lipid and a membrane-associated protein). For clarity, only the cytoplasmic membrane leaflet is shown. Abbreviations: +ALPS, amphipathic lipid packing sensor; BAR, Bin-amphiphysin-Rvs; BKI1, BRI1 KINASE INHIBITOR1; LBD, lipid-binding domain; PA, phosphatidic acid; PH, Pleckstrin homology; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate; PIP, phosphoinositide; PS, phosphatidylserine; PUB13, PLANT-U-BOX13.

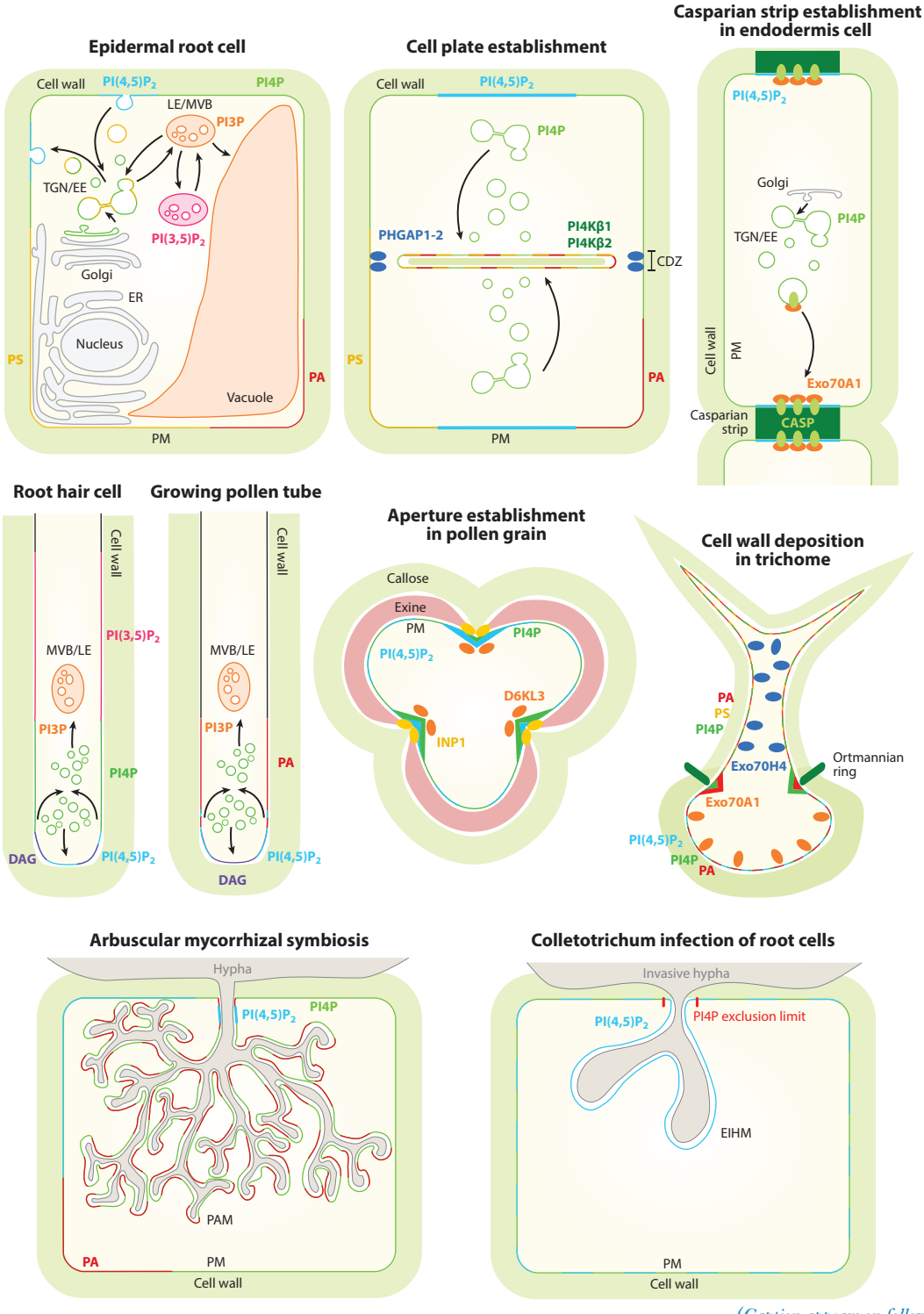

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Figure 3 (*Figure appears on preceding page*)

Anionic lipid distribution in plant cells. The localization of anionic lipids between the different cellular compartments can change depending on the developmental and physiological context. Anionic lipids can accumulate in specific zones of the PM during polarized cell growth (in the pollen tube and root hair cell for instance), when interacting with another organism (e.g., during symbiosis or infection), and to determine the position of developmental structures (such as aperture of the pollen grains, Casparian strip, and trichomes). Abbreviations: CASP, CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN; CDZ, cortical division zone; D6KL3, D6 PROTEIN KINASE-LIKE3; DAG, diacylglycerol; TGN/EE, *trans*-Golgi network/early endosome; EIHM, extra-invasive hyphal membrane; ER, endoplasmic reticulum; Exo70, exocyst complex component 70; INP1, INAPERTURATE POLLEN1; MVB/LE, multivesicular body/late endosome; PA, phosphatidic acid; PAM, periarbuscular membrane; PHGAP, Pleckstrin homology GTPase activating protein; PI3P, phosphatidylinositol 3-phosphate; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; PS, phosphatidylserine.

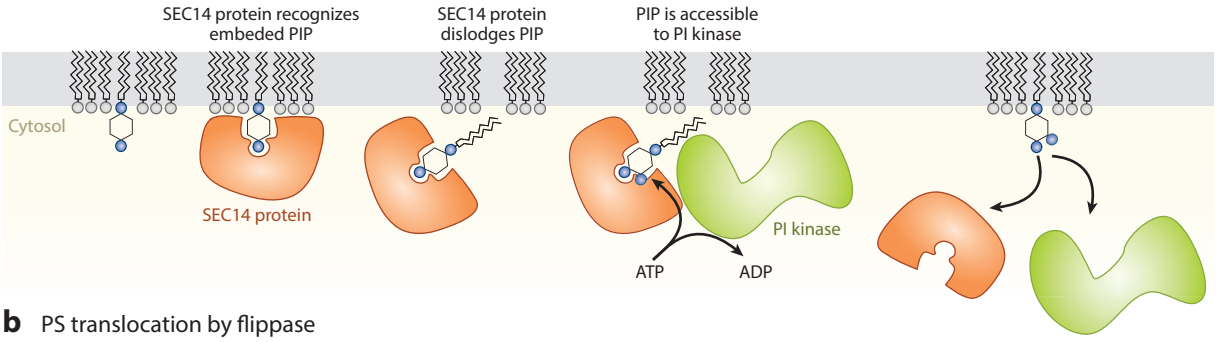
(99, 126). Multiple pathways involving different enzymes at various locations can lead to PA synthesis and are summarized in **Figure 1*b***. The rapid accumulation of PA is induced by numerous abiotic stresses, including salt, cold, heat, and osmotic stress, but also by biotic stresses, as in the case of pathogen infection (126). Moreover, PA synthesis is regulated in physiological and developmental contexts, including hormonal responses such as salicylic acid, abscisic acid, and auxin (69, 126). In each case, PA accumulation leads to different adapted cellular responses, raising the question of how PA can integrate different inputs to lead to specific outputs relevant to the physiological situation. One of the main hypotheses is that PA accumulation never occurs alone but rather that PA is one second messenger among others that together lead to concerted cellular responses (99). The different hypotheses proposed to answer this question are discussed by Pokotylo et al. (99) in a recent review.

2.2.2. Phosphoinositides and rapid interconversion. The phosphorylation of the inositol ring of phosphatidylinositol is mediated by phosphatidylinositol kinases that are specific to the phosphate position (**Figure 1*d***). Plant genomes do not encode for type-I and type-II PI3-kinases (PI3Ks), which are able to phosphorylate PI4P or $PI(4,5)P_2$ (88). As a consequence, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ likely do not exist and have not been detected in plants. The dephosphorylation of PIPs is mediated by phosphatidylinositol phosphatases that belong in plants to three main families: the SUPPRESSOR OF ACTIN (SAC) domain–containing proteins (SAC1–9), the 5PTase family (5PTase1–15), and the PHOPHATASE AND TENSIN homolog (PTEN) family (PTEN1–3) (26) (**Figure 1*d***). Phosphatidylinositol kinases and phosphatases are highly efficient enzymes in vivo, and PIPs can thereby be quickly interconverted, cycling between two states or creating cascade regulations (90). Because anionic lipid pools can be enzymatically modified within seconds, they are able to respond to various stimuli and act in fast cellular events such as membrane trafficking.

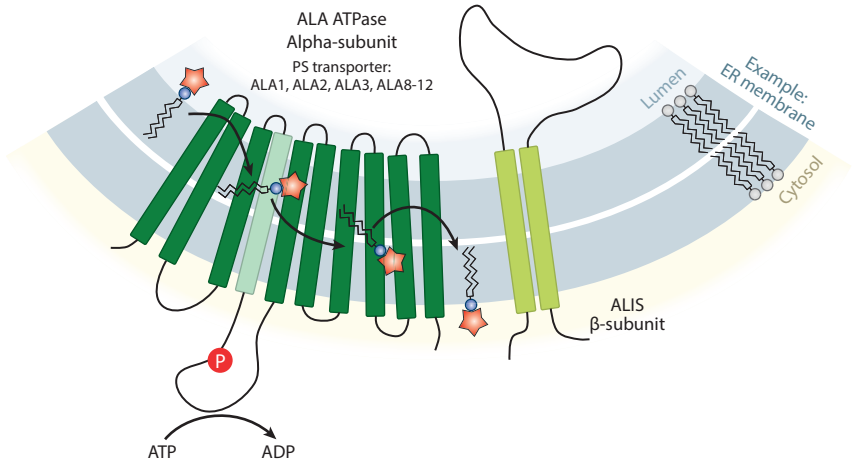
Lipid transfer protein: protein responsible for shuttling lipids between different membranes via nonvesicular trafficking

In vitro experiments testing phosphatidylinositol kinase activity often require the use of detergents, which destroy liposomal structures and free lipid substrates to increase kinase activity (16). These results suggest that phosphatidylinositol kinases have difficulty recognizing their substrate embedded in the membrane. The main candidates to fulfill this function are the SEC14 lipid transfer proteins. The yeast Sec14p protein is able to transfer phosphatidylcholine and phosphatidylinositol from distinct membranes in vitro, possibly bypassing the classical vesicular transport of lipids (16, 108). However, the current model, based on crystallographic data, advocates the idea that Sec14p dislodges the phosphatidylinositol head from the TGN membrane, making it accessible for PI4-kinases (**Figure 4*a***). Based on sequence homology, *Arabidopsis* contains 32 Sec14p homologs (AtSFHs), each containing a highly conserved sec14 domain. The SEC14 domain of several AtSFHs displays the same in vitro activity as the yeast Sec14p and can rescue the *Δsec14*

a Lipid presentation by SEC14 domain-containing protein



b PS translocation by flippase



c PS/PI4P exchanges at ER-PM contact site in human cell

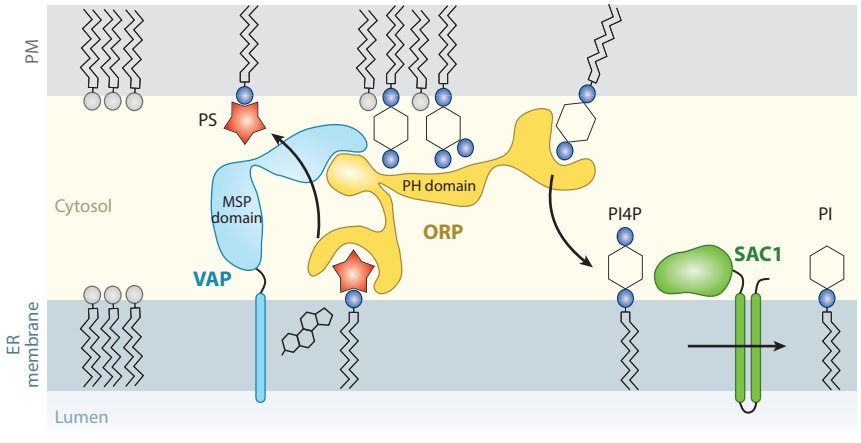


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Figure 4 (*Figure appears on preceding page*)

Mechanisms of lipid presentation and transport. (*a*) A mechanism of PIP presentation has been proposed to explain the highly efficient catalytic activity of PI kinases in vivo, in contrast to in vitro. In this model, SEC14 protein digs up PI or PIP from the membrane and presents it to the PI kinase for phosphorylation. Note that the SEC14 domain also binds to PC. (*b*) The translocation of PS from the luminal leaflet to the cytosolic leaflet of a membrane (ER, Golgi, TGN/EE, PM, or MVB/LE) depends on flippase proteins composed of an α-subunit called ALA, which hydrolyzes ATP and translocates PS, and a β-subunit called ALIS, which specifies 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 and 8 then transfer PS from the ER membrane to the PM via their lipid transfer domain and then countertransport PI4P to the ER. PI4P is then dephosphorylated by SAC1 in the ER membrane, maintaining the PI4P gradient between the two membranes. For clarity, in panels *a* and *c*, only the cytoplasmic membrane leaflet is shown. Abbreviations: ADP, adenosine diphosphate; ALA, aminophospholipid flippase; ALIS, ALA-interacting β-subunit; ATP, adenosine triphosphate; TGN/EE, *trans*-Golgi network/early endosome; ER, endoplasmic reticulum; MSP, major-sperm protein; MVB/LE, multivesicular body/late endosome; ORP, OSBP-related protein; PC, phosphatidylcholine; PI, phosphatidylinositol; PH, Pleckstrin homology; PI4P, phosphatidylinositol 4-phosphate; PIP, phosphoinositide; PM, plasma membrane; PS, phosphatidylserine; SAC1, SUPPRESSOR OF ACTIN 1; VAP, vesicle-associated membrane-associated protein.

yeast mutant (42, 135). So far, the best-characterized AtSFH protein is AtSFH1. AtSFH1 belongs to a subclass of 13 members that contain a C-terminal Nlj16 domain. This domain has two functions: It contains a polybasic patch that localizes to the PM in a $PI(4,5)P_2$-dependent manner and a coiled-coil domain that mediates homo-oligomerization (27). In the model built with the combination of yeast and *Arabidopsis* data, AtSFH1 is targeted at the root hair tip PM by $PI(4,5)P_2$, where it stimulates the activity of phosphatidylinositol kinases (PI4K or PIP5K), thereby enhancing the production of phosphoinositides [directly producing $PI(4,5)P_2$ or PI4P that will then be converted into $PI(4,5)P_2$]. $PI(4,5)P_2$ recruits new AtSFH1, which oligomerizes and further creates $PI(4,5)P_2$ clusters at the root hair tip PM (**Figure 3**).

2.3. Phosphatidylserine Synthesis and Trafficking

In contrast to PA and PIPs, which can be synthesized by several enzymes, a single enzyme produces PS in *Arabidopsis*: the PHOSPHATIDYLSERINE SYNTHASE1 (96, 98, 145) (**Figure 1*b***). This synthase is an ER transmembrane protein that synthesizes PS in the luminal leaflet of this compartment (145). Therefore, in contrast to phosphoinositides, 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 nonexclusive mechanisms may explain the localization of PS.

Flippase: membrane transporter that translocates phospholipids from the luminal to the cytosolic membrane leaflet, thereby maintaining lipid asymmetry across the bilayer

2.3.1. Flippases and vesicular trafficking. As PS faces the lumen in the ER but faces the cytosol once at the PM or endosomes, it must switch membrane leaflets at some point. Flippases are proteins able to flip phospholipids from the luminal membrane leaflet to the cytosolic membrane leaflet (89) (**Figure 4*b***). The *Arabidopsis* genome encodes for 12 aminophospholipid flippases (ALAs), and ALA1, ALA2, ALA3, and ALA10—and possibly others—are able to flip PS in yeast (29, 73, 74, 89, 102). ALA proteins localize not only in the ER (ALA1, 2, and 3) but also all along the endocytic pathway (ALA1 at the PM, ALA2 in MVBs/LEs, and ALA3 in the Golgi and TGN/EEs) (9, 73, 74, 78, 101). Their localization and function depend on ALA-interacting β-subunits. These localizations suggest that PS could be directly flipped in the ER and then transported through vesicular trafficking to the PM and endosomes. Another hypothesis is that PS is first transported by vesicular trafficking on the luminal side of the organelle and then flipped in specific compartments, such as TGN/EEs or the PM. In yeast, the activity of the PS flippase Drs2p is promoted by PI4P (4, 128). As PI4P is present in the same compartments as PS along

the endocytic pathway in plants, this could be an argument in favor of the latter 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 (98, 118).

2.3.2. Phosphatidylserine 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 nanodomains where membranes from two different organelles are brought together (at a distance of less than 30 nm) and stabilized by tethering proteins (47, 94, 112). In animal and yeast, MCSs are major sites of lipid flow and are particularly important in the control of the localization of nearly all anionic lipids (143). Lipid transfer proteins present at MCSs 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 MCSs via interactions with suppressor of choline sensitivity (VAPs in animal cells) in the ER and phosphoinositides at the PM (3, 143). Several ORPs transfer PS (among other lipids) from the ER to the PM and then countertransport PI4P from the PM back to the ER (**Figure 4*c***). The PI4P phosphatase SAC1, an ER resident protein, hydrolyzes the incoming PI4P, thereby maintaining the PI4P gradient between the PM and ER membrane and fueling PS export from the ER (3). Note that PS transfer at MCSs has not been described so far in plants but that ORP proteins are conserved in plant genomes.

Membrane contact site (MCS): close (less than 30 nm) and stable apposition via tethering elements of two membranes, allowing interorganelle communication without membrane fusion

Nanodomain or nanocluster: small membrane zone less than 300 nm in diameter, enriched in one or several lipid species or proteins

Electrostatic membrane territory: within the endomembrane system, corresponds to negatively charged membranes whose cytosolic leaflet harbors anionic phospholipids (post-Golgi compartments), as opposed to neutral membranes (ER-derived)

3. ANIONIC LIPIDS AS DETERMINANTS 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 (80) (**Figures 1*a*** and **2*b***). The strength of this electrostatic field depends on the relative concentration of anionic phospholipids and their respective negative charges, which vary from −1 for PS to −5 for $PI(4,5)P_2$. In plants, TGN/EEs are less electronegative than the PM but more electrostatic than MVBs/LEs, whereas ER-derived compartments (e.g., ER, nuclear membrane, peroxisomes) are neutral (98, 118). Consequently, an electrostatic gradient is formed from the PM to the tonoplast, which follows the endocytic route and specifies an electrostatic membrane territory within the endomembrane system (98). The extensive use of biosensors locating anionic lipids and assessing membrane surface charge in vivo shows that the high electronegativity of the plant PM relies concomitantly on PI4P, PS, and PA, with a major role of PI4P, whereas the surface charge of TGN/EEs depends on both PI4P and PS (98, 118).

The surface charges of membrane compartments are a key determinant in the control of the localization of many membrane-associated proteins. For example, proteins that contain polybasic patches interact with the negative charges of the anionic phospholipids of the membrane by nonspecific electrostatic interactions (**Figure 2*d***). Very often, such motifs also contain hydrophobic residues or are found adjacent to hydrophobic lipid anchors. For example, the plant AGC kinases PINOID, D6PK, and PROTEIN KINASE ASSOCIATED WITH BREVIS RADIX (PAX), which are well-characterized regulators of auxin efflux transporters from the PIN family, localize at the PM through this mechanism (5, 63, 76, 118). Another prominent example of plant proteins that rely, at least in part, on electrostatic interactions with anionic lipids for localization are the small GTPases from the Rho-of-plant (ROP) family, which have a polybasic stretch adjacent to a prenylation site (i.e., a geranylgeranylation lipid modification) (96).

LIPID MOLECULAR SPECIES AND LIPID PACKING DEFECTS

Anionic lipids are defined by their head group. However, they are also composed 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 (7). 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 (7). Membrane curvature and conically shaped 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 specifically target membranes with packing defects by inserting hydrophobic residues into the membrane. In animal cells, high packing defects are found in the ER and *cis*-Golgi membrane, while TGN and PM present low levels of packing defects except in highly curved membranes. The relevance of lipid packing defects for protein recruitment in plants is largely unexplored.

There is no precise study on the number of plant proteins that might contain such polybasic stretches and localize to electrostatic membranes through this mechanism, but this is likely a general mechanism, which contributes to the localization of many proteins within the electrostatic membrane territory.

3.2. Curvature

Anionic phospholipids present different geometries (**Figure 2*a***). Indeed, phosphatidylinositol and phosphoinositides 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 phosphoinositides enhances positive curvature, while the inverted conical shape of PA and PS favors negative curvature (**Figure 2*a***). Membrane curvature is of prime importance when endocytic vesicles are formed at the PM, secretory vesicles are formed at the TGN/EE, or proteins are internalized in MVBs, for instance (10, 82). Similar to surface charges, the curvature of the membrane can also induce lipid-protein interactions via curvature-sensing domains such as Bin-amphiphysin-Rvs (BAR) domains (7, 65) or the amphipathic lipid packing sensor (ALPS) motif, which recognizes packing defects found in curved membranes (see the sidebar titled Lipid Molecular Species and Lipid Packing Defects). 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 (144). In plants, the PI4-kinase PI4Kβ1 relies on such a motif to localize to the curved and electrostatic membrane of TGN/EEs (98).

3.3. Clustering

Secretory vesicle: specific subdomain of the TGN/EE made of uncoated vesicles involved in secretion

Proteins and lipids can also laterally segregate into membrane domains, thereby creating subcompartmentalization inside the membranes (47) (**Figure 2*c***). Such clustering is often required for the protein to fulfill its biological function. While sterol has long been associated with membrane domain formation, it recently emerged that anionic lipids are also key determinants of protein clustering.

For example, remorins (REMs) are proteins that laterally segregate at the PM in domains of about 100 nm in size (hence termed nanodomains) (47, 49) (**Figure 5*a***). REMs are involved in biotic and abiotic responses, although their exact molecular function is still unknown (50). For

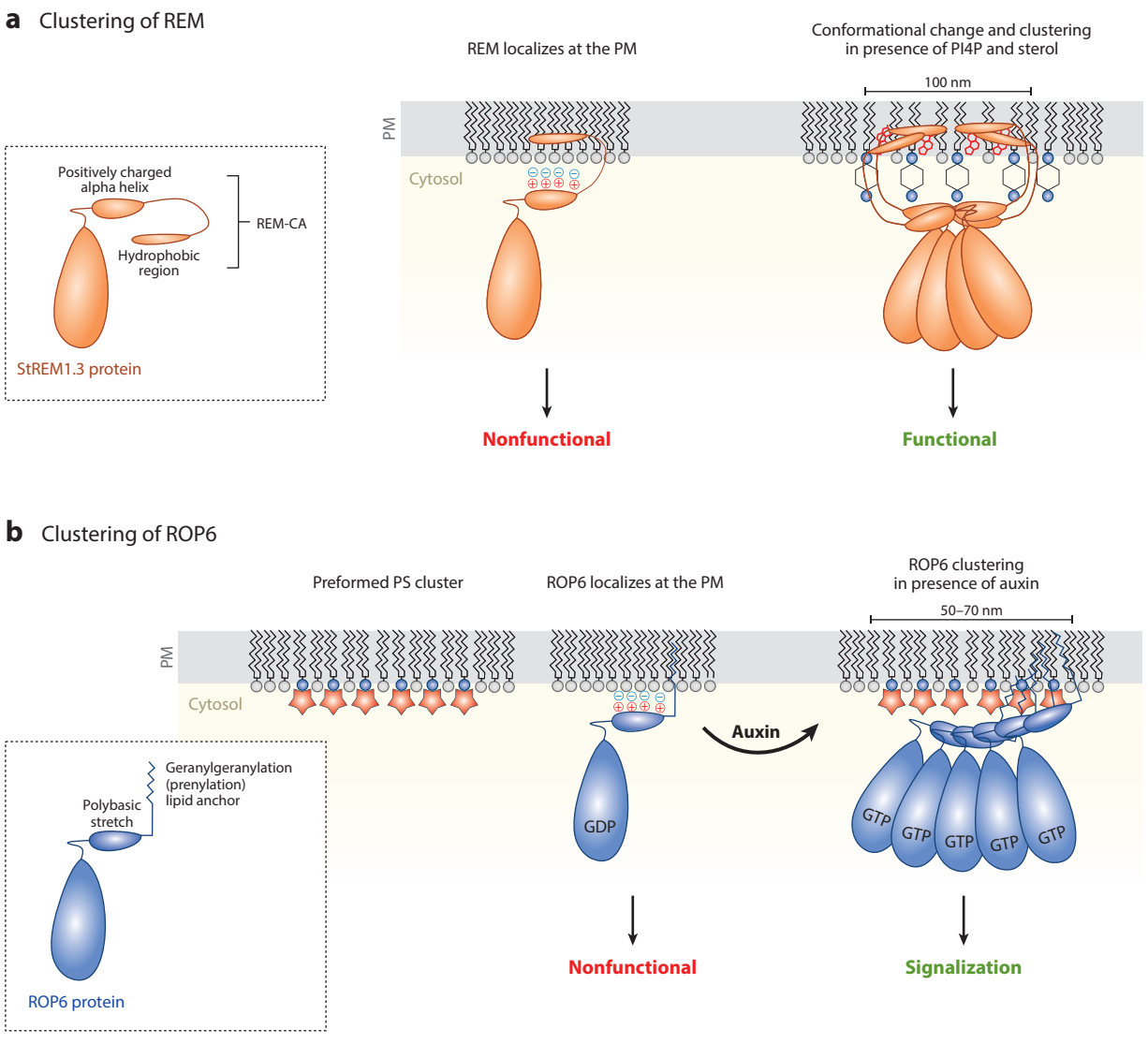

Figure 5

Protein and 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 the presence of PI4P and cholesterol, StREM1.3 undergoes a conformational change that leads to its clustering. The distribution of StREM1.3 in clusters appears necessary for it 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 is stabilized in pre-existing PS clusters. The clustering of ROP6 into PS nanodomains is necessary for ROP6 signaling. For clarity, only the cytoplasmic membrane leaflet is shown. Abbreviations: GDP, guanosine diphosphate; GTP, guanosine triphosphate; PI4P, phosphatidylinositol 4-phosphate; PM, plasma membrane; PS, phosphatidylserine; REM, REMORIN; REM-CA, REM C-terminal anchor; ROP6, Rho-of-plant 6; StREM1.3, *Solanum tuberosum* REMORIN group1 isoform3.

Plasmodesmata (PD): pores that connect plant cells by passing through their cell wall, thereby allowing cytoplasmic, PM, and ER continuity from cell to cell

example, *Solanum tuberosum* REMORIN group1 isoform3 (StREM1.3) limits the spreading of *Potato virus X* and regulates plasmodesmata (PD) permeability (105). A combination of super-resolution microscopy and biophysical experiments showed that StREM1.3 targeting into nanodomains is dependent on the presence of both sterol and PI4P and is required for function (31) (**Figure 5*a***). PI4P is not the only anionic lipid involved in protein nanoclustering into membrane nanodomains. Indeed, PS stabilizes the small GTPase ROP6 into nanoclusters of about 50–70 nm in diameter (96) (**Figure 5*b***). In 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 (96). ROP6 localization into nanoclusters is essential for function, since ROP6 signaling 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 PM suggest that PS itself, not only ROP6, is present in PM nanodomains (**Figure 5*b***). However, unlike ROP6, PS is present in PM nanodomains in both the absence and presence of auxin (96). Therefore, there are prepatterned nanoplatforms of PS at the PM that recruit GTP-loaded ROP6 upon auxin treatment. Future studies are needed to uncover whether such PS nanoplatforms contain additional lipids, such as sterol or PI4P, and how they are formed. The cell wall and the cytoskeleton influence protein diffusion into membranes (47, 77, 79). 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 (26).

All the mechanisms described above converge on the idea that anionic lipids are key determinants 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 are not individually reviewed here. Instead, we dedicate the rest of this review to one of the key questions of cell biology: How do compartments maintain a specific identity despite the constant flow and exchange of proteins and lipids between them? In particular, we highlight how anionic lipids may contribute to MCS establishment and analyze the interplay between membrane trafficking and anionic lipids.

4. ANIONIC LIPIDS AND ENDOPLASMIC RETICULUM–PLASMA MEMBRANE CONTACT SITES IN PLANTS

Several reports put ER-PM MCSs (EPCSs) at the center of essential cellular processes, including the immune secretory pathway, viral movement at PD, stabilization of the cortical ER network, endocytosis, membrane integrity, and pollen, seed, and root hair development (55, 66, 67, 95, 109, 139, 146). 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-SYTs)] (6, 95); the V-EPCSs that contain the vesicle-associated membrane protein–associated protein 27s (VAP27s) and VAP27-related proteins (orthologs of the mammalian VAPs and yeast Scs2) (107, 137, 139); and the PD-EPCSs, which are specifically enriched at PD and contain MULTIPLE C2 AND TRANSMEMBRANE DOMAIN-CONTAINING PROTEIN (MCTP) proteins (11). Importantly, all three classes of tethering molecules—VAP27s, SYTs, and MCTPs—directly interact with anionic phospholipids and likely require these lipids to interact with the PM. Nonetheless, S-EPCSs and V-EPCSs are spatially separated within the same cell, suggesting a tethering signature model in which the abundance of one type of EPCS at the expense of

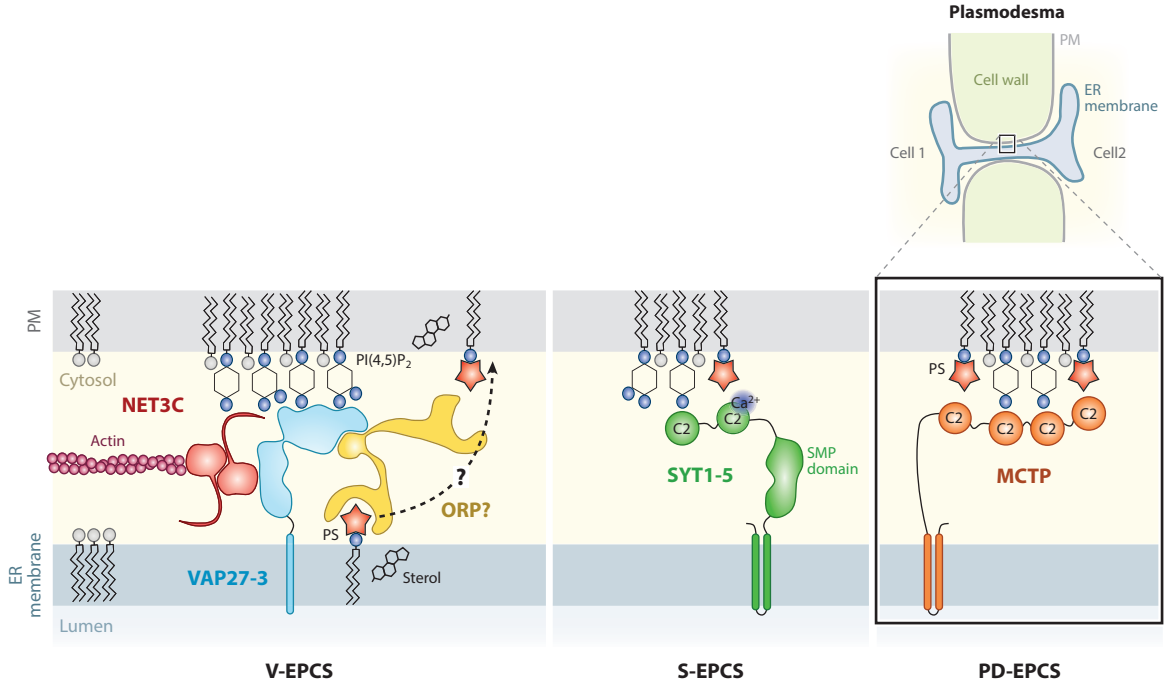

Figure 6

The three different classes of EPCSs in plant and their relation with anionic lipids. For clarity, only the cytoplasmic membrane leaflet is shown. Abbreviations: ER, endoplasmic reticulum; EPCS; ER-PM membrane contact site; MCTP, MULTIPLE C2 AND TRANSMEMBRANE DOMAIN-CONTAINING PROTEIN; NET3C, NETWORKED 3C; ORP, OSBP-related protein; PD, plasmodesmata; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; PS, phosphatidylserine; S-EPCS, SYT-containing EPCS; SMP, synaptotagmin-like mitochondrial-lipid-binding domain; SYT, SYNAPTOTAGMIN; VAP27, vesicle-associated membrane protein–associated protein 27; V-EPCS, VAP27 and VAP27-related proteins containing EPCS.

another could vary in response to environmental or developmental cues (116). Another model, the tethering arrangement model, proposes that the organization of EPCSs could change in response to stimuli such as lipids or calcium fluxes (28). These two models are not mutually exclusive.

4.1. VAP27-Containing Endoplasmic Reticulum–Plasma Membrane Contact Sites

VAP27s are ER transmembrane proteins that are conserved among eukaryotes (143). Ten homologs of VAP27 exist in *Arabidopsis* (139). At least three of them localize at the EPCSs: VAP27-1, VAP27-3, and VAP27-4, together with the actin binding protein NETWORKED 3C (NET3C) (138) (**Figure 6**). VAP27-1 and VAP27-3 interact with phosphoinositides in vitro (121). Moreover, VAP27-3 is able to interact with a lipid transfer protein from the ORP family, ORP3a, that binds sterol in vitro, suggesting possible lipid transfer similar to what has been described in yeast and animal cells (3, 107).

4.2. SYT-Containing Endoplasmic Reticulum–Plasma Membrane Contact Sites

The mammalian E-SYT1 is able to trigger glycerolipids exchanges between liposomes in vitro (110). E-SYTs contain C2 domains that bind anionic phospholipids from the PM in a

Clathrin-coated pit: clathrin-coated vesicles at the PM, corresponding to the initial step of membrane deformation during endocytosis

Ca^{2+}-dependent manner (149). In favor of the tethering arrangement model, E-SYTs Ca^{2+} binding leads to clustering of E-SYTs proteins and an increase in the interactions between E-SYTs and PM anionic lipids (28). This results in stabilization and tightening of S-EPCSs (28, 143, 149). In *Arabidopsis*, ionic stress triggers the expansion of S-EPCSs in association with accumulation of $PI(4,5)P_2$ at the PM, supporting the tethering arrangement model (64). Furthermore, the C2 domains of *Arabidopsis* SYT1 bind PS in a Ca^{2+}-dependent-manner in vitro (95) (**Figure 6**). The relevance of this Ca^{2+}-dependent PS binding in vivo is currently unknown but also argues in favor of the tethering arrangement model.

4.3. Plasmodesmata-Localized Endoplasmic Reticulum–Plasma Membrane Contact Sites

PD pores interconnect virtually all cells within the plant body, establishing direct PM and cytoplasmic continuity. A striking feature of PD is that a strand of ER runs through the pores and is tethered tightly to PM, making PD a unique case of EPCSs in eukaryotes. While SYT1 localizes to PD to some extent (66, 95), several MCTP proteins are specifically enriched at PD and likely contribute to ER-PM tethering at the PD pore (11) (**Figure 6**). Molecular dynamics suggests 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. PD have a specific composition of sterol and sphingolipid (30). 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 occurs 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 is detailed in the following section of this review.

5. TRAFFICKING BETWEEN THE PLASMA MEMBRANE AND THE *TRANS*-GOLGI NETWORK

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 $PI(4,5)P_2$ produced from PI4P by PI4P 5-kinases (PIP5Ks) (85). $PI(4,5)P_2$ allows the initiation and stabilization of clathrin-coated pits by recruiting several components of the endocytic machinery, including the adaptor protein AP2 (54, 100). Moreover, PIP5Ks are directly recruited at the site of endocytosis by AP2, creating a local pool of $PI(4,5)P_2$, which induces a high rate of clathrin-coated pit nucleation (85).

5.1.1. $PI(4,5)P_2$ is 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 $PI(4,5)P_2$ in endocytosis has been demonstrated indirectly using PIP5K overexpression and loss-of-function approaches (45, 151). Notably, a *pip5k1 pip5k2* mutant has abnormal clathrin-coated pits, which are less dense but bigger than those in the wild type (45). This mutant, as well as *PIP5K1* and *PIP5K2* overexpression lines, has defects in the endocytic trafficking of the PIN-FORMED (PIN) polar auxin efflux carriers (45, 83, 125). These defects in endocytosis impact PIN accumulation at the PM and PIN polar targeting. Abnormal PIN localization ultimately leads to altered distribution

of the hormone auxin, leading to various degrees of auxin-related developmental defects (45, 83, 125). Overexpression of *PIP5K* and the *pip5k1 pip5k2* mutant also have a phloem differentiation phenotype (32, 76). *PIP5K* overexpression redirects the trafficking toward the vacuole, leading to protein degradation and vacuole biogenesis, which in protophloem induces vascular discontinuities (32). 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, $PI(4,5)P_2$ levels are constantly kept in check in the root phloem by two PM-localized $PI(4,5)P_2$ 5-phosphatases, COTYLEDON VASCULAR PATTERN 2 (CVP2) and CVP2-LIKE 1, whose double mutant shows phloem discontinuity similar to that of PIP5K overexpression lines or the *pip5k1 pip5k2* mutant (15, 76, 106). In addition, PIP5K1 and PIP5K2 were recently shown to be part of a polarity module in protophloem cells (76). In these cells, PIN1 is localized on the basal end of the cell in a polar domain shaped like a donut located close to the cell edge. PIP5K1 and PIP5K2, together with BREXIS RADIX (BRX) and PAX, localize in a muffin-like polar patch inside the donut-shaped PIN1 domain. Researchers proposed that PIP5Ks/BRX/PAX would act as a self-reinforcing polarity module, since PIP5Ks are directly required for PAX localization in the polar patch (and indirectly required for BRX), while PAX/BRX together recruit PIP5Ks in this domain (76). Interestingly, a component of the endocytic machinery, the dynamin-related protein 1A, accumulates in the polar patch domain together with PIP5Ks/PAX/BRX. Because PIP5Ks were proposed to positively promote endocytosis in plants, it is possible that the localized PIP5K targeting favors endocytosis from the polar patch rather than the PIN1-containing donut domain, perhaps further reinforcing the polarity of protophloem cells, which is critical for their proper differentiation (76).

Clathrin-coated vesicle: vesicle decorated with a membrane-deforming clathrin coat; involved in many vesicular-mediated trafficking pathways within the cell

In the growing pollen tube, exocytosis at the tip is balanced by endocytosis in the subapical regions (**Figure 3**). These regions are marked by $PI(4,5)P_2$ locally produced by PIP5Ks and restricted by degradation into diacylglycerol by phosphoinositide-specific phospholipase C (19, 34) (**Figure 3**). Loss-of-function mutants of PIP5Ks show reduced endocytosis, which leads to a low pollen germination rate and slow tube elongation (43, 120). Conversely, pollen overexpressing *PIP5K6* shows PM invagination and the formation of several tip branches due to a higher rate of endocytosis at the tip of the pollen tube (151). The expression of a clathrin-dominant negative version can rescue the PIP5K6-overexpressing phenotype, indicating that $PI(4,5)P_2$ synthesis enhances clathrin-mediated endocytosis (151). A similar mechanism was proposed in response to salt treatment, which increases the level of $PI(4,5)P_2$ and induces the formation of clathrin-coated vesicles in a PIP5K1- and PIP5K2-dependent manner (45, 58).

5.1.2. Phosphatidic acid 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 treatment (87). In addition to inducing membrane curvature, PA is able to bind clathrin heavy chain and domain clathrin assembly proteins (81). Upon salt treatment, PA production by the phospholipase PLDζ 2 induces the internalization of PIN2 in TGN/EEs (23), whereas in normal conditions, PA and PLDζ 2 are necessary for the proper cycling of PIN2 in root cells (68). 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 (23). These data suggest that PA could act as a mediator of the auxin response by activating the endocytosis of PIN proteins in high-salinity conditions.

To conclude, a role for $PI(4,5)P_2$ in clathrin-mediated endocytosis is established in different cell types and developmental contexts. Nevertheless, the proteins regulated by $PI(4,5)P_2$ 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

Tethering complex: protein complex that brings together two membranes, either transiently before fusion or in a more sustained manner at contact sites

Polar secretion: delivery of proteins or material steered in one or several specific directions within the cell

Exocytosis is led by a tethering complex called the exocyst, which is conserved among eukaryotes and targets exocytic vesicles to the PM (92). In many cases, exocytosis does not occur homogeneously along the PM but is polarized. Polar exocytosis has been reported in growing tip cells such as root hair and the pollen tube (8, 14, 70, 113, 123, 141), for the polar localization of auxin efflux carriers from the PIN family (20, 93, 124), and in the establishment of the Casparian strip (51) or the Ortmannian ring in trichomes (59–61).

5.2.1. $PI(4,5)P_2$ targets the exocyst to the plasma membrane. The exocyst complex is targeted to the PM via two subunits (SEC3 and EXO70) known to bind anionic phospholipids, in particular $PI(4,5)P_2$. In *Arabidopsis*, SEC3 contains a Pleckstrin homology domain that binds to $PI(4,5)P_2$ 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 $PI(4,5)P_2$ (43, 44, 59, 62, 63, 120, 122, 151) (**Figure 3**). Interestingly, the localizations of SEC3 isoforms and EXO70 isoforms often correlate with the $PI(4,5)P_2$ subcellular accumulation pattern, suggesting that $PI(4,5)P_2$ contributes to their localization (8, 51, 113). In the pollen tube, SEC3 and EXO70A1 partially colocalize with $PI(4,5)P_2$ biosensors (8, 113). Since PIP5K mutants display slower tube elongation and reduced secretion of cell wall materials (43, 120), $PI(4,5)P_2$ likely participates in targeting exocyst complexes to specific PM domains.

5.2.2. $PI(4,5)P_2$ and phosphatidic acid: working together to confine exocytosis. EXO70A1 and EXO70B1 are expressed in the growing pollen tube and partially colocalize with both $PI(4,5)P_2$ and PA biosensors (113). Therefore, it is possible that the coincidental detection of $PI(4,5)P_2$ and PA in the 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 the pollen tube, the PM membrane domain that contains a combination of both $PI(4,5)P_2$ 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 *Trans*-Golgi Network

Exocytosis requires not only 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 (52, 104, 127, 148). PI4-kinases PI4Kβ1 and PI4Kβ2, as well as the PI4P phosphatase ROOT HAIR DEFECTIVE 4 (RHD4/SAC7), localize to the TGN. *pi4kβ1β2* and *rhd4* mutants display short or bulged root hairs (52, 104, 127). Ultrastructural studies by electron tomography showed the presence of enlarged, likely deficient, secretory vesicles in the TGN of the *pi4kβ1β2* double mutant, suggesting that PI4Kβs are indeed involved in secretion (52). PI4Kβs and RHD4/SAC7 have opposite catalytic activities yet apparently locate to the same compartment. PI4Kβs and RHD4/SAC7 possibly localize in different TGN subdomains. Consistent with this scenario, PI4Kβ1 localizes specifically in the secretory vesicle subdomain of the TGN (52). According to this hypothesis, PI4-kinases could be necessary for the production of the pool of PI4P at the TGN that confers the proper physical properties (mild electrostatics and high curvature) necessary for secretory vesicle formation. By contrast, RHD4/SAC7 could localize in a different TGN subdomain, perhaps one directly connected to

endocytosis. In such a model, RHD4/SAC7 would degrade incoming PI4P from the PM to maintain the PI4P gradient along the endocytic pathway (118).

5.4. Cell Plate Establishment and the Creation of a New Plasma Membrane

Cell plate: organelle formed by delivery of cell wall and membranes by Golgi-derived vesicles at the plane of cell division in plants

The cell plate is an organelle formed from the secretion and fusion of vesicles from the TGN and forms 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 that of 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 as, or even higher than, at the PM (18, 98, 118) (**Figure 3**). However, PI(4,5)P_2 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 (18, 118, 132). Given that PI(4,5)P_2 is a major regulator of both exocytosis and endocytosis, it is therefore likely that specific trafficking mechanisms occur at the cell plate compared to at the bulk PM, although these have yet to be fully 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 phenotypes, cytokinesis phenotypes, or both. Those include mutants for PI4P production (*pi4kβ1β2*), PI4P/PI(4,5)P_2 interconversion (*sac9* and *pi5k1k2*), and PI3P/PI(3,5)P_2 interconversion [*formation of aploid and binucleate cells*1 (*fab1a fab1b*) and *fra7/sac1*)] (52, 125, 136, 142, 153). Some of the corresponding enzymes directly act at the cell plate. For instance, PI4Kβ1 localizes at the cell plate, and the absence of PI4Kβ1 and PI4Kβ2 directly affects the vesicular trafficking along phragmoplast microtubules (71). However, others might act indirectly on cell division, such as FAB1A and FAB1B proteins that do not localize at the cell plate (38, 142). It is therefore likely that the *fab1a fab1b* cell plate phenotype results from a broad alteration of membrane trafficking. We refer the reader to a recent review on this subject that details the potential involvement of each anionic lipid during cytokinesis (13).

5.5. Anionic Phospholipid Remodeling at the Plant-Fungal Interface

Fungal infection and mycorrhizal symbiosis induce PM reorganization, which includes lipid patterning at the PM, with likely consequences for both endocytosis and exocytosis.

5.5.1. Periarbuscular membrane. During the endosymbiotic association of roots with arbuscular mycorrhizal fungi in *Medicago truncatula*, the fungus develops intracellular branched hyphae called arbuscules (46). In response, the root cortical cells form a new membrane in continuity with the PM called the periarbuscular membrane (PAM) (33). The PAM envelops the hyphal branches and is thought to be generated by polar exocytosis. Indeed, the subunit of the exocyst complex and SNARE proteins known to be involved in exocytosis and membrane fusion are required for PAM formation (33, 150). 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)P_2, and PA have been documented during arbuscular mycorrhizal interaction (46) (**Figure 3**). Both PI4P and PA are found in the PAM to a similar extent as in the PM. However, in the trunk part of hyphal branches, discrete regions where PI4P, PI(4,5)P_2, and PA reporters coaccumulate can be observed (46). This accumulation could correspond to an accumulation of membranes before formation of small bulges. Furthermore, anionic lipid accumulation could influence membrane curvature and thereby physically contribute to the establishment of the PAM.

5.5.2. Extra-invasive hyphal membrane. Using another model of fungus-plant interactions, researchers recently showed in *Arabidopsis* infected by *Colletotrichum* that extra-invasive hyphal membrane (EIHM), which is formed in continuity with the PM, strongly accumulates $PI(4,5)P_2$ and PIP5K3 all along the membrane (**Figure 3**) (41, 114, 115). The EIHM also accumulates subunits of the exocyst complex, which are likely $PI(4,5)P_2$ effectors. Thus, $PI(4,5)P_2$ 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.

Protein sorting: delivery process of proteins to their appropriate cellular destination, based on information contained in the protein sequence, structure, or both

Phagophore: a double membrane that encloses and isolates cytoplasmic components during autophagy

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 (21). Then TGN/EEs mature into MVBs/LEs. During this maturation, endosomal-sorting-complex-required-for-transport (ESCRT) internalizes membrane proteins into intraluminal vesicles. Once the external membrane of the MVB/LE fuses with the tonoplast, the intraluminal vesicles containing the proteins are released into the vacuole and degraded. Nonubiquitinated cargo can be retrieved in MVBs/LEs by the action of the retromer complex that antagonizes the ESCRT complex (90).

6.1. From PI4P to PI3P and $PI(3,5)P_2$: Phosphatidylinositol Phosphate Cascade Leading to Protein Sorting

One of the main characteristics of PIPs is that they are quickly interconverted by kinases and phosphatases (**Figure 1*c***). This is well illustrated along the road leading to protein degradation in the vacuole, in which the different maturation steps correlate with a phosphoinositide interconversion (**Figure 7**). $PI(4,5)P_2$ 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 VACUOLAR PROTEIN SORTING34 (VPS34). Some MVBs/LEs exhibit $PI(3,5)P_2$ owing to the action of the PI3P5K from the FAB1 family (38, 40, 117) (**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/LE by interacting with PI3P (24, 57). Similarly, SORTING NEXIN 1 (SNX1), a component of the retromer complex, also localizes at the maturing TGN/EEs and MVB/LE through interactions with PI3P, $PI(3,5)P_2$, or both (103). In addition, PI3P, $PI(3,5)P_2$, or both also mediate the association of endosomes with the cytoskeleton, since the loss of FAB1 releases MVBs/LEs from cortical microtubules (37, 38) and SNX1 interacts with the microtubule regulator CYTOPLASMIC LINKER ASSOCIATED PROTEIN (1).

6.2. Autophagosome and Protein Degradation

Cytosolic cargoes can also be degraded by autophagy (22). In this process, cargoes are encapsulated by a double membrane to form autophagosomes. The outer membrane of the autophagosome fuses with the tonoplast, and the internal membrane, together with its luminal content, is subsequently degraded in the vacuole (22). 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 via genetic screens in yeast, but most of the actors of autophagy are

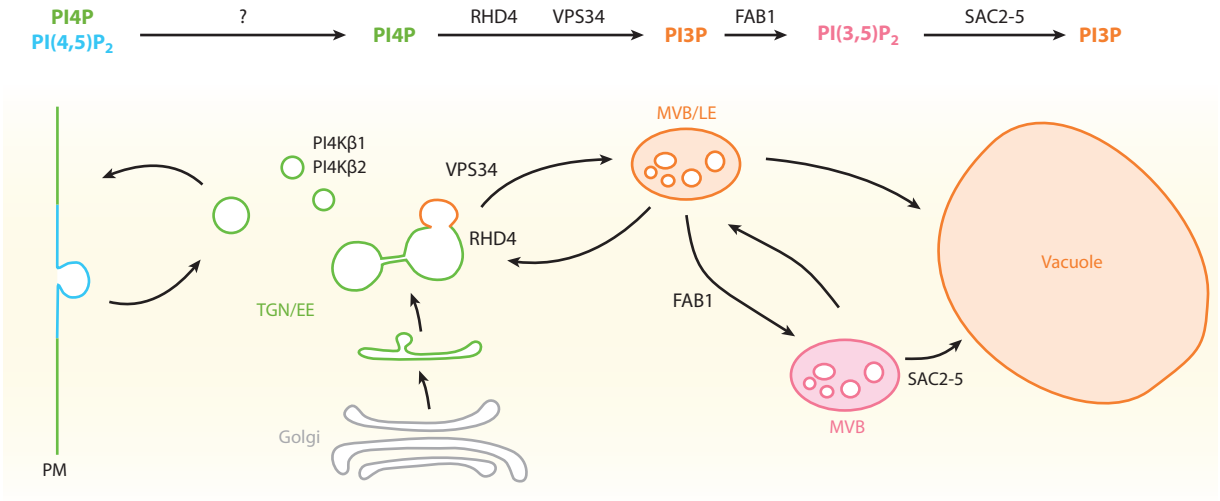

Figure 7

Phosphoinositide cascade during endocytic trafficking to the vacuole. Kinases and phosphatases create a phosphoinositide cascade along the endocytic route. Abbreviations: TGN/EE, *trans*-Golgi network/early endosome; FAB1, FORMATION OF APLOID AND BINUCLEATE CELLS 1; MVB/LE, multivesicular body/late endosome; PI3P, phosphatidylinositol 3-phosphate; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; RHD4, ROOT HAIR DEFECTIVE 4 (also called SAC7); SAC, SUPPRESSOR OF ACTIN; VPS34, VACUOLAR PROTEIN SORTING34.

conserved among eukaryotes (22). 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 (22). A member of the complex, ATG14, targets the PI3K complex to the phagophore (17). 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 in plants, which raises the question of how the PI3K complex is recruited to the phagophore (22). Yet inhibition of PI3Ks blocks autophagy in plants (147), suggesting that, like in other eukaryotes, PI3P is important for this process.

In addition to stabilizing ATG proteins 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 (154). 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 colocalizes with ATG8 in the phagophore in a VPS34-dependent manner (154). SH3P2 interacts with ATG8 and the PI3K complex. The *fyve1/free1* mutant presents an autophagy-related phenotype with accumulation of autophagic structures (24, 154). FYVE1/FREE1 interacts with ATG8, the PI3K complex, and SH3P2 (25). 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, from a missorting of SNARE proteins (53).

6.3. Phosphoinositides and Vacuole Morphology

Mutants for the retromer and the ESCRT complexes present small, unfused vacuoles (24, 25, 48, 56, 57). This phenotype is also found with the loss of function of vesicle transport v-SNARE11 (VTI11), a SNARE protein involved in the docking and fusion of MVB/LE with the tonoplast (129). PI3P-dependent protein sorting may be important for the proper localization of SNARE proteins and thus vacuole fusion. Moreover, the vacuole phenotype of the *vti11* mutant can be rescued by inhibition of PI3K activity, suggesting a role of PI3P in fusion of homeotic vacuoles (152). The homotypic fusion and vacuolar protein sorting (HOPS) tethering complex is required for vacuole morphology (12). VPS33 and VPS45, two HOPS complex members, bind phosphoinositide in vitro and localize to the vacuole in a PI3P-dependent manner (12). SAC2, SAC3, SAC4, and SAC5 are likely PI(3,5)P_2 5-phosphatases that localize at the tonoplast and could hydrolyze the incoming PI(3,5)P_2 from MVBs/LEs into PI3P (91) (**Figure 7**). Loss of function and overexpression of these SAC proteins lead to abnormal vacuolar morphology, consistent with a deficit in vacuole fusion due to lack of PI3P (91). PI4P is also involved in the morphological changes of the vacuole that are regulated by auxin in the root (72, 111). However, the molecular mechanisms 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 presents several specificities compared to yeast or animal cells, starting with the high and low abundance of PI4P and PI(4,5)P_2 at the PM, respectively. Thus, the establishment of the anionic lipid distribution in plants 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 gene expression. Recently, MCSs appeared as a new path of lipid exchange independent from vesicular trafficking. The mechanisms of this exchange, the nature of the exchanged lipids, and the relevance for the cell and plant life remain to be fully appreciated.

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

SUMMARY POINTS

1. Anionic lipids influence the physicochemical properties of cell membranes, such as their surface charges, curvature, packing, and nanoscale organization.
2. Anionic lipids define an electrostatic membrane territory in plants, which corresponds to post-Golgi membranes.
3. Enrichment of specific anionic lipid combinations in different compartments participates in the establishment of organelle identity and the specific recruitment of proteins.
4. The distribution of anionic lipids inside a membrane is not homogeneous in terms of lateral segregation and membrane leaflet accumulation. Anionic phospholipids organize in nanoclusters, together with other lipids and proteins, and are mostly oriented toward the cytosolic leaflet.
5. Most of the enzymes related to anionic lipids are conserved among eukaryotes, while anionic lipid effectors tend to be plant specific.
6. Anionic lipids can be transported via either vesicular trafficking or lipid transport proteins at membrane contact sites (MCSs). Conversely, anionic lipids are themselves master regulators of both vesicular trafficking and MCSs.
7. Phosphoinositide kinases and phosphatases allow the fast production and interconversion of phosphoinositide species, which ensure relatively stable patterns of phosphoinositide subcellular accumulation despite the constant flow of lipids between organelles.
8. Anionic lipid repartition is a dynamic feature of the cell and can change according to its physiological (e.g., infection, symbiosis, stress) or developmental (e.g., polar growth, cytokinesis, aperture establishment, Casparian strip formation, trichrome) status.

FUTURE ISSUES

1. How does the cell maintain lipid homeostasis at both cellular and developmental levels? Addressing this question will require a deep understanding of how lipid production and degradation or consumption are coupled and regulated and how each anionic lipid may work in functionally and physically independent pools within the same cell.
2. Most enzymes involved in anionic lipid homeostasis have been described, but how their activity is regulated is still unknown. Investigations into the role of post-translational modifications (e.g., phosphorylation) have been spearheaded by Ingo Heilmann's group, and this is an area of research that is likely to expand in the future (35, 84).
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 chain of events links defects in anionic lipids to the observed cellular or whole-plant phenotypes. One of the keys to unlocking such a 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, sterols, 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/plasma membrane/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 MCSs, 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 (PD), and *trans*-Golgi network (TGN).
7. The development of anionic lipid sensors compatible with live-imaging and super-resolution microscopy techniques has allowed analysis of 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 made to improve the current methods of anionic lipid identification by mass spectrometry and cellular fractionation.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

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

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