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Review

Plant lipids: Key players of plasma membrane organization and function

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ABSTRACT

The plasma membrane (PM) is the biological membrane that separates the interior of all cells from the outside. The PM is constituted of a huge diversity of proteins and lipids. In this review, we will update the diversity of molecular species of lipids found in plant PM. We will further discuss how lipids govern global properties of the plant PM, explaining that plant lipids are unevenly distributed and are able to organize PM in domains. From that observation, it emerges a complex picture showing a spatial and multiscale segregation of PM components. Finally, we will discuss how lipids are key players in the function of PM in plants, with a particular focus on plant-microbe interaction, transport and hormone signaling, abiotic stress responses, plasmodesmata function. The last chapter is dedicated to the methods that the plant membrane biology community needs to develop to get a comprehensive understanding of membrane organization in plants.

1. Introduction

The Plasma Membrane (PM) is a key structure protecting the cell, regulating nutrient exchanges and acting as a control tower allowing the cell to perceive signals. Plasma comes from the greek πλάσμα meaning "which molds", meaning that the PM takes the shape of the cell by delimitating it. The PM harbors the appropriate signaling cascades allowing adaptive responses ensuring proper cell functions in a

Abbreviations: ASG, Acylated Steryl Glycosides; BY-2 cell, tobacco Bright-Yellow 2 cell culture; CAZyme, CArbohydrate-active enZymes data base; CER, CERamides; DOPC, 1,2-DiOleoyl-sn-glycero-3-PhosphoCholine; DPPC, 1,2-DiPalmitoyl-sn-glycero-3- PhosphoCholine; DRM, Detergent-Resistant Membrane fraction; DRP1A, Dynamin-Related Protein1A; ER, Endoplasmic Reticulum; EVs, Extracellular Vesicles; FRET, Fluorescence Resonance Energy Transfer; G proteins, Guanine nucleotide-binding proteins; GIPC, Glycosyl Inositol Phosphoryl Ceramides; gluCer, glucosylCeramide; GT, GlycosylTransferases; GUV, Giant Unilamellar Vesicles; hVLCFA, 2-hydroxylated Very Long Chain Fatty Acid; ISO, Inside Out Vesicles; K-Ras, Kirsten RAt Sarcoma virus; Ld, liquid-disordered; Lo, liquid-ordered; MCS, Membrane Contact Site; MSC, Membrane Surface Charge; PALM, Photo-Activated Localization Microscopy; PAMPs, Pathogen-Associated Molecular Patterns; PC, Phosphatidylcholine; PdBG2, Plasmodesmata Beta-1,3-Glucanase 2; PDCB1, PlasmoDesmata Callose Binding 1; PDs, plasmodesmata; PIN, PINformed; POPC, palmitoyl-oleoyl-PC; PRR, Pattern Recognition Receptor; REM, REMorin; RGS1, Regulator of G-protein signaling; RSO, Right Side Out vesicles; SG, Steryl Glycosides; SIM, Structured Illumination Microscopy; SMLM, Single-Molecule Localization Microscopy; So, solid-ordered; STED, Stimulated Emission Depletion Microscopy; STORM, Stochastic Optical Reconstruction Microscopy; VLCFA, Very Long Chain Fatty Acid

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continuously fluctuating environment, crucial for cell survival. To address this challenge, the PM needs to be both stable and robust yet incredibly fluid and adaptable. This amazing combination of long-term stability and short-term dynamics in order to adapt to signals relies on its fascinating molecular organization. PMs are extremely complex systems, harboring many different molecular species of lipids in which heterogeneity is more likely to occur than homogeneity. In plants as in animals, the recent development of proteomics, lipidomics and methods to visualize lipids and proteins *in vivo* has greatly increased our knowledge of the PM.

The combination of biophysical, biochemical, and cell biology approaches, recently including super-resolution imaging both of the PM's physical state and of the nanometric distribution of its constituents has significantly broadened our vision of PM organization. In this update review, we will present the current state of knowledge of the plant-PM lipid composition, then we will examine how lipids govern the nano- and micro-scopic properties and organization of the plant PM. We will illustrate how the available data show that lipids are not distributed homogeneously within and between each leaflet of the PM. The role of interdigitation and registration between these two leaflets will be also discussed. Finally, we will show how lipids contribute to the organization of the PM, and how this organization plays a decisive role in a certain number of essential processes of plant cell physiology including immunity, abiotic stress and cell-to-cell communication through plasmodesmata. The involvement of lipids as signaling second messenger molecules are not reviewed in details here, except for plant microbe interactions, and we prompted the readers to refer to reviews [1].

2. Update on the lipid content of plant PM: how to visualize them?

The PM is an asymmetric proteo-lipidic matrix. The lipid-to-protein ratio (mass/mass) was experimentally determined to be close to 1.3 in tobacco PM [2]. Therefore, one can estimate a molar ratio of 1 protein for 50–100 molecules of lipids. Proteomic data on purified plant PM identified *ca.* 500–1000 proteins in the PM, and the lipidome is theoretically made up of thousands molecular species of glycerophospholipids, sphingolipids and sterol-based structures [3]. A conserved feature of cellular organelles is the distinct lipid composition of their membranes, essential to specify their identity and function.

Highly purified RSO (right side out) PM vesicles are easily obtained using a two-phase aqueous polymer partition system from various plant material [4]. Enzymatic reactions or western blotting are generally used to address the purity of the PM fractions and the absence of contaminants. In parallel, development of high-throughput lipidomic methods by LC-MS allow the complete characterization of the main class of lipids present in the plant PM [5,6]: phospholipids [7], phosphoinositides [8], sphingolipids [9,10] and sterols [11,12]. Such procedures allow the characterization of the molecular species of each lipid class at a level of detail including the fatty acid position for glycerolipids, the nature of long-chain bases for sphingolipids and the many classes of phytosterols [13]. Besides these biochemical tools, strategies have been developed to visualize lipids *in vivo* using biosensors showing affinity for lipids. Imaging lipidomics have also been developed, particularly in seeds [14,15] but the resolution is not yet high enough to allow the characterization of lipids inside a given membrane. Recently, "Imaging lipidomics: automated MS imaging of tissue with lipid structure identification" by Ellis et al. (Nature Methods) reported a method that enables the acquisition of lipid tandem mass spectrometry data in parallel with a high-resolution mass spectrometry imaging experiment. Authors developed a lipidome-per-pixel approach able to identify in rat cerebellar tissue hundreds of lipid molecule species and their spatial locations [16,17]. Nano-SIMS (Secondary-ion mass spectrometry) has also been developed with labeled lipids allowing the deciphering of lipid segregation in the plane of the PM in animal cell culture with a lateral resolution of 90 nm [18]. This high-resolution method is yet to be introduced in plants as the cell wall could strongly impair access to the PM.

2.1. Glycerolipids: galactolipids, phospholipids and phosphoinositides

Phospholipids represent *ca.* 30% of tobacco PM lipids [2]. As can be expected, Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) are the major phospholipids of plant PM with palmitic and linoleic acids as main acyl chains [7,19–24]. Phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylserine (PS) are minor phospholipids. Among these phospholipids, only PS is associated with a high proportion of very long chain fatty acids *e.g.*, behenic C22 and lignoceric C24 acid.

Polyphosphoinositides or phosphatidylinositol-phosphates (PIPs) represent a minor fraction of total phospholipids; they are composed of a PI backbone with up to 3 phosphorylations on the inositol moiety. PIPs are involved in many regulatory processes, such as cell signaling and intracellular trafficking. Membrane compartments are enriched or depleted in specific PIPs, providing a unique signature for these compartments. The precise subcellular localizations and dynamics of PIPs were revealed in plants thanks to the design of genetically encoded biosensors with distinct relative affinities [25,26]. Recently, a full set of phosphoinositide biosensors was generated in *Arabidopsis thaliana* called "PIP-lines" [27]. This library extended the range of available PIP biosensors and allowed rapid progress in the understanding of PIP dynamics in plants as well as its monitoring *in vivo*, see below. Hence, not only phosphatidylinositol-4-phosphate (PI4P), phosphatidylinositol-3-phosphate (PI3P), phosphatidylinositol-4,5-bisphosphate (PI4,5P_2), phosphatidylinositol-3,5-bisphosphate (PI3,5P_2), phosphatidylinositol-3,4,5-triphosphate (PI3,4,5P_3), but also PS and PA can be visualized *via* these biosensors [27–30]. Quantitative imaging analysis revealed that there is a gradient of PI4P throughout the cell, with the highest concentration at the PM, intermediate concentration in post-Golgi/endosomal compartments, and the lowest concentration in the Golgi apparatus. A similar gradient of PI3P was observed from high concentrations in late endosomes to low concentrations in the tonoplast. Inside the PM, polyphosphoinositides (PI4P and PI4,5P_2) were enriched in detergent resistant membranes (DRMs, see below) compared with the whole PM, suggesting that PIPs could be present inside domains at the PM [31]. This hypothesis was further supported by the visualization of nanodomain-like clustering by immunogold labeling [31]. Importantly, PIPs and PS influence membrane biophysical properties, which emerge as important features in specifying cellular territories; this is discussed in the chapter 2.

Note that Digalactosyldiacylglycerol (DGDG), present in plastids, is also found in the plant PM particularly in response to phosphate deprivation [32,33]. Neutral lipids like diacylglycerol (DAG) were also visualized *in vivo* [34] and showed to be present at the PM of root epidermal cells in the transition zone, at the trans-Golgi network, the cell plate during cytokinesis, and the apex of growing root hairs.

2.2. Sphingolipids

Sphingolipids are ubiquitous in eukaryotes with a sphingoid backbone called the long-chain amino-alcohol base (LCB). They are abundant and essential components of biological membranes and they can represent up to 10% of total lipids in plants [35] Detected for the first time in 1870 in brain samples, their name comes from the greek Σφίγξ "to squeeze, to strangle" related to the strong amide bond that composes the link between their two lipophilic moieties and with an allusion to the Sphinx for the cryptic nature of these lipids at the time of their discovery. In animal PMs, the main sphingolipid class is sphingomyelin, which is not present in plants. Minor sphingolipids called gangliosides are a class of acidic glycolipids that play an important role in immunity and modulate cellular signal transduction events [36].

Plant sphingolipids are of four major classes: ceramides (CER), glucosylceramide (gluCer), Glycosyl Inositol Phosphoryl Ceramides

(GIPC) and free Long Chain Bases (LCBs), representing *ca.*, 2%, 34%, 64% and 0.5% of total sphingolipids, respectively in *Arabidopsis thaliana* [10]. In addition to the PM, sphingolipids are enriched in endosomes and tonoplasts, representing around 10 to 20% of total membrane lipids [37]. The complex structural diversity of plant sphingolipids arises from the possible occurrence of three very diverse building blocks: the polar head, the fatty acyl chain linked by an amide bond (forming a ceramide) to the LCB [38].

In this review, we will mostly focus on GIPC because recent discoveries on the role of these lipids in the organization of the PM [39] and as toxin receptors [40] swung them into the spotlight. GIPC are representatives of a class of acidic glycolipids from plants, possibly analogous to the acidic gangliosides found in animal cell membranes. They have been discovered during the late 1950's by Edward Carter [41], studied during 20 years and later forgotten until the beginning of the 2000's, see below. GIPC are composed of a ceramide and a glycan polar head group. The diversity of GIPC resides in: 1/the length, the number and position of hydroxylations and unsaturations in the FA chain; 2/the hydroxylation degree, saturation and position of double bond(s) in the LCB; 3/the nature and the number of glycans, and the type of glycosidic links between the glycans that compose the polar head group [38,42]. In general, the ceramide moiety of plant GIPC consists mainly of a t18:0 (trihydroxylated saturated LCB of 18 carbon atoms) or a t18:1 (trihydroxylated and monounsaturated LCB of 18 carbon atoms) as LCB, amidified to a Very Long Chain Fatty Acid (VLCFA) or 2-hydroxylated VLCFA (hVLCFA). Hence, 95 mol% of PM VLCFA and hVLCFA are amidified in GIPC [39]. The polar head of GIPC is made up of a phosphate linked to an inositol to which glycan moieties are bound. The degree of glycosylation of the GIPC polar head groups defines the different GIPC series.

The basic structure of the GIPC polar head is an inositol phosphoryl ceramide (IPC) backbone linked to a glucuronic acid (GlcA). A sugar unit bound to GlcA-IPC forms the series A GIPC. Only a few structures have been fully resolved with the exact sugars and the nature of the sugar bond: tobacco series A GIPC has the most basic known structure: GlcNAc(α1- > 4)GlcA(α1- > 2)inositol-1-O phosphorylceramide [43]. Additional sugar moieties such as glucosamine (GlcN), *N*-acetylglucosamine (GlcNAc), arabinose (Ara), galactose (Gal) and mannose (Man) may lead to glycan patterns of three to seven sugars, so-called series B to F GIPC, see Fig. 1. GIPC found in corn seeds display branched polar heads, see for review [44]. These series are species- and tissue-specific [10,45–48]. In Arabidopsis, series A Man-GlcA-IPC is predominant in leaves [45,49], and GlcN(Ac)-GlcA-IPC is mainly in seeds and leaves [50], seeds [51], as well as in cultured tissues of rice [52] and tobacco [45]. The core structure of series B, predominant in monocots is yet to be deciphered. A broad study of the GIPC polar head of 23 plant species from algae to monocots [46] further showed that polar head structures are largely unknown and versatile for the different biological taxa. Kaul and Lester calculated the ratio between carbohydrate/LCB/Inositol in purified polyglycosylated GIPCs and showed that GIPCs may contain up to 19–20 sugars [53].

The polar head also accounts for the high polarity of the GIPC and its subsequent insolubility in traditional lipid extraction solvents, such as chloroform/methanol (2/1, v/v). Hence, even 50 years after their discovery, the structure and character of GIPC remain elusive. GIPC are not commercially available but different purification procedures have been published [45,49,53–55]. With the emergence of more comprehensive extraction techniques and technological advances in the field of sphingolipidomics over that past decade, more accurate quantification of sphingolipids and the discovery of novel structures are underway.

While the synthesis pathway of gangliosides, their animal counterparts, are well studied, that of plant GIPC remain uncharacterized. The biosynthesis of sphingolipids starts with the condensation of serine and palmitoyl-CoA in the Endoplasmic Reticulum (ER), catalyzed by serine palmitoyl transferase (SPT) forming the 3-ketosphinganine [38]. The second step is the reduction of 3-ketosphinganine by the enzyme 3-ketosphinganine reductase (KSR) generating sphinganine (d18:0), the most basic LCB. The condensation of an LCB with a fatty acyl chain (in the case of GIPC a VLCFA) by ceramide synthases also known as Lag 1 Homolog or LOH 1,2 and 3 produce a ceramide. The specificity of these enzymes relies on the length of the acyl chain and the hydroxylation degree (di- or tri-hydroxylation) of the LCB [56]. The next steps are the modifications of LCBs by the LCB C-4 hydroxylase, Δ4 desaturase, and Δ8 desaturase arising up to nine different LCB structures [9]. LCB unsaturation steps are estimated to occur after condensation into ceramide (discussed in [57]). Although this is not fully evidenced in plants, but free LCB composition in plants supports this statement (much lower unsaturation rate than complex ceramides) and animal delta-4 desaturase does catalyze unsaturation of [58].

Ceramides can also be phosphorylated in the ER by ceramide kinases CERK or ACD5 [59]. There can also be a hydroxylation of the alpha-carbon of the fatty acyl chain [35,45] yielding hydroxyl-ceramide. The hydroxylation of sphingolipids likely plays a role in the interaction of the hydroxyl group between GIPC and with sterols in the PM [60,39]. The enzymes responsible for the hydroxylation have been identified in Arabidopsis, named FAH 1 and FAH2. As biosynthetic intermediates, ceramides are used in the synthesis of the two major PM sphingolipids: GluCer and GIPC accounting for 5–10% and 40 mol% of PM lipids, respectively [2]. The synthesis of GluCer is located in the ER and is catalyzed by plant a glucosylceramide synthase (GCS) with sterol glucoside (SG) acting as a glucosyl donor [61]. The ceramides are converted to GIPC by several glycosylation steps in the Golgi apparatus. The first enzyme involved in the synthesis of GIPC is the inositol phosphorylceramide synthase (IPCS) converting ceramide into inositol phosphorylceramide (IPC). In plants and fungi, PI is the donor of the phosphorylinositol moiety [62]. This enzyme first identified as ERH1 (enhance RPW8-mediated Hypersensitive response-like cell death) in plant holds a key role in modulating plant programmed cell death associated with defense [62]. The pool of ceramide for GluCer or GIPC synthesis is determined by the hydroxylation state of the LCB and acyl chain length. In Arabidopsis seedlings, trihydroxy-LCBs (mostly t18:1) are predominant in both GIPC and GlcCers. GIPC are characterized by the presence of t18:0 largely associated with VLCFAs while GlcCer are composed of dihydroxy-LCBs (d18:1 Δ8) in association with 16–18 carbon atom fatty acids (C16) [9]. In both cases, the KO mutation of GCS or IPCS leads to dramatic functional and developmental impairments.

The second enzyme of the GIPC synthesis pathway is the inositol phosphoceramide glucuronosyltransferase (IPUT1). IPUT1 encodes an IPC glucuronosyltransferase, transferring an alpha-glucuronic acid (GlcA) residue onto the IPC backbone. It was the first GIPC glycosylation enzyme to be characterized. The silencing of IPUT1 triggers the accumulation of IPC in *Nicotiana benthamiana*, as well as ceramides and GluCer. Its overexpression increases GIPC content. In Arabidopsis, IPUT1 is essential for pollen tube viability. The major defect of the *iput1* mutant pollen is a disfunction in tube guidance and ovule fertilization [63]. Further glycosylation patterns of GIPC and glycosyltransferases involved are still not well documented. So far only three more proteins involved in the glycosylation process have been characterized. Understanding the diversity of sugar moieties of the polar head and all the biosynthetic pathways involved remain a challenge. Golgi-localized nucleotide sugar transporter (GONST1) was shown to be indirectly involved in GIPC synthesis by specifically supplying GDP-mannose to the Golgi lumen for GIPC glycosylation. Interestingly, in *gonst1* mutants, only mannosylation of GIPC is defective, while that of the cell wall polysaccharides remain unchanged [47]. The mutants also have a dwarfed phenotype and display spontaneous Hypersensitive Response highlighting the importance of GIPC sugar head groups in different plant functions such as defense signaling. Alongside GONST1, GIPC mannosyl-transferase (GMT1) of the GlycosylTransferases (GTs) found in the Carbohydrate-Active Enzymes data base (CAZyme, http://www.cazy.org), located in the Golgi and specifically targeting GIPC has

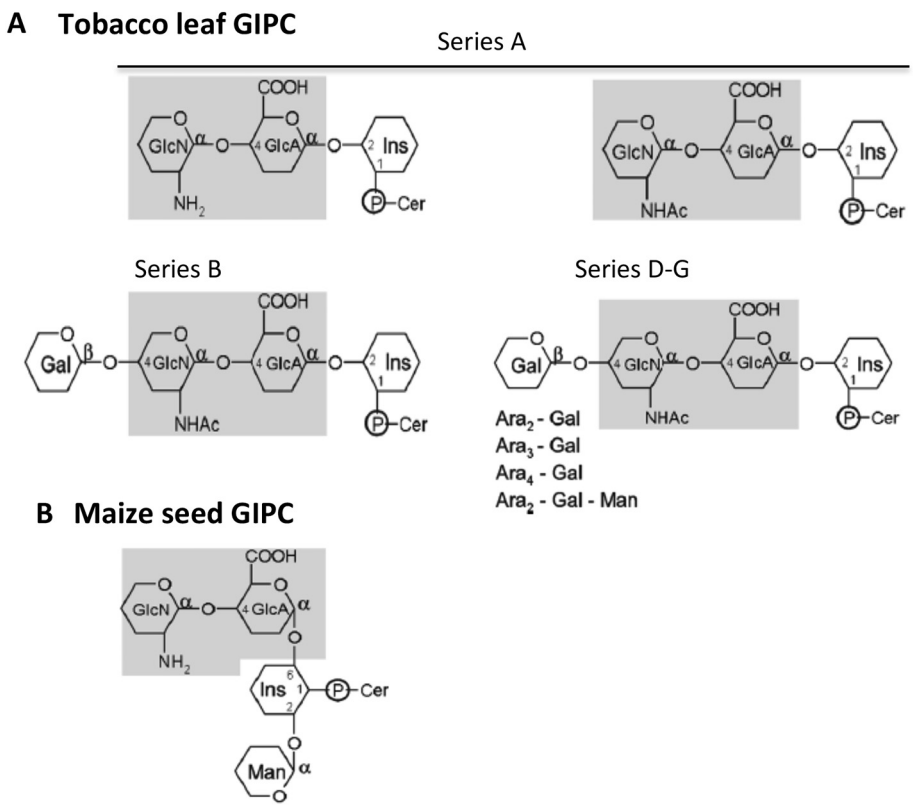

Fig. 1. Determined structures of GIPC glycosidic polar head from tobacco and maize.
A, tobacco GIPC of series A are major in tobaco leaves (top) with glucuronic acid (GlcA) and either glucosamine (GlcN) or *N*-acetyl glucosamine (GlcNAc). Other minor polar head of series B and higher glycosylated GIPC with arabinoase (Ara), galactose (Gal) and Manose (Man) have been identified, but the precise structure remains to be determined. Grey part is the conserved glycan moitie of glucuronic-Hex. Cer indicates the ceramide moitie, in tobacco, with t18:0 and t18:1 for LCB, and VLCFA mostly alpha 2-hydroxylated; B, GIPC found in corn seeds with branched polar head.

recently been reported to transfer a mannose (Man) onto the GIPC head group by Mortimer team [49]. The phenotype of *gmt1* mutant is fairly similar to that of *gonst1* affecting GIPC mannosylation level, displaying a constitutive plant immune response and reducing cellulose content. In plants, GIPC are highly glycosylated with the most common pattern being a GlcA-IPC to which additional glycan moieties such as Man but also glucosamine (GlcN), *N*-acetyl glucosamine (GlcNac) and arabinose (Ara) can be attached [42,45]. The most recent GT identified is glucosamine inositolphosphorylceramide transferase1 (GINT1). It is involved in GIPC glycosylation in seeds and pollen yielding GIPC containing GlcNac and/or GlcN. The study also showed the importance of GlcN(Ac) GIPC in rice seedling survival suggesting once again the importance of GIPC glycan patterns in essential and specific plant functions [64].

Beside biochemical methods (*i.e.* purification of PM coupled with lipidomic analyses), the location of sphingolipids is hardly known *in vivo* due to the lack of appropriate biosensors and fluorescently-labeled lipid-probes. Only one publication reported lipid staining protocols and the use of several fluorescent lipid analogues in Arabidopsis leaf tissue and protoplasts [65]. As stated earlier, tobacco PM contains GIPC representing up to 40 mol% of total tobacco lipids, enriched in the outer-leaflet and interacting with sterols in the formation of microdomains of *ca.* 35 nm. GIPC are further enriched in the DRMs. GIPC in DRMs are in their polyglycosylated forms [39]. Modeling approaches of the organization of lipids in the plant PM proposed that acyl terminal ends (six to seven carbon atoms) of the apoplastic leaflet (h)VLCFA of GIPC penetrate within the inner-leaflet and interdigitate with carbon chains of the inner-leaflet phospholipids [39]. This remains to be fully determined *in vivo*. Interdigitation is an interesting phenomenon which could explain the limited diffusion of proteins in the PM and thermal adaptation [66]. All these aspects of GIPC function will be further discussed in this review.

2.3. Free and esterified phytosterols

The amount of sterols is relatively stable among plant species *i.e.* 2–3 mg of total sterols per dry gram plant. Synthesized in the ER, sterols accumulate in the PM and reach up to 30 mol% of PM lipids [2]. Higher-plant cells contain a vast array of sterols: *e.g.* 61 sterols and pentacyclic triterpenes have been identified in maize seedlings [67]. Sterols are characterized by a planar sterol backbone made up of four condensed aliphatic rings. Phytosterols are mainly C28 and C29 desmethylsterols with nine or ten carbons side chains. In most cases, the second ring has a double bond between carbon C5 and C6. Phytosterols mainly differ from mammalian cholesterol on the side chain by an extra alkyl group in the C24 position (Fig. 2A, B). For example, campesterol is the phytosterol whose chemical structure is the most similar to that of cholesterol, with only an additional methyl group. In contrast, the Δ^5-sterols, with an ethyl group, are represented by β-sitosterol and stigmasterol which contain an additional double bond in the C22 position. The most abundant plant sterol is sitosterol in most species reported including the plant model Arabidopsis, except for a few cases such as stigmasterol in tobacco, spinasterol in Medicago and isofucosterol (delta-5 avenasterol) in many plants [68]. Proportions of other pathway end-products are genetically defined in higher plants [69]. In comparison with cholesterol, the interesting fucosterol that is the major sterol in green algae exhibits modifications at the hydrocarbon tail with a branched chain and a double bond at position C24. Biosynthesis of phytosterols is described in recent reviews [68].

Steryl glycosides (SGs) and acylated steryl glycosides (ASGs) are derivatives of a typical membrane-bound sterol molecule (Fig. 2C). The composition of sterols in SG reflects usually the free sterol composition of the plant. The sugar moiety, the number of sugar and the configuration of its linkage to the sterol may vary. The sugar moiety, most common being the pyranose form of D-glucose, is attached to the 3-hydroxy group at the C3-atom of a sterol and a hydrocarbon side chain at C17. SGs generally carry one or more sugar residues, the steryl D-monoglucopyranoside being the most abundant SG in plants. Finally, an

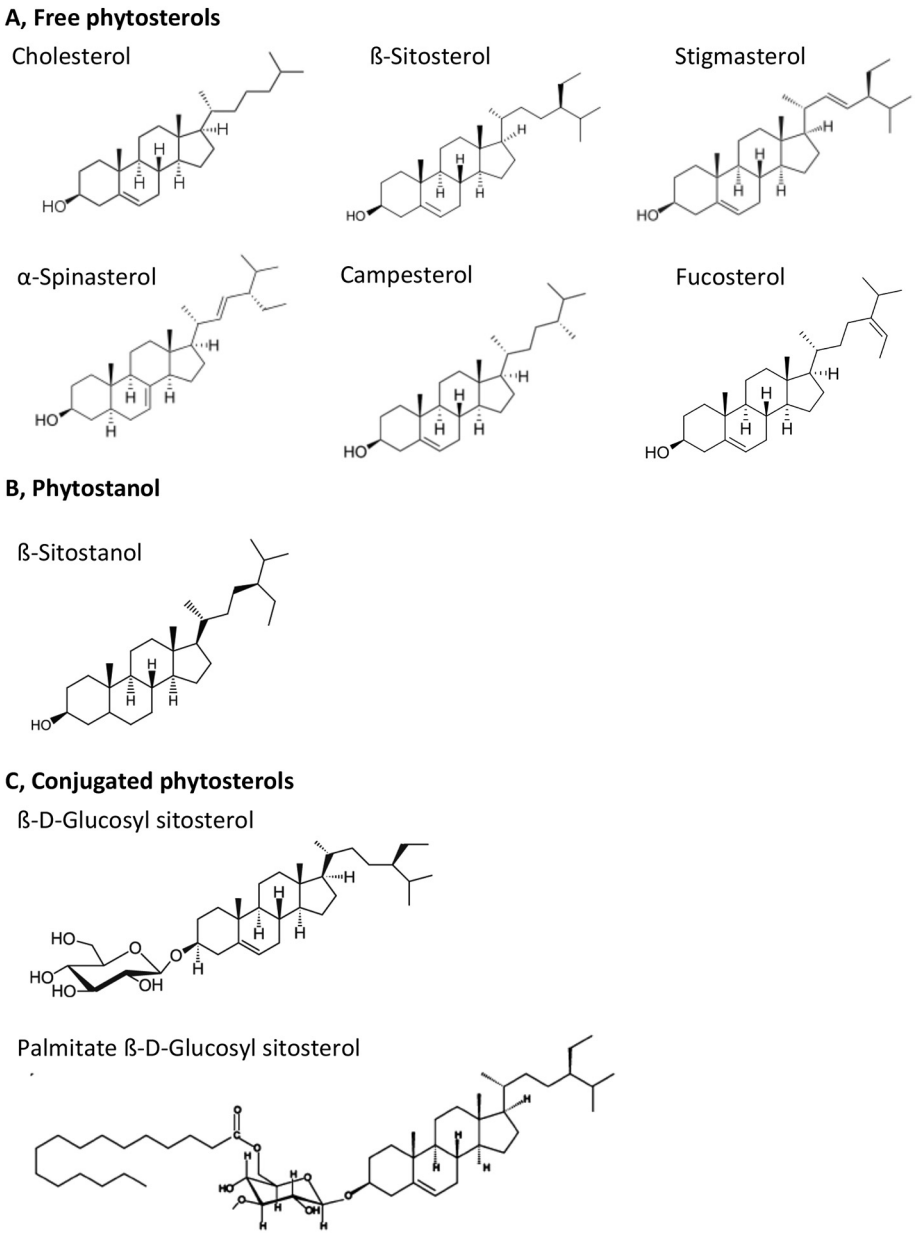

Fig. 2. Structures of specific plasma membrane phytosterols compared with animal cholesterol.
A, free phytosterols; B, phytostanol; C, conjugated phytosterols.

acylation of the sugar moiety could increase SG diversity producing ASG forms. Indeed, SGs may be acylated, usually at the C6-atom of the sugar moiety with palmitic, oleic and less frequently with stearic, linoleic, and linolenic acid. Interestingly, the proportions of SG and ASG in PM differ extremely depending on the plant species and the growth conditions [22,70]. The biological role of conjugated sterols has been discussed recently in [68,71].

Elucidation of sterol function benefit from the development of tools for *in situ* visualization, together with forward and reverse genetic approaches are reviewed in. Several methods to visualize sterols have been developed, for review [72]. Filipin has been extensively used as a specific probe for detection of fluorescent filipin-sterol complexes, including on fixed samples. It is the only established tool for sterol visualization in plants [73–76]. Although powerful to visualize domains enriched or deprived in sterol, filipin has also been used to measure the asymmetrical distribution of sterol using purified Right Side Out (RSO) *vs.* Inside Out (ISO) PM oat vesicles [77]. Recently, imaging method using tunable orthogonal cholesterol sensors allowed simultaneous *in situ* quantification of cholesterol in two leaflets of various mammalian cell PM [78]. This study revealed a marked transbilayer asymmetry of PM cholesterol, with the concentration in the inner leaflet being 12-fold lower than that in the outer leaflet. The asymmetry was maintained by active transport of cholesterol and its chemical retention in the outer leaflet [78]. Development of such sensors for phytosterols (free and conjugated) is of great importance in order to address the role of these lipids in plant biology.

3. Lipids govern global properties of the plant PM

3.1. Fluidity of PM

Confined in a restricted two-dimensional space, PM constituents are mobile and animated with membrane fluidity reflecting the dynamic organization of biological membranes [79]. Hydrocarbon chains perform balance and bending movements, giving elasticity to the PM. These undulations are sources of fluidity and can be measured by

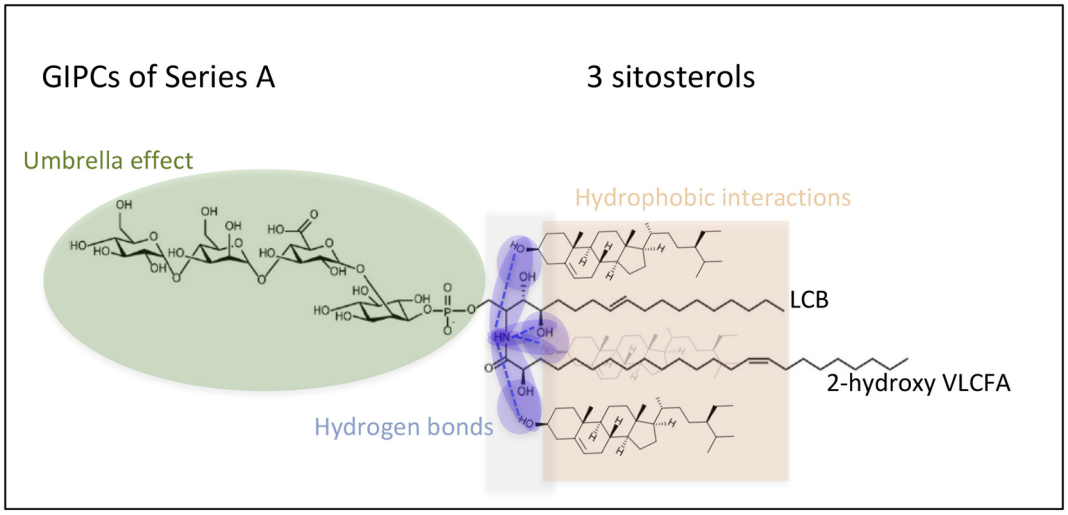

Fig. 3. Biophysical features involved between a GIPC of series A and three molecules of sitosterols.
These interactions are important for nanodomain formations in the PM. LCB, Long Chain Base; VLCFA, Very Long Chain Fatty Acid.

atomic force microscopy that regrettably shows its real limits of use in plants due to the presence of the cell wall. Lipids and proteins by rotating around their axis or moving in the plane of the PM, lead to increase fluidity. Notably, lateral diffusion within plant PM was firstly evaluated around the order of 0.02 $\mu m^2.sec^{-1}$ for the agglutinin receptor of the PM of wheat grain cells [80], whereas more recent data gives a more than ten-fold higher value of 0.34 $\mu m^2.sec^{-1}$ for the flagellin receptor FLS2 in Arabidopsis protoplast [81]. Indeed, PM proteins exhibit distinct relatively low short-distance lateral mobility within plant PM [82,83]. Depending on the lipid environment, the diffusion of labeled tracer molecule also varies from 0.1 to 6 $\mu m^2.sec^{-1}$ in model membranes [84] highlighting the effect on lipid dynamics of unsaturated and saturated PC and cholesterol. Thus, several factors affect PM fluidity, notably the steric hindrance and the interactions of its constituents. The huge diversity of plant lipids, many of which deviate from the canonical cylindrical form, would thus imply that the PM is bound to be very heterogenous in its geometrical arrangement [85]. For example, PC occupies similar volumes at both its extremities *i.e.* its polar head and its two acyl chains, corresponding to a cylinder, this geometry generates a spontaneous organization in lamellar phase [86]. Furthermore, the level of unsaturation results in a larger steric hindrance of the carbon chain, and therefore a greater disorder in the arrangement of the lipids.

Alterations in lipid composition during cold acclimation have been known to be associated with increase in tolerance of plants to cold stress *e.g.* [87]. In particular, accumulation of *N*-acylphosphatidylethanolamines (NAPEs) that is related to high lipid unsaturation degree is critical to maintain membrane fluidity. Indeed, changes in lipid composition regulate cryobehavior of the PM [88] by contributing to maintain the membrane phase transition temperature below the chilling temperature [89]. In cold conditions, plant cell PMs accumulate unsaturated fatty acids to decrease membrane viscosity [90]. A similar positive effect on membrane stability is achieved by a decrease in the unsaturation level of individual phospholipids and total lipids during water deprivation [23].

3.2. Phytosterols are crucial regulators of membrane order

Sterols are known to favor the packing effect in the membrane bilayer as firstly described for cholesterol in animal membranes [91]. Phytosterols are the major component contributing to plant PM rigidity [92–94], Interestingly, major free phytosterols differentially modulate the level of membrane order [95,96]. Indeed, campesterol shows a high potency to organize lipid bilayers [97,98] which could be attributed to its short hydrocarbon tail. Stigmasterol exhibits a much weaker ordering effect than other sterols [99], even if it is a somewhat controversial question [100]. This phytosterol carries an extra carbon-carbon double-bond on the side chain in the C22 position, similarly with α-spinasterol [101] and brassicasterol [95] that both also display a feeble ordering capacity. Ultrasound velocimetry studies [102] and thermodynamic analysis [103,104] propose a better condensing efficiency for β-sitosterol than for stigmasterol. Such variable ability to pack lipid bilayer has been explained by differential interactions between plant sterols and unsaturated or saturated lipids [105,106]. It is worth noting that phytosterols exist as a mixture within plant PM, and as such allows to finely control the level of membrane order in artificial membrane [99], in model PM of soybean [106] and in native PM of Arabidopsis mutants [107].

Conjugated forms of phytosterols, SG and ASG present in plant PM (Fig. 2) have also been shown to have a strong ability to order membranes [108,109]. Furthermore, free and conjugated phytosterols work in synergy to order the membrane [99]. Regardless of the distribution of SG and ASG between different phases of the PM, it seems very likely that the proportion of these lipids in certain membrane domains clearly exceeds those of phospholipids and thus could locally participate in the control of the biophysical properties of membrane domains. Regarding the scarcely documented properties of phytostanols *i.e.* the saturated analogues of sterols reduced in the double bond in a ring skeleton, Langmuir monolayer studies have evidenced that β-sitostanol (Fig. 2B) exhibited a similar ability to β-sitosterol in strongly interacting with saturated phospholipids [110]. Accordingly, incorporation of β-sitostanol into artificial membranes is able to modify their packing level as well as their behavior [111]. Overall, the multiple phytosterols are essential regulators of membrane order.

3.3. Involvement of sphingolipids in PM membrane order

Plant sphingolipids also interact with phytosterols to increase the level of plant PM order, with the notable exception of stigmasterol-GluCer association [92–94,112]. In mammalian models, cholesterol appears to interact preferentially with sphingolipids over phospholipids [113]. Several parameters have been proposed to explain such affinity: 1/sterols emphasize a better shielding from water by the bulky sphingolipid head group; 2/a pairing between the two lipids *i.e.* hydrogen bonding between these lipid species, with the low amount of water at the PM interface increasing the stability of these bonds (Fig. 3). The interface within the region of the membrane of the amide bond of the sphingolipid LCB can both donate and accept a hydrogen bond as well

as the hydroxyl groups of the LCB and Fatty acids; 3/the saturation of sphingolipid hydrophobic tails which increases the order level [114].

In plants, GIPC also show an ability to increase, in a sterol-dependent manner, the lipid packing of the PM [99] and both mechanisms could be similarly proposed (Fig. 3). First, the major GIPC polar head is composed of Hexose–glucuronic acid-inositol-phosphate, and up to seven sugar moieties can be added [45]. Thus, the volume occupied by the head group of GIPC is far much bulkier than phospholipid head groups, and, as a general trend the volume occupied by the phosphoinositol-sugar head group increases with the complexity of the oligosaccharide chain. Predictions based on the geometrical properties of glycosphingolipid molecules indicated accordingly that local enrichment of such bulkier head group strongly favors phase separation and is concomitantly accompanied by spontaneous acquisition of a positive membrane curvature (for review [36]). Moreover, GIPC LCB profiles are abundant in tri-hydroxylated LCB species in widely varying proportions (for review, see [38]), and one hydroxyl residue is very often present at the 2 position of the fatty acid. One may hypothesize that the presence of these three hydroxyl and the amide groups at the interface between the polar phase and hydrophobic phase of the bilayer may be of importance for sphingolipid/phytosterol interactions, see Fig. 3, but also sphingolipid/sphingolipid interactions [60]. Similar mechanisms have been experimentally confirmed, showing strong interactions between phytoceramides and POPC (palmitoyl-oleoyl-PC) into a highly packed gel phase (32.1 $Å^2$/molecule) [115], and between GIPC and sitosterols [2].

3.4. Electrostatic charge and pH domains of the PM

In all eukaryotes, the PM cytosolic-leaflet is the most electronegative compartment of the cell [116]. Electrostatic territories are controlled by a combination of negatively charged lipids that are organized as a gradient along the endocytic pathway. Membrane surface charge (MSC) is critical for the specific recruitment to membranes of proteins with polybasic regions. Thus, PM electrostatics is fundamental parameter in signaling, intracellular trafficking and polarity. For example, MSC controls the PM localization and function of the polar auxin transport regulator PINOID as well as proteins from the BRI1 kinase inhibitor 1 (BKI1)/Membrane-associated kinase regulator (MAKR) family, which are involved in brassinosteroid and receptor-like kinase signaling [117]. MSC can be probed by biosensors constituted of a fluorescent protein fused to an unstructured peptide of varying net positive charges [117]. Negatively charged lipids regulate the MSC in plant PM. By contrast to yeast and animals, PI4P strongly accumulates at the PM establishing a negative inner surface potential of this membrane [117]. In addition, it was recently shown that PM surface potential varies according to other negatively charged PM lipids such as PA and PS which are separately required to generate the electrostatic signature of the plant PM [118]. Therefore, the combinatorial lipid composition of the cytosolic leaflet of PM not only defines electrostatic territory but also distinguishes different compartments within this territory by specifying their MSC. How the spatiotemporal pattern of PIPs is established and maintained within plant cell is one of the many future challenges to tackle.

A recent study showed that the pH on both sides of the plant PM is different *in vivo*. Genetically encoded fluorescent pH sensors enable access to membrane-associated pH and transmembrane differential pH values from the surface of the root to the deepest cell layers beyond the Casparian strip barrier [119]. This study demonstrated that the apoplastic pH close to the PM was maintained at values ranging from 6.0 to 6.4 in mature root cells despite direct contact with the soil. By contrast, the overall pH in the apoplastic space is far more acidic [119]. The role of lipids in this observation remains to be determined.

4. Plant lipids are unevenly distributed within the PM and able to organize into domains

4.1. Asymmetric composition of inner and outer leaflets

During the 1970's, alongside the fluid mosaic model proposed by Singer and Nicholson (1972), experimental evidences showed that proteins and specially lipids could segregate forming a heterogeneous membrane with both lateral and transversal asymmetry. It is also well established in animal cells that there is a compositional heterogeneity of PM lipids between the two leaflets of the PM. In human erythrocyte membranes, the prototype of animal cell PMs, the outer-leaflet is composed of mostly PC and sphingolipids, while the inner-leaflet of PS, PE, and PI as described in [120]. Minor lipids such as PIPs and PA are located in the inner-leaflet, whereas glycosphingolipids face the outer surface. This out-of-equilibrium is maintained by the activity of lipid translocases (namely flippases, floppases and scramblases), which compensate for the slow spontaneous diffusion of lipids. Because of the heterogeneity of lipids, the two monolayers display different physical properties: the inner-leaflet has a lower average viscosity than the outer-leaflet. The importance of membrane asymmetry is well studied in animals, see for recent reviews [121,122].

In plants, only two publications experimentally address the asymmetry of lipids in the PM: by using a phospholipase A2 treatment, filipin labeling and immmuno-labeling with antibodies against DGDG and gluCer on purified oat PM, it was shown that DGDG was exclusively located in the inner-leaflet together with 60% of phospholipids, and the GluCer and sterols were enriched in the outer-leaflet [77]. Unfortunately, GIPC and the exact phospholipid content were not addressed in this study. GIPC are synthesized inside the Golgi apparatus, with their polar heads inside the lumen, see Fig. 4, therefore it is very likely that these lipids are located in the outer-leaflet of the PM after fusion of the secretory vesicles. Moreover, the large size of the GIPC' polar heads likely prevents spontaneous flip between the two leaflets. Immunogold labeling on tobacco PM vesicles showed that polyglycosylated GIPC mostly locate in the outer-leaflet of the PM [39].

By taking together these scarce experimental evidences, we recently proposed a model for the distribution of lipids in the plant PM: 1/GIPC and GluCer are exclusively located in the outer-leaflet; 2/sterols (free and conjugated) are enriched in the outer leaflet; 3/phospholipids are enriched in the inner-leaflet with PIPs, 4/PS, PA exclusively in the inner leaflet [39]. Future work should be dedicated to the in depth analysis of the lipid composition of each PM leaflet with special focus on deciphering the diversity of the various molecular species *i.e.* fatty acid content (unsaturation and length) of each class of lipids will be done and the different forms of sterols will be characterized. Numerous methods are available on RSO *vs.* ISO purified PM vesicles or on live protoplasts cells to address this delicate question [123].

4.2. Membrane phases in model and biological membranes

4.2.1. Membrane phases, dyes and modeling approaches

Assembly of lipids can adopt different physical states, the so-called phases. Following the nomenclature introduced by Ipsen [124], lipid organization of lamellar bilayer structures can be divided in three main phases: the solid-ordered (So), liquid-ordered (Lo) and liquid-disordered (Ld) phases depending on the lipid species, acyl chain unsaturation, temperature, pressure and several additional parameters. In So phases, lipids are tightly packed and lateral diffusion is very slow. In Ld phases, lipids are much less condensed, acyl chains are mobile and loosely packed, and lateral diffusion coefficients are high, especially at high temperatures [125]. In Lo phases, like in So phases, a high degree of acyl chain order is observed, but lateral diffusion coefficients are comparable to those of Ld phases. Phase formation in lipid mixtures has been extensively studied *in vitro* with liposomes and giant unilamellar vesicles (GUV) as models. In most reports, GUV membranes exhibit

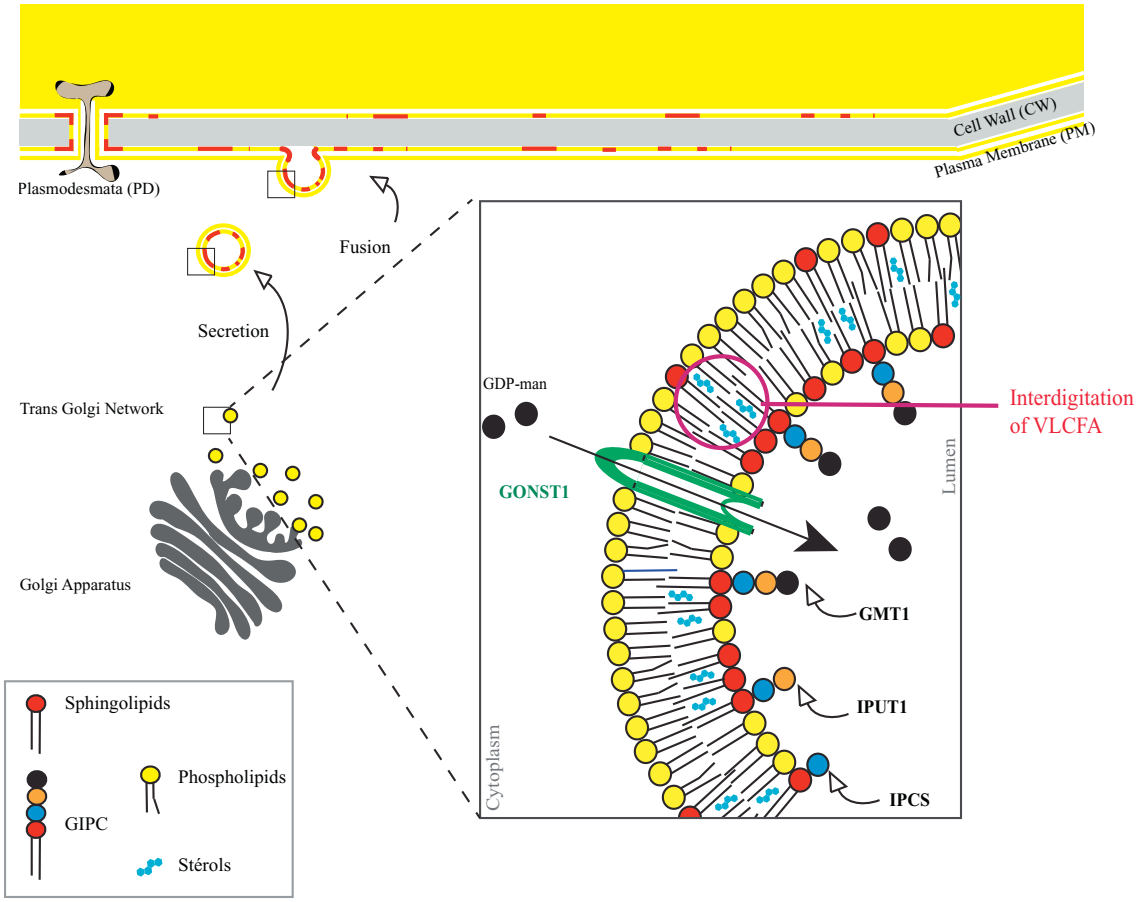

Fig. 4. Formation of GIPC- and sterol-enriched domains along the secretory pathway.
GIPC are synthesized in the lumen of the trans Golgi network (TGN) by grafting on the ceramide sequentially inositol-phosphate (IPCS, inositolphosphorylceramide synthase), glucuronic acid (IPUT1, inositol phosphorylceramide glucuronosyltransferase) and mannose (GMT1, GIPC mannosyl-transferase1). Golgi-localized nucleotide sugar transporter (GONST1) is responsible for the import of GDP-mannose to fuel GIPC synthesis. After vesicular fusion to the PM, GIPC polar heads face the apoplasm. Polyglycosylated GIPC form nanodomains in the PM (in red) [99].

micrometer-size liquid immiscibility over a wide range of temperatures. GUVs with such properties contained a minimum of three components: high-melting temperature lipids (*e.g.* saturated VLCFA-containing sphingolipids), low melting temperature lipids (*e.g.* not necessarily but often unsaturated phospholipids), and sterols, for more details see review [126]. Lo phases are also referred to as cholesterol-dependent phases because cholesterol was used in most studies on the subject, [127,128] for reviews. In the mammalian model, lateral partitioning of Lo and Ld phases is thus explained by a preferential interaction of sphingolipids with cholesterol over phospholipids, likely due to better shielding from water by the sphingolipid headgroup [99].

Lo phases are also observed in the presence of various free- and conjugated-phytosterols such as SG and ASG [109,129,130]. Use of environmental fluorescent probes sensitive to membrane order such as di-4-ANEPPDHQ and Laurdan [131] allow the analysis of phase separation on GUVs made of various mixtures of plant lipids. This reveal contrasted abilities of free-phytosterols to control phase separation on model membranes. Although stigmasterol added to 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)/1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) vesicles fail to induce the lateral segregation of lipids into domains of different order levels, GUVs containing sitosterol and campesterol promote the formation of Lo domains at the surface of model membranes [99]. Noteworthy, SG and ASG added separately exhibit the same ability as the corresponding free sterols, increasing the amount of Lo phase. This effect is reinforced when used in combination and increases strikingly when free and conjugated sterols are present in the mixture [99]. The same study indicates that GIPC, the major plant sphingolipids [2] are not able alone to promote the formation of a liquid-ordered phase within a phospholipid bilayer revealing no extensive phase separation in the binary sphingolipid/phospholipid system. By contrast, this study shows the ability of GIPC to increase the amount of Lo phase of the membrane in presence of phytosterol and interestingly this remains still the case even in the absence of saturated phospholipids such as DPPC. These *in vitro* studies expose the complex association of different classes of lipids necessary to form distinct phases that are *in vivo* linked to various membrane functions such as cell signaling or development. Yet there is a real benefit to be able to observe this partitioning *in vivo via* fluorescent probes and dyes.

The partitioning of lipid fluorophores between coexisting Lo and Ld phases for different ternary lipid mixtures has been extensively performed by comparing fluorescence intensities in coexisting domains. These labeled lipids have a fluorophore (*e.g.* NBD, Texas Red, Bodipy, *etc*…) attached either to the head group or to the hydrocarbon chain. Studies using fluorescently labeled lipid analogues in different mixtures must be analyzed cautiously for several reasons: 1/the fluorophore might alter the distribution of the lipids on which it is grafted *i.e.* a large fluorophore attached to an acyl chain might hamper the incorporation of the labeled lipids into the Lo domains, as found in the case of fluorescent ganglioside probes [132]; 2/It has been shown that the same fluorescent probe might have different partitioning preferences depending on the chosen lipid mixture [133]. Nevertheless, an important finding from this body of research is that partitioning in ordered-phases is increased for fluorophores with saturated chains that approximately match the thickness of one leaflet of the host membrane

[134]. Fluorescent lipid probes with unsaturated chains are found to partition into the Ld phase. These studies indicate the ability of different molecular species of lipids to partition selectively in the different phases of a complex model membrane, according their structure [135,136].

There is a current lack of such fluorescent probes designed from typical structures of plant PM lipids. This prevents unambiguous assessing of specific lipid behaviors in different complex mixtures. Non-perturbing specific-labeling of PM nanodomains in plant cells has been, and remains, one of the foremost challenges in the field.

Modeling approaches based on simulation can also bring grist to the mill of such experimental evidence. For example, very recent work by Ingolffson et al., in a pioneering *in silico* study of PM-lipid assembly mimicking the complexity of the animal PM, confirmed the non-ideal lateral mixing of the different lipid species [137]. Based on large-scale molecular dynamics simulation, this study provided a high-resolution view of the lipid organization of the PM at an unprecedented level of complexity since the model consists of 63 different lipid species, 14 types of head groups and 11 types of aliphatic moieties asymmetrically distributed across the two leaflets. This closely mimics an idealized mammalian PM. A general non-ideal lateral mixing of the different lipid species was observed together with the formation and disappearance on the microsecond time scale of transient domains with liquid-ordered characteristics: in the outer leaflet, distinct nanodomains consisting of gangliosides were observed, and PIPs showed preferential clustering in the inner leaflet of PM. Nonetheless, the lack of biophysical parameters for plant lipids necessary for the calculation of molecular dynamics impairs the use of these approaches to modelize plant PM (see section "Conclusions").

4.2.2. Solubilization by detergents: evidences from model membranes

Detergents are amphiphilic molecules, most of them consisting of a polar head and a hydrophobic chain. These molecules have a conical shape and spontaneously form micellar structures displaying a positive curvature in aqueous solution. Detergents have thus the ability of incorporating themselves into membranes and of solubilizing proteins by replacing their lipid environment. Pioneering work evidencing correlations between resistance to detergent solubilization of a fraction of the PM and its peculiar lipid and protein composition suggested the possible existence of lipid domains in the PM of mammalian cells [138].

Detergent-resistant membrane fractions (DRMs) could be isolated from a variety of eukaryotic cells and gave birth to the hypothesis that such fractions are present within native PM as a distinct phase within the bilayer. DRMs are rich in saturated phospholipids, sphingolipids and sterols, and display the properties of the Lo phase previously described in model membranes. Such a hypothesis received strong support from parallel studies on lipid vesicles constructed to mimic the lipid composition of these membranes [139]. In particular, [140] demonstrated that when mixtures of sphingolipids, unsaturated phospholipids and cholesterol were treated in the cold with nonionic detergents such as Triton X-100, the lower-melting phospholipids were readily solubilized while the higher-melting sphingolipid species, and to a lesser extent cholesterol, were largely recovered in an insolubilized and sedimentable fraction. Similar results were obtained using analogous lipid mixtures without cholesterol, or in which long-chain saturated phospholipids replaced the sphingolipid component. Measurements of diphenylhexatriene fluorescence polarization have suggested that the existence of a DRM fraction was correlated with the presence of Lo phases in the original bilayers. Since that, numerous studies have addressed the differential sensitivity of Lo and Ld domains to detergent solubilization. Nevertheless, only a few reports, like that of [141] compared the effect of distinct detergents on Ld and Lo domain solubilization within model bilayers. Most works indeed focused solely on the impact of Triton X-100. Atomic Force Microscopy (AFM) observations on vesicles containing dioleoyl-PC/sphingomyelin/cholesterol provided evidence for both Triton X- 100-insoluble domains composed of sphingomyelin and cholesterol, and Triton X-100-soluble areas surrounding them [142]. Similarly, using real-time AFM imaging of dioleoyl-PC/sphingomyelin/cholesterol mixtures, [143] found that detergent did not affect sphingomyelin/cholesterol Lo phases, while dioleoyl-PC Ld phases were completely solubilized. Developing the same experimental approach on dioleoyl-PC/dipalmitoyl-PC vesicles, authors showed that Triton X-l00 concentrations right above the critical micellar concentration (CMC) enabled the solubilization of the dioleoyl-PC matrix, but prevented dipalmitoyl-PC domains from solubilization [144]. [145] proposed a model based on equilibrium thermodynamics showing that resistance to solubilization only depends on the target lipid affinity with the micellar phase. The effects of cholesterol on the resistance of lipid mixtures to solubilization have also been investigated. Ahyayauch et al. demonstrated that cholesterol facilitates PC solubilization better than sphingomyelin [146]. Furthermore, cholesterol was found to induce higher resistance to solubilization of dipalmitoyl-PC vesicles, with a notable exception at 4 °C. Interestingly, cholesterol also induced higher resistance of palmitoyl-oleoyl-PC bilayers to detergent solubilization on a broader range of temperatures (from 4 to 15 °C) [147]. In addition, sterols are not the only lipid family determining detergent insolubility. For example, it was shown that addition of 5–30 mol% ceramides prevented Triton X-100 from completely solubilizing sphingomyelin-containing bilayers [148]. It is noteworthy that microscopic observations carried out by Staneva et al. [147] revealed neither domain formation, nor domain coalescence in response to Triton X-100 treatment in heterogeneous GUV systems. Such observation supports the idea that isolation of DRM from biological membranes by detergent-induced extraction is not an artifact and moves the question of DRM controversy back to the central importance played by the used methodology.

Cholesterol is not the only sterol to induce resistance to detergent solubilization. Plant sterols, had similar capacity even if a weaker effect than cholesterol, when incorporated in phospholipid bilayers [100], with the lowest efficiency being observed with stigmasterol, see below. All these results, based on biophysical analysis performed on model membranes indicate: 1/that animal and plant PM-mimicking lipid mixtures undergo a segregation between different phases corresponding to different physical states; 2/that the different lipids present within the bilayer partition differentially in these phases according to their chemical structure; 3/that there is a close correlation between the composition and physical characteristics of the DRM fraction isolated from model membranes and the ones displayed by the Lo phase.

4.2.3. Isolation of detergent resistant membranes from PM, biochemical fractions with a specific lipid composition

Based on the conceptual framework exposed in the previous sections, a tremendously high number of publications (about 2000 in the last 40 years) reported the isolation and characterization of DRMs from biological membranes from a wide variety of animals, plants and microorganisms [149]. Virtually all protocols rely on a similar experimental procedure: treatment of either intact cells or purified membranes with a nonionic detergent (most frequently Triton X-100, but Triton X-114, Brij or Lubrol have also been used), generally at low temperature (4 °C) followed by ultra-centrifugation on a sucrose (or Ficoll) gradient to recover the insoluble fraction [150]. Parameters which have been proven to be crucial and were carefully adapted to each material concern mainly the concentration of detergent and the detergent-to-membrane ratio used. The protein yield recovered in the insoluble fraction may vary between 5 and 20% of the initial amount of membrane proteins, depending on the biological material and the experimental conditions. In animal cells, extensive characterizations of lipids associated to DRMs consistently revealed a 3- to 5-fold enrichment in lipids associated to the Lo phase of model membrane, in particular cholesterol, saturated phospholipids, gangliosides and sphingomyelin [151,152]. Analyses of the phospholipids content of DRMs classically exhibit a decrease in anionic phospholipids compared to the

whole membrane [153], an increase of the proportion of saturated fatty acids [154], and a typical enrichment in GM1 gangliosides [155]. The biochemical analysis of such DRMs extracted from animal cell membranes have been extensively performed using proteomics approaches (see for review [156]). It emerged from this huge amount of data that some typical proteins, such as caveolin, or protein families, such as kinases of the Src family or small G proteins (Guanine nucleotide-binding proteins), are in a systematically enriched in such fractions, together with some proteins harboring particular post-translational modifications such as GPI-anchored proteins. We can note here that some detergent-free methods to isolate sub-fraction of the PM have also been developed. [157–159].

In plants, DRMs were first purified in tobacco, with the first isolation reported by [160] and the characterization of DRM-associated proteins and lipids provided by [19]. Similar studies revealing by mass spectrometry the catalogue of proteins were then performed on different species such as *Arabidopsis thaliana* [161–165], *Medicago truncatula* [166,167], *Oryza sativa* [168], *Avena sativa* and *Secale cereale* [169] or more recently *Beta vulgaris* [170]. According to the methodology used, the amount of proteins may differ between the studies but the continuous improvement of performance and sensitivity of the mass spectrometry approaches, led to an increase of the number of proteins identified which reached > 300 proteins in the more recent studies [163,164,171]. The latest extensive analysis published on plant PM proteome, performed in rice, allowed the identification of > 3900 proteins, which is quite consistent with the yield of PM-derived DRM proteins which is typically around 10% of total proteins in most studies [19,172]. The family of proteins identified in the different studies is also quite consistent, with a high proportion of proteins involved in signal transduction, responses to different stress, and plant-microorganism interactions. Accordingly, a few studies implementing quantitative proteomics approaches based on different methodologies, clearly revealed a qualitative and/or quantitative modification of the proteins associated to DRMs upon environmental modifications, for instance in the early steps of plant defense signaling [171,164,168], or following abiotic stress [173,170]. Note that by contrast with proteins where genetically encoded fluorescent tags or specific antibodies are available, generating DRMs is the most used technique in order to study the potential segregation of lipids. In the next chapters, we will discuss the lipids found in DRMs purified from plant PMs.

4.2.3.1. Glycerolipids in plant DRMs. As expected from biophysical work, major structural phospholipids, *i.e.* PC, PE, PS, PA are markedly depleted in plant DRMs when compared to the total PM [19,162]. The case of phosphoinositides (PIPs) deserves a particular attention. Despite the real challenge related to the detection of such a minor class of lipids in very reduced biological sample such as DRMs, the combined use of Thin-Layer Chromatography to separate the different classes of phosphoinositides prior to quantitative Gas Chromatography-Mass Spectrometry analysis [174] allows to quantify PI4P and PI(4,5)P_2 in DRMs isolated from tobacco leaves and tobacco Bright-Yellow 2 cell culture (BY-2 cells) [31]. It was shown that both PIPs isomers represent < 5 mol% of the total lipids of tobacco PM. However, their relative amount is 11-fold higher in DRMs compared to PM from which they originate. Hence, it is estimated that 50 mol% of PM phosphoinositides likely segregate in BY-2 cell PM-domains. A lower increase in PI4P and PI(4,5)P_2 was also observed in tobacco leaf DRMs: 43 and 31 mol% of the isomers were found to concentrate in this fraction respectively. Moreover, PIPs display highly saturated fatty acids in both DRMs and PM, with 16:0, 18:0 and 18:1 being the major fatty acids, which is all the more consistent with the packed lipid environment or liquid-ordered phase characteristic of this fraction. This work has demonstrated that PIPs are the only glycerolipids enriched in plant DRMs, which is in agreement with biochemical studies performed on animal cells. In good agreement with this result, the nanodomain-protein marker from the potato REM group 1 isoform 3 (StREM1.3 or REM) was shown to cluster in the PM inner-leaflet nanodomain by specific binding to PI4P [175]. The question arises whether PI4P is clustered in PM-nanodomains before the anchoring of REMORIN, or whether REM's C-terminal anchor promotes PI4P clustering. In line with the results obtained for PIPs, striking evidence related to the characteristics of the fatty acyl chains associated to DRM glycerolipids have been obtained. Indeed, the comparative analysis of polar lipids from tobacco leaves or BY-2 cells revealed a very significant enrichment in saturated fatty acids (C16:0 and C18:0), in agreement with a DRM phospholipids double-bond index lower than that of the overall PM [19]. This was consistently observed in maize embryos and bean leaves with the total amount of saturated long-chain fatty acids being higher in the PM than in DRMs. Similarly, the saturated/unsaturated ratio PM/DRM also rised in the DRMs compare with PM [176]. These characteristics perfectly fit with the direct relationship classically observed in model membranes between the proportion of lipids with saturated fatty acyl chains and the global order of the bilayer which has been confirmed using lipids from plant PMs [99].

4.2.3.2. Sterols in plant DRMs. In all plant tissues tested, free sterols are major components of isolated DRMs. Quantitative analyses showed a clear increase in the sterol-to-protein ratio to around 1.7-fold in DRMs prepared from both tobacco leaves and *Medicago truncatula* roots [19,166] or even 2.7- and 4-fold enrichment in DRM fractions from bean leaves and Arabidopsis cell cultures [161,177]. On the other hand, maize embryo [177] and Arabidopsis seedlings [173] exhibited a smaller free-sterol accumulation with only an 1.3-fold enrichment in DRM fractions indicating a range of plant sterol enrichment factors as broad as that observed in animal cells. In general, relative abundances of individual free-sterol species such as stigmasterol, spinasterol and sitosterol fractions are similar in DRM and PM [19,161,162], with the noticeable exception of maize embryo membranes [177]. For the first two sterols that showed very low even not clear ability to order membrane, such observation is surprising. However, their differential ability to order membrane had been measured in artificial membranes, a context in which i) they are the only one sterol among the lipid mixture (a crucial difference with PM sterol mixture, where an additive effect of ordering-capacity of each sterol was furthermore demonstrated [99]) and ii) their specific capacity to segregate into a DRM fraction was not assessed to date, to the best of our knowledge.

Moreover, additional results reinforced the possible role of phytosterol in the structuration of plant PM domains suggested by their enrichment in DRMs. They were essentially provided by the use of the pharmacological compound methyl-β-cyclodextrin. This cyclic oligosaccharide able to trap sterols from artificial and biological membranes has been widely used to lower the content of membrane cholesterol in various types of animal cells and to assess sterol-associated membrane structuring, see for review [178] to read about the specific and non-specific effects of cyclodextrins. This molecule has been proven to remove from isolated plant PM, with a comparable efficiency to that associated with cholesterol, the free phytosterols (campesterol, stigmasterol, sitosterol and isofucosterol) [93]. Such a treatment resulted in a decrease by about 50% of BY-2 cell PM sterols, without affecting PM-content in conjugated sterols, phospholipids, sphingolipids and proteins. Importantly, methyl-β-cyclodextrin treatment totally abolished the recovery of any DRM fraction after PM solubilization at 4 °C with Triton X-100 [93]. Moreover, the use of environment sensitive fluorescent probes allowed to associate this depletion in free-sterols with a decrease in liquid phase heterogeneities, and particularly in Lo phases [93]. This work on isolated PM has been further corroborated by similar results obtained on living BY-2 cells showing a clear decrease of the proportion of ordered PM-domains by cyclodextrin treatment that reduced by *ca.* 20% the amount of PM sterols [92]. Finally, the combination of cyclodextrin and extensive quantitative proteomic characterization of DRMs isolated from Arabidopsis PM identified a subset of proteins, whose association to DRMs is sterol-dependent [163,165].

As exposed in Section 1.3, phytosterols can be conjugated with sugars, which in turn can be acylated to form SG and ASG (for a review see [71]). Lipidomic analyses have shown varying amounts of SG and ASG in plant DRMs. In *M. truncatula* roots, the same enrichments in SG, ASG and free-sterols were observed in DRMs compared to PM fractions [166]. Conversely, while free-sterols did not show any significant enrichments in DRMs from *Arabidopsis thaliana* seedlings and plants, SG and ASG were found considerably enriched (> 4-fold) in these fractions [162,173]. In oat roots, conjugated-sterols were observed in similar proportions in PM and DRMs whereas a clear enrichment of free-sterols was reported [77]. Furthermore, total sterol amounts were markedly increased in tobacco leaf DRMs, mainly because of the high-enrichments in free-sterols and ASG [31]. As exposed in section 2.1, conjugated-sterols in combination with free-phytosterols are potent modulators of the order level of model membranes, suggesting again a close relationship between the composition of the DRM fraction and the presence of a Lo phase in plant PM.

4.2.3.3. Sphingolipids in plant DRMs. By contrast with DRMs extracted from animal cells, plant DRM sphingolipid content has only been investigated in very few studies. A first line of evidence of the enrichment of sphingolipids in DRMs relies on the characterization of their VLCFA content. By analyzing highly-purified PM from bean leaves and germinating maize embryos, a 3- to 4-fold increase of VLCFA relative amount in DRMs compared to PM was evidenced [176]. Moreover in the two plant species considered, VLCFAs harboring 20- to 32-carbon chains were present in DRMs. A significant enrichment of VLCFAs (from C20:0 to C26:0) in DRMs compared to PM was also observed in tobacco leaves and BY-2 cells [2]. Among the hundreds of sphingolipid species that exist in plants many possess 2-hydroxy fatty acids, containing a hydroxylated C-2 position [9] may contributes to the rigid binding of sphingolipids between themselves and other lipids through hydrogen-bonding between hydroxyl groups, as evidenced in artificial membranes [179,180]. It is thus likely that 2-hydroxy sphingolipids contribute to the ordered structure of PM domains. Using mutant rice lines in which the levels of sphingolipids containing 2-hydroxy fatty acids were decreased by knocking-down two genes encoding fatty acid 2-hydroxylases (FAH1 and FAH2), [52] demonstrated that the DRM/PM ratio was altered in these lines. This result suggested a role for such lipids in structuring Lo phases within the PM, which was further confirmed by the observation using the environmental probe ANEPPDHQ that the PM in OsFAH1/2-KD1 was significantly more disordered than in the wild type.

GluCer belongs to the monosaccharidic cerebroside family, and many GluCer species have been reported in plants, and lipidomics approaches showed that GluCer can exhibit a wide range of LCB and fatty acid composition [38]. Their relative abundance in plant PMs has not been unequivocally determined, varying significantly form one species to another and also according to the photosynthetic activity of the tissue considered [181]. In line with those observations, various quantitative data have been reported concerning the enrichment of GluCer in DRMs. In DRMs isolated from tobacco leaves and BY-2 cells [19] or *Medicago truncatula* roots [166], GluCer was only slightly enriched. On the other hand, GluCer was found significantly enriched in Arabidopsis and leek DRMs prepared from PM and microsomal membranes [161,173]. DRMs isolated from leek Golgi membranes displayed a 4-5-fold in GluCer compare th the PM [162]. Similarly, DRMS isolated from tobacco pollen tubes harbored a percentage of GluCer which increased up to two fold with the detergent/protein ratio [182]. Taking into account these data, it is difficult to conclude whether or not GluCer enrichment can be considered as an essential component of plant DRMs, even if its relative proportion increased in this fraction compared to the PM, in most studies performed.

The first indication of an enrichment of GIPC in plant DRMs was reported by [161], showing an approximately 5-fold higher LCB-to-protein ratio in DRMs extracted from Arabidopsis microsomal fractions. The relative decrease of (8Z)-4-hydroxy-8-sphingenine (abbreviated t18:1c) in the DRMs compared with microsomes and the increase of the ratio of this LCB compared with its stereoisomer (8E)-4-hydroxy-8-sphingenine (t18:1t) in the DRMs suggested that DRMs might contain a high proportion of GIPC, which have a greater 8Z:8E ratio than cerebrosides [161]. However, although GIPC belong to one of the earliest classes of plant sphingolipids that were identified [41], their study has for long been impaired by their limited solubility in typical lipid extraction solvents, and very recent progress concerning their structural characterization and role in membrane organization relies on the development of efficient protocols of purification [42,54]. By taking advantage of such methodological developments, [39] showed that the hVLCFA and VLCFA contents were highly comparable between DRMs and purified GIPC, with an even higher proportion of hVLCFAs in DRMs purified from BY-2 cells, suggesting that hVLCFA-containing GIPC are most likely present in this fraction. Moreover, levels of the two LCBs t18:0 and t18:1, which are mostly present in GIPC [45], strongly increase in DRMs when compared with PM, reaching 80% of total LCBs in DRMs. A further characterization indicated that series A GIPC were found in both PM and DRM fractions of tobacco leaves, whereas for BY-2 cell series B GIPC were 3-fold enriched in DRMs when compared with the PM, reaching 17% of total GIPC in BY-2 DRMs [39]. When the global lipid composition of the PM and DRM fractions was recalculated taking into account these data, it appeared that GIPC represent 45 and 30 mol% of total PM lipids isolated from leaves and BY-2 cell suspensions, respectively, and up to 60 mol% of the DRM fraction, suggesting that the contribution to sphingolipid-enrichment in PM Lo phases is mainly due to GIPC [39].

4.2.4. The use of DRMs to study the segregation of lipids in plant PM; some limits but significant contributions

The "raft hypothesis" states that specific PM-lipids, mainly sterols and saturated sphingolipids, interact together to form dynamic nanoscale clusters by recruiting lipids and proteins that are present in signaling and trafficking platforms in the PM [114]. Experimentally, the nonionic detergent Triton X-100 is used to separate Lo phases from the rest of membrane preparation by isolation of DRMs isolated in the upper-phases of a sucrose density gradient after ultracentrifugation. DRMs are considered by many as *in vitro* counterparts of membrane rafts [150]. The characterization of lipids found in DRMs isolated from PM fractions of various plant species gave rise to a overall features such as a global decrease of glycerolipids content, with the noticeable exception of phosphoinositides; a strong enrichment in lipids containing saturated fatty acyl chains; an increase in free- and conjugated-sterols; and a strong enrichment in sphingolipids, and in particular in GIPC. These characteristics are consistent with the canonical description of DRMs isolated from a plethora of animal PMs whilst taking into account the specificities of plant PM lipids [183]. Moreover, it appears that such a composition is typical of the Lo phase in model membranes, as detailed in Section 3.2.1. Yet, as stated in several publications (*e.g.* [184–187]) the use of DRMs to evidence PM-associated dynamics should be accompanied with great precaution as DRM fractions should not be considered as direct equivalents to PM-domains. However, the numerous convergent correlations obtained on many different biological materials and model membranes have indicated for instance that 1/a consistency between the composition of the Lo phase *in situ* and the DRM lipid content; 2/a relationship between the presence and abundance of DRMs and the order of biological membranes; 3/the association of particular proteins to DRMs and their clustered distribution within the PM make them valuable tools to progress towards a better understanding of plant PM organization. An example of this last point is the PM-associated NADPH-oxidase RbohD, which was demonstrated to be responsible for the oxidative burst observed in the very early steps of the plant immune signaling cascade, was proved to be exclusively associated to DRMs in tobacco [19]. This characteristic could be related to the immunoelectron microscopy observation that this protein is

Table 1
Examples of inhibitors used to modify *in vivo* the pools of lipids, and some recent related references.

The used concentration of the inhibitors is indicative, and must be tested for each plant species or tissues. To address the modification of the PM lipid pool, a phase partition to purify PM vesicles must be conducted coupled with a dedicated lipidomic approach. PLD, Phospholipase D; PLC, Phospholipase C, DAG, Diacylglycerol; VLCFAs, Very Long Chain Fatty Acids; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase.

	Inhibitors of:	Name	References
Phosphoinositides	PI3-Kinase (50–100 μM)	LY-294002	[398]
	PI3P 5-Kinase (1 μM)	YM-201636	[399]
	PI4-Kinase (30–60 μM)	Phenylarsine oxide (PAO)	[117,398]
	PI3-Kinase (1 μM)	Wortmannin	[283,31]
	PI3-Kinase + PI4-Kinase (30 μM)	Wortmannin	
Sphingolipids	Ceramide synthase (1 μM)	Fumonisin B1 (1 mg)	[400,401]
	Glucosylceramide synthase (50 μM)	DL-THREO-PDMP	[402]
	VLCFAs/sphingolipid (50–100 nM)	Metazachlor	[235]
	Serine palmitoyltransferase (SPT)	Myriocin	[56,403]
	Inositol phosphorylceramide synthase (fungi)	Aureobasidin A	[404]
Diacylglycerol/phosphatidic acid	Lyso PA Acyl transferase	CI-976	[405–407]
	PLD-derived PA formation (50 μM)	(*R*)-(+)-Propanolol hydrochloride	[408]
	PLD-derived PA formation (0.2–0.4%)	1- butanol	[409,410]
	PLC-derived DAG formation (5 μM)	U73122 (active analog)	[31,411]
	PLC-derived DAG formation (5 μM)	U73343 (inactive analog)	[411]
	PLC-derived DAG formation (50 μM)	Edelfosine	[283]
	DAG-Kinase (50 μM)	R59022	[283]
Sterols	Cyclopropylsterol isomerase 1, CPI1	Fenpropimorph	[7,175,162]
	HMG-CoA reductase	Lovastatin	[7]

organized within the PM in clusters of about 20 nm in diameter [188]. Upon activation, NADPH-oxidase products (the Reactive Oxygen Species) were also present as discrete nanometer sized patches along the PM [189]. Similarly, the enrichment of the flagellin receptor FLS2 in DRMs observed a few minutes after treatment of Arabidopsis cells with the bacterial elicitor flagellin flg22 is fully consistent with recent results observed using super-resolution microscopy indicating its clustered distribution in the PM. Noteworthy, a significant modification of its dynamics within PM, namely an increased population of long-lived receptor clusters and a reduction of its lateral displacement was observed a few minutes after flg22 treatment [190]. Moreover, group 1 Remorin proteins were the first biochemical markers of plant DRMs and observed as forming PM-associated clusters of about 75 to 100 nm in diameter [191,192]. Such nanodomain-organization was shown to be sterol-dependent as it was strongly impaired by the use of sterol-chelator methyl-β-cyclodextrin [191] or by inhibitors of sterol biosynthesis, see Table 1 [175]. More recent data have confirmed the confinement of group 1 Remorin in PM-nanodomains to be sterol- and PI4P-dependent and by using *in vivo* single-particle tracking microscopy that the size of group 1 Remorin-associated domains were of *ca.* 80 nm in diameter [175].

One must note that the colocalization studies of Remorins of different phylogenetic groups, namely groups 1, 3, 4 and 6 have shown the coexistence of highly-distinct membrane domains in the plane of the PM [193]. These results have demonstrated that the use of biochemical approaches such as DRMs cannot be sensitive enough to accurately represent the biological complexity of membrane-compartmentalization *in vivo*. A decisive milestone will be the ability to describe, in an extensive and comprehensive manner, the distribution of the various lipids together with proteins within PM, and to identify key causal mechanisms underlying such an organization. To do so, super-resolution microscopy with one or more fluorophores can be used for proteins to visualize *in vivo* whether the protein of interest is enriched in nanodomains; this kind of method is currently at its infancy for lipids due to the few fluorescently-labeled lipids available and because it is hard to insure a proper intake of such dyes in plant cells (see below). The use of chelator of lipids, fluorescent lipid probes or lipid-biosynthesis inhibitors (Table 1) are the next steps to address the role of lipids in PM-nanodomain formation and maintenance.

5. Spatial and multiscale segregation of lipids and proteins: a complex picture emerging from the combined use of various imaging techniques

Since the publication of the fluid mosaic model for biological membranes in 1972, a lot of experimental evidences revealed the outstanding complexity of the PM. Rafts characterized by tight lipid-packing are involved in a wide variety of cellular processes: regulation of *endo-* and exocytosis, hormone signaling, membrane trafficking in polarized epithelial cells, T-cell activation, cell migration, life cycle of influenza and HIV viruses [194]. As expected for biology as an experimental science, understanding the organization of the PM strongly relies on the evolution of microscopic methods. Thanks to the development of new efficient methodologies, among them imaging lipids and super-resolution microscopy [114], very important results have been published refining this organization but at the same time raising new questions. Currently, we can clearly state that the PM is organized in domains that differ in the nature of their components, their stability and their size from the nanometer to the micrometer scale. This complex remodeling is highly-dynamic and respond to various abiotic and biotic stresses. Particular interest of the scientific community for cell surface signaling processes in the past decade has led to an improved vision of the PM's membrane organization: a complex multi-component and multi-scale heterogeneity with a high degree of subcompartmentilization into micro- to nano-domains have been evidenced *in vivo* and deserve to be clearly qualified. Domains were originally referred as to "lipid-rafts" but the designation has very much evolved since. It is now known that lipid-rafts do not cover a single type of domain but rather include a collection of domains differing in their protein and lipid composition and their resident time (aggregation/disaggregation).

5.1. Micro- and nano-domains coexist in the plant PM

5.1.1. Microdomains in plant cells

Cell polarization-induced PM-microdomains (above 1 μm) are easily observed by classical confocal fluorescence microscopy in leaf, root and pollen tube cells [185,195]. The accumulation of proteins and lipids into microdomains is involved in defining cells' fate, functional specialization for cell polarity and specialization of host membranes for defense [196,197]. These include the polar distribution of PINFORMED (PIN), AUX1–Auxin transporter protein 1, ABCB (ATP-binding cassette

protein subfamily B)/P-glycoprotein, Auxin binding protein 1 (ABP1) or RAC/Rho of plants GTPases (ROP) that localize to the apical or basal pole of a cell [198] and the lateral and equatorial domains in plant endodermal cells populated by DYNAMIN-RELATED PROTEIN1A (DRP1A) or CASPARIAN STRIP MEMBRANE DOMAIN PROTEINs (CASPs) [185,199]. Similarly, tip-growing cells like pollen tubes and root hairs are also of particular interest for the study of membrane microdomains. For instance, the pollen-specific H^+-ATPase is located in the shank whereas Phospholipase C, G proteins and phosphoinositide kinases are located at the apex of the pollen tube (for review see [200]. Lipids are also segregated into microdomains *i.e.* sterol-, phosphoinositide-, PI4,5P_2 and diacylglycerol-rich microdomains are shown to be especially concentrated in the apex of the pollen tube [182]. The presence of microdomains enriched in phosphoinositides (PIPs) plays an important role in polar tip growth by regulating the machinery maintaining polarity and by controling cytoskeletal dynamics and the remodeling of vesicle trafficking [201]. In root endodermal cells, EXO70A1 exocyst subunits colocalize with PI4,5P_2 [202]. Finally, in plants, plasmodesmata (PDs), which are channels characterized by the apposition of the ER and the PM possess a specific lipid composition [203]. These PM-lined PD (PD-PM) have been shown to contain definite microdomains where not only proteins such as Plasmodesmata-located protein 1 (PDLP1) and PD callose binding proteins (PDCBs), but also lipids such as sterols and sphingolipids are enriched [204,205,7].

Importantly, besides local enrichment of specific lipids and proteins within microdomains, the characterization of the biophysical state of pollen tube microdomains has shown that they are highly-segregated in the cell, *i.e.* they are especially concentrated at the PM of the cellular apex but also present as a ring-like distribution around the tube [74,182]. Similarly, the cell plate of Arabidopsis contains highly-ordered membrane microdomains which rely on sterols and DRP1A-dependent endocytosis [206]. Yet, how the localization of proteins and lipids in microdomains relies on the cooperativity of multiple mechanisms is not yet understood [207,208,200].

5.1.2. Nanodomains in plant PM

The development of new methods of high- and super-resolution imaging has provided the ability to observe membrane domains at the nanoscale level, termed nanodomains and defined by a size below 1 μm [209,210]. These methods include mainly stimulated emission depletion microscopy (STED), structured illumination microscopy (SIM) and single-molecule localization microscopy (SMLM, including methods such as Photo-Activated Localization Microscopy (PALM) and Stochastic Optical Reconstruction Microscopy (STORM)). These techniques have been routinely used in animal cells but have only recently emerged in plant studies. Super-resolution microscopy techniques allow the acquisition of high-density super-resolved nanoscale maps of individual fusion-protein localizations and trajectories in the PM [211]. Single-molecule tracking combined with photoactivated localization microscopy (spt-PALM) allows not only the description of the supramolecular organization of proteins at the PM level far below the resolution limit of confocal microscopy but also allows the determination of the mobility dynamics of single molecules or particles in the PM.

Super-resolution microscopy methods have shown that PM-associated proteins are sub-compartmentalized within nanodomains, to only name a few: Hypersensitive Induced Response HIR1 [212] PIN2 [207], Borate efflux transporter (BOR1) [213], Dynamin-related protein 1a (DRP1A), Cellulose synthase A6 CESA6 [214], IDQ family of calmodulin–binding proteins [207,213,215] S-type anion efflux channel/Calcium protein kinase SLAH3/CPK21 and REM1.3 [192,175], flagellin receptor FLS2 [190], Brassinosteroid insensitive 1/Somatic embryogenesis receptor kinase 1- BRI1-associated receptor kinase 1 BRI1/SERK1-BAK1 [216], Flotilin [217] and the NADPH oxidase [218]. Notably, the study of group 1 REM mutants has revealed that the protein mobility rate (measured by the Mean Square Displacement) and protein supramolecular organization are not necessarily coupled. These results have shown that proteins displaying the same mobility rate can however assemble into clusters of different sizes [175]. Differential combinations of multiscale organizations have been evidenced as for instance PIN2, which is preferentially targeted in a polar fashion to PM-microdomains in Arabidopsis roots, locates at a lower scale into PM-nanodomains as shown by STED microscopy [207]. By contrast, REMORIN StREM1.3 localizes in nanodomains of *ca.* 80 nm observed by SPT-PLAM [175], but without a particular polar localization in *N. benthamiana* leaf epidermal cells.

Lipids are also found in nanodomains both in the inner-leaflet and in the outer-leaflet. Immunogold-electron microscopy has shown that PI(4,5)P_2 is found clustered in the inner-leaflet of the PM [31]. Interestingly this cluster formation was not significantly sensitive to sterol depletion [31]. More recently, immunogold-electron microscopy strategy has revealed that the distribution of polyglycosylated GIPC, likely in interaction with phytosterols, form nanodomains of *ca.* 40 nm in the outer-leaflet of tobacco PM [39]. These two results strengthen the idea that lateral nano-segregation of lipids also takes place at the PM in plants. Yet, tools dedicated to the study the dynamic of plant lipids at the nanoscale level are still lacking, impairing progress in understanding their molecular distribution, behavior and dynamics of PM lipids.

Therefore, the plant PM must be acknowledged as a fluid yet highly-compartmentalized mosaic wherein numerous membrane domains with different compositions and biophysical properties co-exist at different scales [195,219]. The challenging questions now reside in clearly defining the essential mechanisms governing specific interactions between the different molecular species of PM intrinsic components. Currently microcopy methods are able to localize and track single molecules with a resolution of 1 nm achieving an ultimate resolution limit in fluorescence microscopy: MINFLUX [220,221], subdiffusive motion at the single trajectory level [222] or motion transition state [223]. Such methods must be adapted to plant cells to address the specific question of plant PM biology and will pave the way to a better understanding of the PM's dynamic organization.

5.2. PM lipids are critical regulators of plant PM organization at the nanometer scale

Lipid-lipid interactions and protein-lipid interactions are believed to be key regulation parameters governing plant PM organization. In pollen tubes, various isoforms of the exocyst complex colocalize with either PI4,5P_2 or PA, resulting in the formation of PM domains [224]. The localization of EXO70A1 not only coincides with, but is also required for the accumulation of PI4,5P_2 [202]. The targeting of REMs to inner-leaflet PM nanodomains is independent of the secretory pathway, although it is still mediated by direct interactions with PI4P in a sterol-dependent manner [175]. This understanding of the anchoring mechanisms of REMs confirms the impairment of clustered distribution of REMs by phytosterol depletion [191,225]. The use of raster image correlation spectroscopy (RICS) has shown that the lateral mobility of auxin transporters PIN is dependent on the amount of sterols in tobacco cell PM, arguing in favor of a sterol-dependent protein organization within the plant PM [82].

In PD, modulations of the sterol composition alter callose-mediated PD permeability and reversibly impaired the PD localization of the glycosylphosphatidylinositol-anchored proteins Plasmodesmata Callose Binding 1 (PDCB1) and the Plasmodesmata beta-1,3-glucanase (PdBG2). This study emphasizes the importance of lipids in defining PD membrane microdomains and is in line with the lipid-raft model postulating the existence of nanoscopic assemblies of sphingolipids and sterols in the outer-leaflet of the PM. Finally, it is important to acknowledge that nanodomains exist in both leaflets and the lipid content of each of them regulates the clustering of proteins and lipids. The possible interaction between nanodomains across the two leaflets (a process called pinning or registration) will be discussed in the following

section.

5.3. PM heterogeneity might originate from a tight control along the secretory pathway

The neo-synthesis of lipids found at the PM results from intricate pathways that originate at the ER, which is certainly the most ancient eukaryotic endomembrane compartment. From the ER, lipids are transported to the Golgi apparatus where they are further assembled and modified before reaching the PM, see Fig. 4. At the PM, it is thought that the auto-association of glycerolipids, sphingolipids and sterols drives membranes close to a demixing point (phase separation) and induces sorting of lipids to either Lo phases or Ld phases of the membrane [226]. The Lo phase is enriched in sphingolipids and sterols. A higher proportion of free-hydroxyl groups in the long-chain-bases and acyl-chains of sphingolipids as compared to glycerophospholipids, allows more interactions with sterols and thereby ensuring the stabilization of sphingolipid-enriched membrane domains (see Section 3). The characteristic length of sphingolipid acyl-chains is a particularity among lipids that confer special physical properties to biological membranes. This particularity increases the melting-point of sphingolipids as compared to other lipids and causes strong hydrophobic mismatches between sphingolipid acyl-chains and the polar heads of other lipids with smaller acyl-chains. Hence, sphingolipids are segregated and more physically-ordered microdomains are created within the membrane [227]. Acyl-chain length also induces the formation of interdigitated phases (interdigitated lipid-leaflets) and plays a role in membrane stiffness and thickness [227,66].

Considering that these different phases are observed at the PM, one might ask how this complexity is implemented. Does this PM-lipid heterogeneity already occur within secretory pathways that lead to the PM and does it have a role in the secretion of proteins? In mammalian cells, a protein secretion model called the rapid-partitioning model, proposes a *cis*-to-*trans* gradient of the sphingolipid/glycerophospholipid ratio that would account for a partitioning of transmembrane cargos and enzymes into distinct domains of the Golgi: domains enriched in Golgi resident enzymes (low sphingolipid/glycerophospholipid ratio) and domains where transmembrane cargos are progressively enriched (high sphingolipid/glycerophospholipid ratio) at the *trans*-most cisterna of the Golgi until their loading into post-Golgi vesicles [228]. This model is in agreement with the observation that newly arrived cargos exit the Golgi with mono-exponential export kinetics. Moreover, alteration of sphingolipid homeostasis by treating mammalian cells with short acyl-chain ceramides (for further incorporation in sphingolipids at the Golgi) impacts the export of protein cargos from the Golgi, reduces the lipid order in Golgi membranes, and alters the ultrastructure of Golgi cisternae from flat to highly-curled membrane sacs, further supporting the role of very-long-chain sphingolipids in Golgi morpho-dynamics and sorting [229,230].

In plants, inhibition of the condensation of glucose with ceramides (produces GluCer) results in the disaggregation of Golgi cisternae into vesicular structures and in the inhibition of secretion [231]. In animal and plant cells, the *trans*-most cisterna of the Golgi apparatus is continuous with a tubular, branching and reticulated Golgi structure called the trans-Golgi Network (TGN). In yeast, it has been observed that secretory vesicles budding-off of the TGN are enriched in sterols and sphingolipids and possess a high proportion of Lo phases [232]. In mammalian cells, a genetically encoded probe that labels sphingomyelin has revealed that sphingomyelin synthesis at the Golgi promotes sphingomyelin enrichment in a subset of TGN-derived secretory vesicles, where the sorting of a glycosylphosphatidylinositol-anchored protein is in turn also promoted [233]. In plants, extraction of DRMs has revealed that both PM and Golgi yield DRMs suggesting that they can be enriched in sterols and sphingolipids [162]. However, as stated before, this method can generate artificial segregations of lipids within membranes. Therefore, a combination of subcellular remodeling of sterols using the fluorescent probe filipin, and a novel extraction approach to specifically immuno-purify TGN sub-domains and Golgi apparatus without any detergents coupled with quantitative mass spectrometry, has opened new perspectives in defining which lipids are present in these compartments [234,235]. The *in situ* subcellular remodeling of sterols by filipin has revealed that sterols are the most present at the PM and in sub-populations of TGN vesicles [234]. Further on, purification fractions of TGN sub-domains and Golgi have identified an enrichment of sterols and α-hydroxylated VLCFAs, a specific signature of plant sphingolipids. Moreover, this signature was specifically stronger in a sub-domain of TGN: Secretory Vesicles [235]. Not only an enrichment of sphingolipids is observed in TGN-derived vesicles, but the length of the sphingolipid acyl-chains is found to be a critical factor for the correct polarized secretory sorting of the auxin-carrier protein PIN2 in root epidermal cells [235]. Altogether, the enrichment of sterols and sphingolipids at TGN-derived secretory vesicles seems to be a conserved feature in eukaryotic cells, and appears to be required for sorting and vesicle budding. This enrichment is favorable to Lo phase lipid-segregation at TGN and suggests that a gradient of lipids along the secretory pathway is established from the ER where no enrichment of sterols and sphingolipids is observed through the TGN to the PM. This membrane heterogeneity is not only a structuration of pre-PM lipids but it has as well an important role to play in many intracellular trafficking pathways, see Fig. 4.

The lipid heterogeneity within the secretory pathway also exists for lipids other than sterols and sphingolipids. In plants, the TGN-localized choline transporter like1 (CTL1) is involved in PM-recycling of the ion transporter NRAMP1 and the auxin efflux carrier PIN1 [236]. An interesting observation is that free-choline, but not PC, can inhibit phospholipase D (PLD) activity [236]. PLD hydrolyses PC and PE to produce PA, a phospholipid that favors the fission of vesicles [237]. Hence, one hypothesis is that CTL1 transports choline inside the TGN lumen to maintain a low choline concentration on the cytoplasmic side of TGN in order to conserve high PLD activity converting PC and PE into PA. This mechanism would require further characterization, but it could be a possible way to establish membrane heterogeneity between the lumenal and cytoplasmic leaflets of TGN membranes. Another example of phospholipid membrane heterogeneity is with PS in yeast where it has been suggested that PS resides primarily in the lumenal leaflet of the Golgi and is flipped to the cytosolic leaflet in the TGN [238]. This leaflet translocation of PS is operated by PS flippases at the TGN and is thought to control oxysterol-binding proteins (OSBP), which exchange ER-associated sterols with TGN-associated PI4P in unidirectional fashion [238]. The exchange of lipids participates in creating sterol enrichment and membrane lipid order at the TGN [239]. An elegant model has proposed that this exchange of sterols for PI4P occurs at ER-TGN membrane contact sites, where PI4P is generated at the TGN by PI4 kinases (PI4KIIα) which are themselves regulated in an oscillatory (waves of PI4P consumption by OSBPs) fashion by sterols [239]. These studies have revealed a crucial characteristic of membrane heterogeneity at the TGN: its highly-dynamic and oscillatory nature.

In plants, PIPs are localized in a gradient throughout the endomembrane system, PI4P being mainly located at the PM with a secondary pool at the TGN while PI3P is mainly located in late endosomal compartments, see Section 1 [27]. The function of PI4P at the TGN and its relationship with other lipids have not yet been addressed in plants and will definitely be an exciting field to explore in respect to plant trafficking specificity. In plants, unlike animal TGNs, at least two populations of TGNs are observed, one is associated to the Golgi apparatus and one is independent from it, as TGNs detach from the Golgi apparatus to form a highly-dynamic Golgi-independent structure [200], [240–243]. This highly-dynamic TGN can undergo homotypic fusion and can associate transiently with the Golgi apparatus similarly to what is found for early endosomes and TGN in mammalian cells. In addition, it has been observed that the plant TGN can integrate the endocytic tracer FM4-64 relatively fast (a couple of minutes) during endocytosis

before reaching Late endosomes/MVBs [244,242]. Hence, plants have no early endosomes as described in animals, and endocytic vesicles converge directly to the TGN, where endocytic cargoes are sorted for recycling and/or degradation [242,240,200]. As such, plant TGNs can be viewed as a functional equivalent of mammalian early endosomes. Hence, it will be interesting in the future to see how the two systems evolve in respect to lipid heterogeneity regulation at the plant TGN.

5.4. A model for plant PM organization, interdigitation, pinning and registration

5.4.1. A model for plant PM organization: mechanisms at work

All these observations led to a model of plant PM organization that supposes both lipid-driven phase segregation and protein-dependent protein localization. This model is based on experiments on artificial membranes, composed of lipids mimicking plant PMs (lipids and/or proteins), indicating that lipid-lipid interactions strongly order plant membranes [99], whereas protein-lipid interactions could untighten plant PM organization [245]. Several models could explain lipid-lipid interactions. The "condensed lipid complex" model has been stated following the visualization of low-energy free stoichiometric cholesterol-lipid complexes occupying smaller molecular lateral zones than those occupied by each lipid alone [246]. Sphingolipids, originally proposed as preferred partners of cholesterol, cannot form this type of complex. The existence of cholesterol superlattice in lipid bilayers highlights a parallel model proposing long-distance repulsion forces between cholesterol molecules as the source for sterol-lipid interactions [247]. The "umbrella" model states that the shift between the small polar head of cholesterol and its large apolar body determines its preferential association with some adjacent molecules of the membrane [248]. In this model, cholesterol is covered by the polar heads of neighboring phospholipids to limit the unfavorable free energy due to the exposure of the apolar portion of cholesterol to water molecules. Such interactions between cholesterol and "large-headed" lipids provide increased protection. In addition, the free energy needed to cover a cholesterol cluster is larger than the energy required to cover a single cholesterol. An essential property thus emerges from these models, namely the strong tendency of cholesterol molecules not to regroup, which has been accordingly demonstrated by Monte Carlo simulations [249]. By sailing between lipid molecules, proteins increase membrane line tension and modify the mean size of the ordered domains as reported in Lung Surfactant Monolayers [250]. In agreement, it has been demonstrated that short hydrophobic transmembrane peptides decrease the affinity of sterols for neighboring phospholipids.

In animal, all these data led to a model that incorporates the importance of hydrophobic matching between integral membrane proteins and the lipid bilayer thickness considered in the so-called "mattress" model [251]. This thermodynamic model includes the elastic properties of lipids and proteins, as well as indirect and direct lipid-protein interactions expressed in terms of the geometrical variables. The notion of hydrophobic mismatch regions between lipids and proteins is also an important component of the model. This proposal remains speculative in plants and calls for further investigations.

5.4.2. PM asymmetry, interdigitation, pinning and registration

As stated before, the animal cell PM has a highly asymmetric distribution of lipids with PIPs, PE and PS mostly confined to the inner-leaflet, and sterols and sphingolipids to the outer-leaflet [252]. The same observations seem to be true in plant PM [77,2]. In addition, the PM contains dynamic nanodomains involved in a continuous repartitioning of components between different domains. Recent experimental data in the animal field have shown that transient links between lipids and proteins involving both the extracellular matrix and cytoplasmic components may temporarily pin membrane domains, see below. It is becoming increasingly clear that asymmetry and pinning processes, also called registration, play important roles in PM nanodomain formation and coupling between the 2 PM leaflets [253].

For example, a direct interaction between outer-leaflet sphingolipids Lactosylceramides containing VLCFAs, and inner-leaflet nanodomain acylated-protein kinase has been shown. This interleaflet pinning has been shown to specifically modulate neutrophil activity [254]. Similarly, transbilayer pinning between outer-leaflet long-acyl-chain-GPI-anchored proteins and inner-leaflet PS are demonstrated to be pivotal in generating actin-dependent nanoclusters of PM lipid-anchored proteins [255]. These interactions may provide clues to the underlying mechanisms for the registration of functional lipid domains between both leaflets of the PM. Yet, cross leaflet lipid-lipid interactions seem to be the main driving force behind the formation of ordered membrane domains *in vivo* [256].

How asymmetry, pinning, and interdigitation contribute to PM organization is only beginning to be unraveled in animals. Currently, very little is known in plants but this area of research will surely be developed in the next few years of membrane biology. The pending questions are of a fundamentally compelling nature. One may ask whether VLCFA-containing GIPC in PD could register with acylated-proteins across the plant PM, or whether phytosterols in one leaflet influence the fluidity of the other leaflet. This makes PM domains exceptionally challenging to study and even then, much of what is known about membrane domains has been deduced from studies on model membranes at equilibrium. However, living cells are by definition not at equilibrium, PM-lipids are still distributed asymmetrically *in vivo* so model membranes may not be as biased as can be expected. Moreover, each phospholipid group encompasses a wealth of species that vary according to their different acyl-chain combinations, and consequently their lateral distribution is heterogeneous and modulated *in vivo*. It is therefore with a combination of *in vivo* and *in vitro* analyses that these questions clearly need to be tackled in plant membrane biology.

6. Lipids are key players in plant PM function

Proteins and lipids located in PM nanodomains serve as modulators of host–pathogen interactions such as the binding of the cholera toxin to animal PM-located outer-leaflet gangliosides GM1 to form a pore through the PM [257]. The discovery of a high level of saturated sphingolipids and cholesterol in the viral envelope of HIV also proposed that enveloped virus budding is nanodomain-mediated [258,259]. Besides, a large number of proteins and lipids that are associated with cancer, atherosclerosis and immune responses have been found in nanodomains, see the recent review [114]. The example of the K-Ras protein is of particular interest as the molecular mechanisms to understand its precise PM localization have been detailed in recent reviews [260–262].In the next chapter, we will focus on the role of plant PM lipids in different physiological functions.

6.1. Plant-microbe interactions

6.1.1. Membrane lipids in plant-microbe interactions

Plants counteract pathogenic microbes by sensing non-self and modified-self molecules by cell-surface and intracellular localized immune receptors [263]. PM lipids and lipid-derived metabolites have been shown to operate in plant immune signaling [264,265]. As a result of the sensing of a pathogen, enzymes hydrolyzing the polar heads of phospholipids are mobilized to trigger signaling cascades essential for cellular responses. Phospholipases generate crucial messenger molecules such as oxylipins, jasmonates and notably PA which can regulate the activity of defense-associated proteins [266,267]. For example, the activation of phosphoinositide-specific phospholipase C (PI-PLC) is one of the earliest responses triggered by the recognition of several microbe-associated molecular patterns (MAMPs), such as xylanase, flg22, and chitosan or of pathogen effector proteins [268–270]. PI-PLC catalyzes the hydrolysis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (PIP2) to generate water-soluble inositol

bisphosphate (IP2) or inositol triphosphate (IP3), and diacylglycerol (DAG), which remain in the membrane. In plants, DAG produced by PI-PLC activity is phosphorylated by DAG kinase (DGK) to produce PA [271,272]. PA has been implicated specifically in the modulation of immune signaling components, such as MAPKs and PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1, PDK1; [273,274]). Binding of PA to proteins/enzymes has been shown to affect their activity, localization, and binding to other signaling components [275,276,267]. For example, PA binds to the NADPH oxidase isoforms RBOHD and RBOHF to induce ROS formation during abscisic acid (ABA)-mediated stomatal closure [277]. PM-localized PI-PLC2 [278], is rapidly phosphorylated upon flg22 recognition [279] and plays an important role in stomatal pre-invasion immunity and non-host resistance as it associates with RBOHD [280]. This suggests a potentially central regulation of the Arabidopsis NADPH oxidase and, consequently, of ROS-dependent processes induced by PLC2.

In addition, it has been shown that PLC activity is required for ROS production during effector triggered immunity (ETI) responses [281], that NPC2 is involved in the response of Arabidopsis to *Pseudomonas syringae* attack, by regulating elicitor-induced ROS production [282]. Furthermore, it has been demonstrated that DGK-produced PA is required for optimal ROS production in response to cryptogein [283]. Nonetheless, direct regulation of Rboh isoforms by PA binding during immune responses remains to be investigated. In addition, PA binding inhibits regulator of G-protein signaling (RGS1) activity to affect specific immune signaling pathways in Arabidopsis. Interestingly, the central immune receptor cytoplasmic-like kinase BIK1 directly linked nanodomain-localized pattern recognition receptor (PRR) [190] and RBOHD [284]. BIK1 regulates RGS1 activity by direct phosphorylation [285] and by inhibiting PLC activity. FLS2 has been shown to be no longer endocytosed after binding to flg22 [286], pointing PA as a core regulatory component of plant receptor kinase-based immunity. Interestingly, remodeling of cortical actin network in response to elicitors is mediated by the negative regulation of CAPPING PROTEIN by PLD-produced PA [287].

The production of PA by Phospholipase D enzymes (PLD) is involved in ROS production in response to elicitation [288]. This production of PA has been also shown to be essential for phytoalexin biosynthesis [289] yet considering the various subcellular localizations of PLDs this may not be specific to PM-associated PA [290]. PLDδ has been also found to be involved in non-host resistance of *A. thaliana* epidermis against the barley powdery mildew fungus *Blumeria graminis* f. sp. *Hordei* and the pea powdery mildew fungus *Erysiphe pisi.* PM-localized PLDδ is enriched at the penetration sites and PA is supposedly produced and necessary for resistance considering the observed increase in susceptibility after treatment with n-butanol, a PLD inhibiting drug [291]. PLD-produced PA is also involved in plant-virus interactions by promoting the RNA replication of the Red clover necrotic mosaic virus. A viral auxiliary replication protein binds PA *in vitro* and the exogenous addition of PA increase replication rates. This is consistent with the observed increase of PA levels in infected cells [292]. PA could therefore play a central role in viral replication by tethering protein complexes to each other and to the membrane, thereby putatively modulating catalytic activities [293] and membrane curvature [294]. As PA negatively favors curved membranes [295], a local increase in PA levels is likely to impact membrane structure and charge. Nonetheless, stimuli-dependent impact of PA production on membrane organization and the dynamics of plant immune component remain to be studied. What are the molecular function(s) of PA in immunity is still to be further studied.

In addition to PA, both phosphoinositides and lysophospholipids have been shown to play a role in plant defense. Lysophospholipids are derived from glycerophospholipids by the action of PLAs. Examples of lysophospholipids include L-PA, lysophosphatidylcholine, sphingosylphosphorylcholine, and sphingosine-1-phosphate [296]. The signaling activity or specificity of these compounds is dependent on the length and position of the acyl chain, the degree of saturation, and the presence of the phosphate head group. Acyl chain length and degree of saturation have been shown to influence plant-pathogen interactions. The accumulation of C16:1 and C16:2 fatty acids in tomato and eggplant, due to the overexpression of a yeast delta-9-desaturase, resulted in a heightened resistance to powdery mildew *Erysiphe polygoni* DC and *Verticilium dahliae*, respectively. An increase in C18:2 and C18:3 has also been shown to increase resistance to *Colletotrichum gloeosporioides* and *Pseudomonas syringae* in avocado and tomato respectively [297,298]. Moreover, bean resistance to *Botrytis cinerea* induced by a non-pathogenic strain of Pseudomonas has been correlated with an increase of C18:2 and C18:3 [299].

As stated in chapter 1, plant sterols are core components of membrane and accumulate in the PM. Conversely, PM sterols are conserved regulators of membrane organization. Mutants altered in sterol biosynthesis and the use of sterol-biosynthesis inhibiting drugs, affect cell wall composition and induce abnormal callose and lignin deposits (cell wall compounds involved in biotic stress) [300]. Cryptogein is able to induce an increase in PM-fluidity *via* sterol-binding [112]. Highly-hydroxylated sphingolipids increase membrane stability and decrease membrane permeability which are both associated to increased defense against phytopathogenic fungi [35,301]. Rice fah1/2 knock-down mutants, displaying the lack of an α-hydroxyl group on the fatty-acid moiety of sphingolipids, exhibit a decrease in PM order level [52]. These mutants show reduced resistance to the rice blast fungus *Magnaporthe oryzae*, with the delocalization of major actors of innate immunity such as NB-LRRs, NADPH oxidases, Small GTPases and Calcium-dependent kinases [52]. On the contrary, Arabidopsis fah1/2 knock-out mutants display an increased resistance to obligate biotrophic fungi *Golovinomyces cichoracearum* potentially due to a consequential increase in intracellular ceramides and salicylate [302]. Interestingly, a sphingosine analogue produced by the fungal pathogen *Alternaria alternata* f. sp. *Lycopersici* (AAL), serves as a virulence factor that induces PCD in plants and animals [303].

6.1.2. Sphingolipids as receptors of necrotrophic toxins and plant-pathogen elicitors

Glycosylated lipids are often receptors in insect host binding to microbial toxins [304]. One very important glycosylated lipids of the animal kingdom are gangliosides. Their polar heads act as surface recognition markers and surface receptors for bacterial toxins such as cholera and Bt toxins [257]. They are recognized and used by virus to enter and infect cells [305]. Plant GIPC bear structural similarities with gangliosides because they contain negatively charged glycan polar heads located in the outer-leaflet of the PM [306].

A recent study has shown that GIPC were located in the outer-leaflet of plant PM are receptors to Necrosis and ethylene-inducing peptide 1-like (NLP) proteins [40]. In the study of Lenarčič et al. 2017, microbial NLP proteins are used for the identification and characterization of NLP receptors. NLPs are part of a superfamily of cytotoxins produced by plant pathogens such as bacteria, fungi and oomycetes [307]. They can be cytolysins, inducing symptoms on eudicot plants but not monocot plants where no necrotic and cytolytic effects are observed [308]. The secretion of NLPs occurs in the extracellular environment of host plants with the toxins targeting the PM outer-leaflet [307,309].*In vitro*, NLPs were shown to specifically bind to purified GIPC from tobacco and Arabidopsis but not to phospholipids, GluCer or sphingomyelin. Moreover, NLPs also bind to all GIPC, irrespective of the plant clade, with similar affinities [40]. Upon binding to the sugar moieties of the GIPC polar heads, NLPs undergo structural changes triggering the conformational modification of their L3 loop and the incorporation of a Mg^{2+} ion responsible for its cytotoxicity. This results in the interaction of the W155 residue of the L3 loop with the membrane, crucial for cytolysis [40]. The study has also showed that sugar residues exposed on the plant outer membrane surface are important for NLP toxicity, such that Glucosamine, Man/Glucose being GIPC terminal sugars of

tobacco and Arabidopsis respectively, induces membrane damage upon binding to NLPs. Plant mutants impaired in the GIPC biosynthetic pathway, are less sensitive to NLPs, implying the importance of intact GIPC for NLP cytotoxicity [40]. The sensitivity to NLP toxicity occurs only for eudicots but not monocots with the exception of a monocot *Phalaenopsis* species (an orchid). This intriguing fact could be explained by the presence of different GIPC series in these two plant clades. While both have similar terminal sugars with similar affinity to NLPs; eudicots GIPC contain two sugars linked to IPC (series A GIPC) whereas monocot GIPC contain three sugars (series A GIPC), with the exception of *Phalaenopsis* containing both series A and B. Biophysical characterization of GIPC series in monolayer artificial membranes suggests a perpendicular arrangement of the polar head of both series A and B GIPC, such that the terminal hexose in series B GIPC, is located further away from the membrane surface compared to series A GIPC. Hence, NLPs binding to series B GIPC terminal sugars have their L3 loop positioned farther from the plant membrane, preventing NLP contact with the membrane and thereby, cytolysis. Both, the length of GIPC head groups and the structural design of the NLP sugar-binding sites explain the differential sensitivity of host plants to NLP toxins.

6.1.3. Lipid domain-associated proteins in plant microbe interaction

Remorins (REM) are plant-specific nanodomain-organized proteins notably involved in plant-microbe interactions. REMs are anchored by their C-terminal domain to the cytosolic leaflet of the PM. The anchoring and lateral segregation in the PM is PI4P- and sterol-mediated [175]. REM1.3 was shown to be delocalized after sterol disrupting treatments, such as methyl-β-D-cyclodextrin [192,225,163,191,175] or fenpropimorph [175]. Both their presence at the PM and their correct partitioning within their cognate nanodomains, are essential for their cellular function, *e.g.* StREM1.3's role in hindering Potato Virus X cell-to-cell movement [175,310]. Other REM-group proteins have been evidenced as key players in biotic interactions such as SYMREM1 (MtREM2.2) involved in the nodulation process of *M. truncatula* with *Sinorhizobium meliloti* [311] Its role was also shown to be essential in the dynamic stabilization of the LYK3-FLOT4-SYMREM1 PM-nanodomains, important for root bacterial symbiosis [312]. Remorins are found in DRM, the closest biochemical counterpart to PM-nanodomains known today, in virtually all clades of land plants: Poplar [313], Oat and Rye [169], Tobacco [172], Arabidopsis [165]. The role of REMs as actors involved in a wide range of biotic interactions has been established throughout the years: ranging from susceptibility factors to viral infections [314], to oomycetes [315] or on the contrary as resistance factors to Potato Virus X [191]. Their capacity to impact PD permeability [175,316] also demonstrates their implication in innate immunity. Super-resolution microscopy has been used to understand the role of nanodomain dynamics in the context of viral infection, revealing that an optimal partition (*i.e.* size of nanodomains, number of molecules in and out domains, and mobility) of REM-associated nanodomains was necessary for the function of REMs [175].

PM receptors involved in the oxidative-burst response to the perception of Pathogen-Associated Molecular Patterns (PAMPs) are key players in plant innate immunity [317]. Their PM localization gives them the role of gatekeepers capable of dynamically associating to co-receptors complexes and triggering signaling pathways to prepare the cell for immediate and short-to-mid-term defense responses [317]. The best example is FLS2, an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin (flg22) in Arabidopsis [318]. PM-subcompartmentalization *via* raft-like domains is an essential phenomenon in the PAMP perception and response processes. Upon cryptogein treatment on tobacco cells, a quantitative proteomics approach has evidenced the delocalization of various Dynamin and 14-3-3 proteins into the DRM fraction, both involved in PM-based signaling [171]. The reorganization of the PM in response to elicitors is not only observed *via* its protein composition but also *via* the biophysical properties conferred by lipids: upon elicitation with flg22 or cryptogein, PM order and fluidity are altered [92]. As regards to flg22's cognate receptor FLS2, it was shown to relocate to DRM fractions upon flg22 treatment as well as many other key proteins involved in immunity-associated signaling [164], revealing that PM-remodeling, at both the lipid and protein level, is important for a functional immune response.

In good agreement, perception of flg22 in BY-2 cells induces global increase of the order level of the PM. While this modification of the PM properties correlates with signal initiation [319], the potential functional implication and molecular basis of such membrane modification remains to be elucidated. Cryptogein is shown to be able to induce an increase in PM-fluidity *via* sterol-binding [112]. Sterols and their associated micro-environments appear to be crucial for immune responses at the cellular level to the extent that both mutants for sterol biosynthesis and sterol-biosynthesis inhibiting drugs affect cell wall composition and induce abnormal callose and lignin deposits *i.e.* cell wall compounds involved in biotic stress [300].

6.1.4. GPI-anchored proteins & outer-leaflet PM domains

The importance of outer-leaflet PM-nanodomains enriched in sphingolipids and sterols is also underscored by the presence of GPI-anchored proteins in these domains, many of which are implicated in host responses to invading microbes. GPI-anchored β-1,3-glucanases (BGs), responsible for callose degradation, are found in DRM fractions alongside callose synthases [313]. Their presence in microdomains around PD enable a turnover of callose deposits when proper signaling occurs. The localization of BGs at PD is regulated by the presence of sterol-enriched domains at the PM, which share a virtually identical lipid composition with the PD-PM interface to the extent that sterol-biosynthesis inhibitors abolish this targeting [7]. The PD-enriched GPI-anchored protein LYM2 has been found to impact PD conductance in response to chitin treatments [320]. Several GPI-anchored BGs have been shown to associate to PD thanks to their GPI-anchoring motif [321] in a salicylic acid-dependent fashion [322] consolidating the idea that addressing proteins to PM-PD microdomains *via* GPI-anchoring plays a significant role in host responses to pathogens.

6.1.5. Extracellular vesicles & host-induced gene silencing

Extracellular vesicles (EVs) are PM-derived vesicles secreted in the extracellular matrix involved in inter-cellular communication and in response to stress, notably biotic stimuli. The existence of these vesicles has been first observed in plants in the 1960's [323] yet EVs have only recently been better characterized [324] regarding their function during plant-pathogen interactions. They are observed upon xylanase treatment [325] and in response to hormone treatments [326]. EVs are believed to be derived from multi-vesicular bodies (MVB) which accumulate around appressoria and haustoria during defense against fungal pathogens [327,328]. They have recently been shown to contain a number of biotic stress-related compounds such as phytoalexins like glucosinolates [326], small-interfering RNAs that can effectively silence pathogen-associated virulence and/or housekeeping genes [329–331] and signaling-associated enzymes, such as PLD and PLC [332]. The presence of these phospholipases hints to the possibility of EVs being involved in lipid signaling pathways.

Plant EVs contain phospholipids such as PI4P [325,333], PI and PA [326]. The lipid composition may vary upon which organ they are secreted, and upon different stimuli applied to the secreting cells. For example, upon jasmonic acid treatment, sunflower seed-derived EVs will be enriched in PI4P and depleted in PI [326]. The lipidome of plant EVs, in different conditions, has yet to be published and it will surely reveal crucial information on their biogenesis and activity.

6.2. Hormone signaling and transport

Lipid-mediated protein sorting mechanisms at TGN have strong impact on plant development since they are involved in directing the secretion and endosomal recycling pathways of a set of proteins that

includes hormone transporters. Higher plants are multi-cellular organisms able to respond and quickly adapt to their environment. In particular, the plant hormone auxin plays a fundamental role in the regulation of a variety of developmental processes enabling plants to adapt to their environment, including directional growth as gravitropism [334–340]. Auxin mediated control of plant development relies on establishment of concentration gradient of auxin that are generated by the activity of plasma membrane localized auxin carriers [338–342]. Therefore, the mechanisms that control the remodeling of auxin carriers represent a key control point for signals that control plant development and response to abiotic stress. Several studies have shown that the TGN is involved in auxin-carrier trafficking but the sorting mechanisms are poorly understood [343–345]. Several elements related to G proteins are known to be involved in TGN-mediated auxin-carriers' trafficking. Our goal in this review is not to create an exhaustive list of all these elements but we can name a few: the small GTPase protein RAB-A1b, the ECHIDNA protein which interacts with YPT/RAB GTPase interacting protein 4a (YIP4a) and YIP4b, the ADP ribosylation factor (ARF) ARF1 as well as the ARF-guanine exchange factors (ARF-GEFs) GNOM and BIG1-5, and finally the ARF-GTPase activating proteins (ARF-GAPs) SCARFACE/VAN3 [345–350]. On the lipid side, sphingolipids represent a class of lipids particularly interesting since VLCFAs, an imprint of sphingolipids, are enriched at TGN [235]. Shortening of the acyl-chain using pharmacological and genetic tools reveal that the length of sphingolipid acyl-chains is involved in the secretory sorting of the efflux auxin carrier PIN2 (but not in PIN1 or AUX1 trafficking), auxin redistribution during root response to gravity (root gravitropism) and root gravitropism *per se* [235]. Interestingly, it had been shown before that VLCFA-containing sphingolipids are involved in PIN1 and AUX1 trafficking (but not PIN2 trafficking) and lateral root formation [56]. These results are not necessarily contradictory. Indeed, the structural diversity of sphingolipids is wide and has been described in chapter 1 and in several reviews [35,301]. LCBs are not always found included in a ceramide molecule but can also be freely found at non-negligible proportion inside the cell as sphinganine and phytosphingosines. Finally, aside from sphingolipids, VLCFAs can also be included in some phospholipids. On this topic, it has been shown that the *pas2* mutant displays a reduced level of VLCFA-containing PE and show endocytic trafficking defects [351]. Decrease of VLCFA-containing PE in the *pas2* mutant targets the RAB-A2a compartment and the plasma membrane endocytic recycling of the auxin efflux-carrier PIN1 but has no effect on PIN2 localization [351,352].

Coming back on sphingolipids, it has been shown that the *loh1/loh2* double mutant, which is defective in ceramide synthases LOH1 and LOH2, are involved in endocytosis and plasma membrane recycling of the auxin carriers PIN1 and AUX1, but not PIN2, potentially through RAB-A2a compartments [56]. Hence, it is possible that VLCFA-containing sphingolipids are important for endocytic/recycling of certain subset of proteins from plasma membrane through a RAB-A2a-positive subdomain of TGN that would host the recycling pathway. In contrast, the herbicide metazachlor, that drastically modifies both GluCer and GIPC fatty acids composition by replacing VLCFAs in GluCer and GIPC pools by 16–18 carbon atom fatty acids without modifying the global quantity of either GluCer or GIPC, alters PIN2 polarity at plasma membrane while it neither affects PIN1 polarity nor AUX1 localization [235]. Importantly, metazachlor blocks PIN2 predominantly at SYP61/secretory vesicles (SVs) compartments as compared to RAB-A2a/CCVs [235]. Concomitantly, metazachlor treatment neither alters endocytosis nor plasma membrane recycling of PIN2 but rather blocks the secretion of *de novo* synthetized PIN2 at SYP61/SVs compartments. From these studies, it can be postulated that TGN-associated RAB-A2a/Clathrin-coated vesicles (CCVs) compartments could host the ceramide-dependent PM recycling of auxin carriers PIN1 and AUX1 while TGN-associated SYP61/SVs compartments could host VLCFA-containing GluCer and GIPC-dependent secretory sorting of PIN2. However, future challenges on this topic need to address TGN sub-domains' dynamics, interactions and maturation, which could involve a tight regulation of lipid homeostasis and crosstalk to define the identity of each sub-domain.

The VLCFAs of sphingolipids determine a specific physical property of sphingolipids, which is the ability to insert their acyl-chain within the opposing leaflet of the membrane, a phenomenon known as interdigitation, see Fig. 4. Interestingly, it has been shown in animal cells that the coupling of membrane leaflets is cholesterol-dependent [66]. In yeast and in plants, it has been shown by immuno-purification of intact compartments coupled with lipid mass spectrometry analyses, that TGN-derived secretory vesicles are enriched in both sphingolipids and sterols [232,235]. Currently, it is not clear whether the pool of sterols at the TGN would play a role in the trafficking of auxin carriers. Previously, it has been shown that sterols are involved in endocytosis and recycling at plasma membrane of the PINs auxin carriers [353–355]. Sterol-mediated endocytosis is involved in PIN2 polarity establishment after cytokinesis by removing PIN2 from the new basal membrane of daughter cells, while PIN2 at the new apical membrane remains [354]. Sterol-mediated auxin carriers' sub-cellular localization impacts auxin distribution at the tissue-level, which has repercussions on plant development such as the graviresponse of the root [354]. Interestingly, not only are the major sterols: sitosterol, stigmasterol and campesterol involved in plant development, but also some very rare intermediate-sterol compounds such as derivatives of cycloartanol (4-carboxy-4-methyl-24-methylenecycloartanol) and 4α-methyl sterols (24-ethyllophenol and 24-ethylidenelophenol) [356,357]. Future studies will reveal how sterols interplay with other lipids such as sphingolipids and anionic phospholipids to regulate hormone transport.

6.3. Abiotic stress

Lipids have been involved as second messengers in many responses to abiotic stress, see for review [265,358–360,361]. PLD and PLC/DGK-mediated PA formation and it subsequent phosphorylation to form Diacylglycerol Pyrrophosphate (DGPP) are key players in this response [362]. Other lipids participate to the response: oxylipins, PIPs, sphingolipids, fatty acids, lysophospholipids, N-acylethanolamines and galactolipids, see for reviews [1,363]. Studies have started deciphering the different isoforms of enzymes involved in these transduction pathways as well as their tight regulations, for recent reviews see [364]. For example, PA displays different modes of action, including direct target protein binding and biophysical effects on cell membranes. It is puzzling that PA production can be triggered by opposite stressors, such as cold and heat. How PA regulates this diversity of response is discussed in this recent review [267].

PLD-derived PA was shown to recruit the ABA-regulated ABI1 phosphatase 2C (PP2C) to the PM [365]. The identification of ABI1 as a direct target of the PA provides a functional link between the two families of important signaling enzymes, PLD and PP2C. Recently, a study on the regulation of ion transport in plants showed that C2-domain ABA-related (CAR) family of small proteins is involved in the Ca^{2+}-dependent recruitment of the pyrabactin resistance 1/PYR1- like (PYR/PYL) ABA receptors to the PM. CARs are peripheral membrane proteins that cluster at the PM and generate strong positive membrane curvature. These features represent a mechanism for the generation, stabilization, and/or specific recognition of PM discontinuities involved in the recruitment of PYR/PYL receptors and other signaling components in cell responses to salt stress [366].

6.4. Plasmodesmata function

Plasmodesmata (PD) are specialized nano-sized membrane-lined channels, which cross the walls of plants and of some algal cells. PDs enable direct, regulated, symplastic transport of small RNAs and molecules between cells. They are also hijacked by phytoviruses to allow their propagation from cell-to-cell. Ultrastructure of PD has been

deciphered. They are lined by the PM forming what is termed the PD-PM subcompartment (PD-PM) and contain a strand of tubular modified ER called desmotubule, and the space between these two membranes is thought to control PD permeability [203]. A recent study has reconstructed PD three-dimensional ultrastructure with an unprecedented resolution using electron tomography, showing that ER-PM contact sites undergo substantial remodeling events during cell differentiation [367]. Post-cytokinesis PD, called type I PD, present an intimate ER-PM contact along the entire length of the pores whereas during cell expansion, the PD pore widens and the two membranes separate, leaving a cytosolic sleeve spanned by tethers whose presence correlates with the appearance of the intermembrane gaps, called type II PD. Surprisingly, the type II PD allow diffusion of macromolecules despite the apparent lack of an open cytoplasmic sleeve, forcing the reassessment of the mechanisms that control plant cell-to-cell communication [367].

The membrane organization of PDs is therefore characterized by the close apposition of the ER-derived desmotubule and the PM with spoke-like structures linking the two membranes likely defining microdomains of the PM, PD-PM and desmotubules has been recently proposed to be membrane contact sites (MCS). MCS control close appositions between two membranes that form microdomains involved in the control of lipid exchanges or in coupling events (review in [368]). MCS-subdomains are likely to display specific biophysical properties and may cluster proteins and negatively-charged lipids like phosphoinositides, promoting specific physicochemical membrane properties taking part in shaping local membrane electronegativity gradients [368].

Firmly anchored within the cell wall, PDs are difficult to purify. A two-step simple purification procedure (consisting in isolating cell wall fragments containing intact PD and an enzymatic degradation of the wall matrix to release of PD) has been successfully design to obtain highly-purified PD preparations [369]. Hence, analyses of PD fractions have provided valuable information on the functional and structural elements that define PD, particularly for lipids. PD membranes display enrichment in sterols and sphingolipids with saturated VLCFAs (likely GIPC) when compared with the bulk of the PM. This profile is reminiscent of DRMs although the isolation procedure is detergent-free and suggest that lipids are laterally segregated at the PD-PM cell-to-cell junction in *Arabidopsis thaliana* [7]. This study identifies a role for sterols in modulating cell-to-cell connectivity, possibly by establishing and maintaining the positional specificity of callose-modifying GPI-anchored proteins at PD and emphasizes the importance of lipids in defining PD-associated nanodomains. The role played by the other major lipid constituents, such as GIPC and glycerolipids in defining specialized membrane domains in PD, remains to be further studied. This change of paradigm regarding the membrane organization of PD will likely pave the way to a deep understanding of cell-to-cell communication in plants.

7. Conclusions and perspectives: how to get a comprehensive membrane organization in plants?

The deep-seated understanding of how the plant PM is organized in terms of the involvement of lipids and proteins clearly necessitates multiple approaches. Our community needs to develop new dedicated methodologies and dare multidisciplinarity even further that what we have been accustomed to. For example, the extensive use of biophysical approaches to study lipid/protein interactions as reviewed in [370], among them: 1/surface plasmon resonance [40], isothermal titration calorimetry (ITC), Langmuir monolayer tensiometry, liposomes binding and lipid strips and arrays [117] to study the interaction of proteins with lipids; 2/liposome leakage and lipid-mixing assays [310] to investigate how proteins can destabilize membrane bilayers; 3/Circular dichroism [310], Fourier-transform infrared spectroscopy [175], solid-state NMR with labeled lipids or proteins [371] to obtain 3D structures of the membrane-bound interaction-complexes; 4/Atomic force microscopy (AFM) to scan the surface of bilayers and access the topology of different membrane organizations. A deep understanding of the structural aspects of protein/lipid interactions will allow the targeted-mutagenesis of key residues, the biological role of which could be further addressed by reverse genetics approaches.

In all the methods described above, there is an urgent need for biochemistry to obtain purified proteins and lipids, in order to reconstitute proteoliposomes. Concerning proteins, the problem of insolubility of highly hydrophobic proteins could be solved by using different expression systems [372]. As for lipids, most of plant lipids are commercially available with the notable exception of GIPC. The lack of commercially-available GIPC strongly impairs any serious *in vitro* reconstitutions of true plant PM-like vesicles. One must undertake hard-core preparative biochemistry as described during the 1970's to obtain milligrams of purified GIPC from living material [53,55]. Purification of mg amount of GIPC will also pave the way to the development of fluorescently labeled sphingolipid analogs by conjugating a hydrophilic fluorophore to the headgroup or a hydrophobic fluorophore to the amidified fatty acids *e.g.* [373]. Unfortunately, very few plant lipids labeled with a fluorochrome or deuterated are available. Similarly, none of the great diversity of free- and conjugated-phytosterols are commercialized in a labeled form, which strongly impairs NMR studies that would enable to further enquire about the role of each molecular species individually. From purified lipids, the preparation of asymmetrical liposomes will also be very challenging. To have access to the properties of asymmetric vesicles that mimic the plant PM would provide means to understand lipid raft formation or transmembrane helix orientation [374],

Molecular dynamics (MD) is a method that computes the physical movements of atoms and molecules in order to give a view of the dynamic evolution of a virtual chemical system. It can therefore study the interaction dynamics of lipids and proteins, or the conformational changes of molecules, for a fixed period of time. MD computes lipid-lipid and lipid-protein interactions and provides a comprehensive atomistic model of a typical lipid bilayer and gather information on membrane domain formations [375,376]. MD has been used in plant membrane biology to decipher the structural basis of the unconventional lipid-binding motif of REM that confers nanodomain organization [175]. Such approach of “computational microscopy” captures the molecular interactions within a complex system at a spatiotemporal resolution, unmatched by any other conventional experimental methods *e.g.* [137,377]. MD can be of particular interest in grasping the intricacies of plant PM dynamics because the control of the thermodynamic parameters permitted by MD can be used to mimic the constantly varying environmental conditions sustained by plants in their natural habitats, and thereby to understand the biophysical implications of these variations on the PM. Unfortunately, neither plant sphingolipids (GluCer, GIPC), nor phytosterols (free or conjugated) have been, to this day, modelized in the force field, preventing all MD studies with plant PM lipids. The force field refers to the functional form and parameter sets used to calculate the potential energy of a system of atoms or coarse-grained particles; it is the necessary step for MD simulations. The parameters of the energy functions may be derived from experiments in physics (solid-state NMR, Langmuir monolayer, calorimetry…) or chemistry (chemical structure, liquid-state NMR…) [378]. All-atom force fields provide parameters for every type of atom in a system. Coarse-grained potentials, which are often used in long-time simulations of macromolecules and multi-component complexes, provide even cruder representations for higher computing efficiency. Such modelization must be carried for plant sphingolipids and phytosterols as it was described for animal gangliosides in [379].

Other aspects still missing in the plant membrane biology field, are the methods to image lipids *in vivo* necessary to follow their segregation at the nanoscale level, their dynamics and interactions with proteins. Two reasons can be given: firstly, as stated before, very few fluorescently-labeled lipids are available, and secondly, the cell wall strongly impairs the intake of fluorescently labeled molecules. Nevertheless, the

use of *in vivo* bioorthogonal click chemistry could be an elegant approach to circumvent this problem in plants [380,381]. Although genetically encoded biosensors for PIPs, PA, DAG and PS have already been developed [27,117,118], biosensors of plant sphingolipids and phytosterols are crucially missing and will surely be developed using lipid-binding domains found in plant proteins. Inhibitors of lipid synthesis (Table 1), and genetically-modified lipid-using enzymes (phosphoinositides kinase, lipid phosphatase, lipase) able to target the PM and specifically modify *in vivo* pools of lipids, are used to address the question of the role of lipids in nanodomain formation and dynamics [118,175,382]. Similarly, membrane surface charge or pH biosensors must be improved as described in animal literature with intramolecular fluorescence resonance energy transfer (FRET) sensors [383,384]. The development of environmentally-sensitive probes to label the outer- or inner-leaflet and measure independently the fluidity of plant PM leaflets, must also be engaged (see examples of available probes in [385]). Furthermore, super-resolution microscopy will continue to allow a deep understanding of segregation and dynamics of proteins and lipids at the nanometer scale. The development of fluorescent bimolecular tracking in the live-cell PM will reveal whether proteins and lipids may directly or indirectly interact with each other [211].

Finally, state of the art lipidomics [386] and proteomics approaches must be further developed including phosphoproteomics and lipid-modification of proteins (myristoylation, palmitoylation, isoprenylation and GPI-anchoring, review in [387]).

The study of K-RAS protein which controls cell proliferation in animal cells should be exemplified for the combinations of approaches mobilized by Hancock's group to tackle its function in relation to its organization within PM [260–262,388–390]. K-Ras controls cell proliferation, and when mutated, cells continuously proliferate and often develop into cancer. This group tackled the role of K-Ras in PM nanodomains by using in parallel biochemistry, biophysics, modeling, high-resolution imagery, mutagenesis, structural biology, model membranes, transcriptomics, cancerology and genetics techniques. This form of multidisciplinary approach has led to a deep understanding of the anchoring, the clustering of K-Ras with PM lipids, as well as the integration of these molecular mechanisms into higher levels of cell biology, hence determining their consequences on the fate of the cell. For example, a recent paper of this group has shown that K-Ras anchoring sequences can create lipid nanodomains with a remarkable specificity [261], and that lipid nanodomains are not preexisting. A matter of the chicken or the egg causality dilemma! Similarly the works of Katharina Gaus' [383,384,391–394] or Akihiro Kusumi's labs [132,395–397] are exemplary to understand, by developing single-molecule imaging methods, how T-cells initiate an immune response and to better fathom the intricate complexity between cytoskeleton and protein/lipid segregation. These two research groups have been remarkable by developing, in collaboration with biophysicists, state of the art technology to follow single-molecules in the PM.

Taken together, tools and methods must be developed by the plant membrane biology community in the near future to pave the way towards the better understanding of the intimate molecular relationships between lipids and proteins at the basis of domain segregation, dynamics, signaling and function.

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Conflict of interest

No conflict of interest declared.

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