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The function of sphingolipids in membrane trafficking and cell signaling in plants, in comparison with yeast and animal cells

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Abstract

Sphingolipids are essential membrane components involved in a wide range of cellular, developmental and signaling processes. Sphingolipids are so essential that knock-out mutation often leads to lethality. In recent years, conditional or weak allele mutants as well as the broadening of the pharmacological catalog allowed to decipher sphingolipid function more precisely in a less invasive way. This review intends to provide a discussion and point of view on the function of sphingolipids with a main focus on endomembrane trafficking, Golgi-mediated protein sorting, cell polarity, cell-to-cell communication and cell signaling at the plasma membrane. While our main angle is the plant field research, we will constantly refer to and compare with the advances made in the yeast and animal field. In this review, we will emphasize the role of sphingolipids not only as a membrane component, but also as a key player at a center of homeostatic regulatory networks involving direct or indirect interaction with other lipids, proteins and ion fluxes.

Introduction

Sphingolipids constitute one of the largest family of lipids due to their high molecular and functional diversity. The exact count of sphingolipid molecular species is difficult to evaluate, each class of sphingolipids, i.e. long chain bases (LCBs), ceramides and complex glycosylated sphingolipids, contains sub-classes of hundreds of molecular species each. It would not be an overstatement to say that, in the plant model *Arabidopsis*, several hundreds of sphingolipid molecular species co-exist in total. Thus, we are far from understanding the functional role of each sphingolipid species or sub-classes, but recent advances in the plant' sphingolipid field identified novel role for specific sphingolipid species in cellular mechanisms [1–12]. Additionally, one given sphingolipid molecular specie could fulfil distinct functions depending on whether it involves the polar head or the hydrophobic tail of the molecule [3,5,7,8,13–16]. Finally, the interaction network of sphingolipids with specific proteins or other lipids adds another layer of regulation and complexity to the functional impact of sphingolipids on cellular processes and cell physiology ([1–4,17]. Sphingolipids support numerous cell and physiological processes, including but not limited to membrane trafficking, cell polarity, cell differentiation, cell-to-cell communication, signal transduction, immunity and environmental responses [1,6,9,18]. At the structural level, some sphingolipid species differ between animals, yeasts and plants (Figure 1). Common to the three kingdoms are the LCBs that further condensate with a fatty acid through an amide bond to form a ceramide molecule (Figure 1). However, the level and position of unsaturation as well as hydroxylation, both on the LCB or fatty acid chain, might vary between kingdoms. For example, plants mainly use tri-hydroxylated LCB like phytosphingosine as sphingolipid precursor while animals mainly use the di-hydroxylated LCB sphingosine. Glucosylceramide (GlcCer) is also a common molecule, but complex glycosylated sphingolipids greatly differ between animals, yeasts and plants (Figure 1). In animals, ceramide can be grafted with a phosphocholine polar head resulting in sphingomyelin (Figure 1), or be grafted with different glycosidic moieties, with or without esterification to a sialic acid in the case of gangliosides or globosides, respectively (Figure 1). In plants and yeasts, sphingomyelin, gangliosides and globosides are absent. Instead, an InositolPhosphate (IP) group is grafted to ceramide to produce InositolPhosphorylCeramide (IPC) that are further glycosylated to produce the final forms of sphingolipids (Figure 1). In yeasts, one or two mannose residues are added to IPC and result in MannosylInositol PhosphorylCeramide (MIPC) or Mannosyl diPhosphorylInositol Ceramide (M(IP)₂C). In plants, a glucuronic acid (GlucA) followed by a series of various sugars (mainly a mannose in *Arabidopsis*, but in other plant species up to 14 sugar residues can be found) is added to IPC to form the Glycosyl Inositol PhosphorylCeramides (GIPC) (Figure 1) [1]. The idea of this review is to provide a concise view of the most representative and recent advances in the plant sphingolipid field with a focus on membrane-based cell processes and a constant back and forth comparison with the yeast and animal

field. We will more generally describe the involvement of sphingolipids in membrane-based intracellular and intercellular trafficking mechanisms and processes. Aside from the intracellular membrane organelles, the Plasma Membrane (PM) is also admittedly a crucial membrane as it faces both the extracellular space where is perceived developmental and environmental signals, including pathogen' elicitors, and the intracellular space where the signals are encoded and transmitted. Thus, we will describe in this review the signaling events mediated by sphingolipids at the plant' PM.

Sphingolipid trafficking and sorting at the ER-Golgi and post-Golgi stations in animals and yeasts

Intracellular trafficking is a fundamental process that supports the fluxes of both biosynthetic and degradative processes. A crucial intracellular membrane interface is the Golgi apparatus where a wealth of transport and sorting pathways converge. The classical textbook representation of the Golgi is a stack of three distinct cisternae: the *cis*-Golgi that receives and sorts protein cargos coming from the Endoplasmic Reticulum (ER) through ER-Export Sites (ERES) followed by the medial-Golgi and the *trans*-Golgi cisternae where glycosylation occurs. At the *trans*-side of the Golgi apparatus, a tubulovesicular network called the *trans*-Golgi Network (TGN) is differentiated (Figure 2). However, the Golgi apparatus is organized differently between animal and yeast cells. In animal cells, the Golgi apparatus is an assembly of several Golgi stacks connected together by tubular structures and immobilized on the MicroTubule Organizing Centre (MTOC). In the fission yeast *Saccharomyces pombe* or in the saccharomycetes *Pichia pastoris*, the Golgi forms a stack similarly to plant cells. This is in contrast with the budding yeast *Saccharomyces cerevisiae* in which the cisternae are not packed together in one stack but are rather dispersed in the cytoplasm (Figure 3). Despite these differences, sphingolipids have been shown to traffic through the Golgi and TGN in animals and yeasts where they are sorted into different pathways. In addition to the conventional vesicular transport, our current knowledge and understanding suggest that the endomembrane system is connected, notably through membrane contact sites (Figure 2) [19–24]. Both vesicular and non-vesicular transport of sphingolipids has been evidenced at the ER/Golgi/TGN nexus module. Indeed, in mammals, non-vesicular transport of ceramides, that are synthesized at the ER, is performed by the ceramide transport protein CERT at ER/TGN membrane contact sites (Figure 2A) [24–27]. In yeasts, ceramides are transferred from the ER to the Golgi by the Nvj2p and/or Svf1 proteins [28,29]. In animal cells, ER/TGN membrane contact sites involve complex lipid exchanges including ceramides, sterols or phosphatidylserine (PS) from the ER against phosphatidylinositol-4-phosphate (PI4P) from the TGN (Figure 2A) [26,30]. Interestingly in mammals, the synthesis of sphingomyelin, by the grafting of a phosphocholine on ceramide, regulates the level of PI4P at TGN through its consumption by the lipid transfer OxySterol-Binding Proteins OSBPs (Figure

2A) [27]. The homeostatic circuit is very complex but a key is the production of a Di-Acyl Glycerol (DAG) molecule during sphingomyelin synthesis, that will activate downstream enzymes or proteins involved in 1/ the transport of ceramide from the ER to TGN (CERT), 2/ the local production of PI4P (PI4KinaseIII β) at TGN or 3/ its transfer from the TGN to the ER (OSBP) (Figure 2A) [27,31]. These production/consumption mechanisms are bound together to buffer the oscillations occurring in the lipid composition at TGN, thus ensuring a lipid flow through the TGN without impacting its steady-state composition [27,31]. Importantly, it has recently been demonstrated that ER/TGN membrane contact sites are essential to generate a lipid gradient that facilitates polarized cargo sorting at TGN [32]. Interestingly, once synthesized at TGN, sphingomyelin is differentially sorted into a subset of TGN-derived secretory vesicles that are enriched in Glycosyl-Phosphatidyl-Inositol (GPI)-Anchored Proteins (GPI-APs) [33]. In yeasts, sphingolipids also get sorted into a subset of TGN-derived secretory vesicles that are enriched in sterols [34]. Importantly, in animal cells, intra-Golgi non-vesicular transport of glycosylated forms of ceramide, such as GlcCer, is additionally occurring between the *cis*-Golgi and the TGN and is driven by the pleckstrin homology domain-containing family A8 (PLEKHA8)/FAPP2 protein (Figure 2B) [35–37]. Strikingly, the vesicular and non-vesicular transport of GlcCer leads to the production of different complex glycosphingolipids, i.e the synthesis of gangliosides at the Golgi and the synthesis of globosides at the TGN, respectively [35]. FAPP2 binds to PI4P that is enriched at TGN and GlcCer strengthens this interaction providing a coupling mechanism between lipid synthesis and sorting in different trafficking pathways [35].

Sphingolipid trafficking and sorting at the ER-Golgi and post-Golgi stations in plant cells

A deadlock in the plant's sphingolipid field is the absence of a fluorescent tracer, probe or biosensor to label sphingolipids. The structural specificity of the polar head (inositolphosphate, glucuronic acid and a mannose for Arabidopsis) in plants makes the transfer of biosensor tools from the mammalian or the yeast field to the plant field difficult and hazardous. For example, our team repetitively tried and failed to genetically encode in plants the Sphingolipid Binding Domain (SBD) of the Human amyloid β peptide that binds sphingolipids in a cholesterol dependent manner [38,39]. Probably, the SBD probe does not recognize plant sphingolipids due to the difference of the polar head structure between animals and plants. Moreover, the use of sphingolipid fluorescent analogs or fluorescent dyes that could be externally applied to plant cells has been tried before but unfortunately this approach is working only on cells deprived of the plant extracellular matrix called the cell wall [40]. Thus, the visualization of sphingolipids in native plant tissues is not possible for now due to the presence of the tightly packed polysaccharides-constituted cell wall. Establishing an atlas of sphingolipid-interacting proteins or the development of click-chemistry

could help in getting new tools to visualize sphingolipids in the future for the plant field. Despite these limitations, PM purification and immuno-isolation of intracellular compartment, allowed to measure that while sphingolipids are synthesized at the ER they actually get enriched at the PM and the TGN as compare to the whole pool of membranes [8,41]. Interestingly, sphingolipids are enriched at a subdomain of TGN labeled by the syntaxin SYP61 [8]. Thus, these results suggest that sphingolipids are sorted into a subset of TGN-derived vesicles, similarly to gangliosides, globosides and sphingomyelin in animal cells [8,33]. The localization of the enzymes involved in sphingolipid biosynthesis might also provide some clues on the localization of sphingolipids but this has to be taken with cautious due to that the sphingolipid products might be synthesized in one place and accumulated elsewhere. In plants, the main form of sphingolipids is the GIPC, an ER-synthesized ceramide backbone grafted with an inositolphosphate (IP) group plus a glucuronic acid (GlucA) and a mannose residue as a polar head [42–44]. Interestingly, IPCS enzymes (grafting of inositolphosphate on ceramides) are localized at TGN while the IPUT1 (grafting of glucuronic acid on IPC) enzyme, the mannose transporter GONST1 and the GIPC mannosyl transferase GMT1 that transfers mannose on IPC, all localize to Golgi apparatus (Figure 5) [3,14,16,45–48]. At first sight these results could look contradictory, if ceramides are transferred from the ER to the Golgi apparatus and then to the TGN, then why the first enzymes (IPCS) localize to TGN while the last enzymes (IPUT1 and GMT1) localize to the Golgi apparatus? An explanation to this could be that ceramides are transferred directly from the ER to TGN. In plants, an ER-to-Golgi ceramide transfer protein, such as CERT, or Golgi-to-TGN sphingolipid transfer protein, such as FAPP2, have not been identified yet. Indeed, in plants the TGN is highly mobile and can detach from the Golgi apparatus to become a Golgi-independent organelle; the Golgi itself is composed from dozens of individual stacks that are mobile along actin cables (Figure 4) [49–51]. Thus, the characterization of membranes contact sites at the Golgi interface is not trivial and as a consequence there are not yet any convincing evidences for the existence of ER/TGN membrane contact sites. However, we do not want to exclude the possibility of a transfer of ceramide at ER/TGN membrane contact sites since some potential plant homologs of the yeast Nvj2p or Svf1 proteins, or proteins with known function at membrane contact sites, such as the SYNAPTOTAGMIN (SYT) proteins, or other uncharacterized proteins could potentially fulfill this function. In this hypothesis, ceramides would be transferred from the ER to TGN via membrane contact sites, at TGN ceramides would be converted to IPC by IPCS and would then be transported to the Golgi apparatus *via* retrograde trafficking where IPUT1 and GMT1 will convert IPC into GIPC (Figure 5). GIPC would then cross the TGN by anterograde trafficking to reach the PM eventually. Alternatively, if ER/TGN membrane contact sites would not exist in plant cells, then ceramides could be transferred to the Golgi apparatus, like in yeasts, and reach the TGN through canonical Golgi-to-TGN transport or cisternae maturation. The two hypotheses are equally plausible and here again, to solve this issue we would need to

develop in the future a complete subcellular map of the localization of sphingolipid species at endomembrane compartments by either immuno-purification of the compartments coupled with a lipidomic characterization or/and *in situ* localization of sphingolipids using specific probes.

Function of sphingolipids in ER-to-Golgi trafficking in animals and yeasts

Recently, novel twists in animal cells on the trafficking model between the ER and the Golgi apparatus deeply changed our view of trafficking. In this revised model, the ERES that convey proteins to the Golgi apparatus, are not exit-sites in the way they were traditionally viewed previously, but a network of intertwined membrane tubules that connects the ER to the Golgi apparatus through pearled membrane extensions (Figure 2C) [52]. Importantly, the COPII coat complex that is involved in ER-to-Golgi protein sorting is not localized on COPII-vesicles as exposed in the text books but remains associated at the neck region of the ERES where it mediates the entry of neosynthesized cargo proteins to the ERES-derived tubules protrusion (Figure 2C) [52,53]. This mechanism could potentially not only involve coat proteins such as COPII, or COPI protein complex which was found to act downstream of COPII on more distal tubules, but also lipids. In animal cells, protein-lipid Förster Resonance Energy Transfer (FRET) allowed to identify a specific interaction between a sphingomyelin specie containing an unsaturated acyl-chain of 18 atoms of carbon (SM18:0) and the VXXTLXXIY domain of the transmembrane domain of the single-spanning COP machinery protein p24 (Figure 2C) [54]. This interaction induces p24 dimerization and thus promotes the formation of higher-order trafficking machinery structures [55]. Interestingly, sterols are involved in this process, not by acting on p24 dimerization directly, but rather by favoring lipid-ordered phases within the membrane [55]. Thus, it appears that the interaction between membrane lipids and proteins co-creates a higher-ordered specific domain in which crucial elements of the trafficking machinery, such as COP proteins, could get concentrated (Figure 2C). In animal cells, a computational homology screen of a relaxed version of the sphingolipid-binding motif originally identified in p24 further revealed its presence in multi-spanning proteins, especially G-Protein Coupled Receptors (GPCR) in which the sphingolipid-binding domain was mostly located in the 6th transmembrane domain [56]. This identification was further confirmed in the Human serotonin1A receptor, a member of the GPCR family [57]. Moreover, FRET-based *in vitro* lipid transfer assay suggest that membrane-embedded acidic residues adjacent to the sphingolipid-binding motif of the LYSOSOME ASSOCIATED PROTEIN TRANSMEMBRANE 4B (LAPTM4B) could be additionally required for the binding of the protein to sphingolipids through a hydrogen bond [58]. Additional to GPCR, computational screening of the relaxed version of the p24 sphingolipid-binding domain identified the ER-localized GPI-ATTACHMENT PROTEIN 1 (hGAA1) as a putative sphingolipid-binding protein [56]. However, the

binding of sphingolipids to the proteins identified through computational screen should be confirmed experimentally and the relevance of these sphingolipid binding motifs in the function of the targeted proteins should be further explored. For example, the hGAA1 protein cited above is essential for the attachment of the GPI group to proteins to produce GPI-APs, but GPI-APs of mammalian cells do not rely on sphingolipids to ensure their transport from the ER to the Golgi apparatus at least in polarized epithelial cells [59–61]. It could be that the attachment of the GPI group is dependent on sphingolipids while the transport of GPI-APs would not depend on sphingolipids. However, this view has been recently challenged since it was elegantly demonstrated that in mammalian cells, ether lipids (lipids containing an ether bound in contrast to the ester bound of phosphoglycerolipids) act in a coordinative manner with sphingolipids to ensure the correct transport of GPI-APs at the ER-to-Golgi interface [62]. Interestingly, molecular dynamics simulation revealed that p24, an element of the COP machinery that contains a sphingolipid-binding domain and that is involved in the export of GPI-APs, is surrounded by an enrichment of ether lipids (Figure 2C) [62].

In yeasts, ceramide-based GPI-APs are segregated into specific ERES that are distinct from other ERES where transmembrane cargo are sorted, this segregation eventually results in the packing of GPI-APs and transmembrane cargos into distinct COPII carriers [63–68]. Interestingly, proper sorting of GPI-APs at specific ERES requires the presence of ceramide species with very-long chain fatty acids (VLCFAs) of 26 atoms of carbon (C26) in the ER membranes (Figure 3A) [68]. Complementarily, it has been show that these C26 ceramide species are incorporated in the GPI-anchor through lipid remodeling and that a quality control ensures the proper recognition of C26 ceramide-based GPI-APs by the p24 complex (Figure 3A) [68,69]. Interestingly, on one side the p24 complex binds to the particular sphingolipid species SM18 to initiate COPI vesicle budding and retrograde transport from the Golgi apparatus to the ER, while on the other side p24 proteins selectively pack GPI-APs in anterograde ER-to-Golgi COPII carriers in a C24-ceramide dependent manner in yeast or ether lipid dependent manner in mammalian cells. The idea of a selective direct or indirect interaction between lipids and elements of the trafficking machinery is now emerging as a recognition code, or combinatorial password, to induce segregation and transport of cargo through specific membrane subdomains.

Function of sphingolipids in ER-stress response and ER-to-Golgi trafficking in plant cells

In plants, the function of sphingolipids at the ER/Golgi interface is not documented. However, some studies show a role of sphingolipid in ER-associated cellular processes such as the ER-stress responses that include the Unfolded Protein Response (UPR), the ER-Associated Degradation (ERAD), and autophagy [70]. In plants, the degree of lipid unsaturation is crucial in the activation of UPR in plants

since a mutant defective for the ER-localized FATTY ACID DESATURASE 2 (FAD2), that produces both unsaturated phosphatidylcholine (PC) and phosphatidylethanolamine (PE), is hypersensitive to chemical-induced ER-stress [71]. Consistently, the CHOLINE TRANSPORTER-LIKE1 (CTL1/CHER1), that participates to the production of PC and PE, is involved in ER stress signaling by modulating the abundance of the auxin transport facilitators PIN-LIKES (PILS) at the ER, in turn PILS negatively regulate nuclear auxin signaling and growth rate [72]. Interestingly, ceramides are involved in this process since the fungal toxin fumonisin B1 (FB1), that inhibits the synthesis of ceramide at ER, increases PILS abundance at the ER, reduces auxin signaling and differential growth during apical hook development [72]. FB1 triggers several ER-stress related subcellular phenotypes. In the Bright Yellow 2 *Nicotiana tabacum* plant cells culture, FB1 induces the formation of ER-derived tubular aggregates [73]. Consistently, in *Arabidopsis thaliana* root tissues, FB1 treatment resulted in a larger and more branched ER-network and ectopic accumulation of PILS-containing ER structures [5,72]. Furthermore, FB1 induces cell death and stress-induced production of reactive oxygen species (ROS), another indicator of ER-stress [74,75]. However, FB1 experiments have to be cautiously considered as the inhibition of ceramide synthase by FB1 decreases the amount of VLCFA-ceramides but increases the level of its precursor, the long chain bases LCBs [5]. Given that LCBs act as a detergent, the effect of FB1 could be due to LCBs rather than ceramides. Nonetheless, a mutant deficient for the ORM proteins, a negative regulator of SERINE PALMITOYLTRANSFERASE (SPT) enzyme, that synthesizes the LCBs that will be amidified to a fatty acid to produce ceramides, displays ER-stress related defects [76]. Moreover, the yeast *orm* mutants lead to overproduction of sphingolipids, alteration of inositol metabolism and activation of UPR [77,78]. Altogether, there are good indications that sphingolipids are indeed involved in ER-stress but future studies would need to clearly decipher the link between ceramides and UPR or ERAD during ER-stress response. As for autophagy, it is known that ceramide homeostasis can either induces or inhibits autophagy depending on the balance between ceramide and long chain bases (LCBs, an early product of the sphingolipid synthetic pathway) and the type of abiotic stresses applied [79]. As discussed above, the ER is a place for stress-response, but the ER is additionally the first station in the carriageway of the secretory system. In plants, our understanding of the role of lipids in ER-to-Golgi trafficking remains very limited. First, the existence of an ER-Golgi Intermediate Compartment (ERGIC) is heavily discussed in the plant cell community despite the discovery that some part of the Golgi apparatus, called GECCO for Golgi Entry Core Compartment, remains in close association with the ERES upon the Golgi-destructuring drug brefeldinA (BFA); a behavior similar to the animal ERGIC upon BFA [4,49,80–83]. Recently, our team published a preprint in which we used super-resolution microscopy, membrane dynamics in live cells and cargo tracking to unveil the nature of ER-to-Golgi trafficking in plant cells [84]. These results suggest the existence of a highly dynamic ER-Golgi

intermediate tubulo-vesicular network from which more stable Golgi pre-cisternae arise [84].

Interestingly, this study suggests that C24-ceramides are involved in the generation and dynamics of the ER-Golgi intermediate tubular network [84]. Thus, sphingolipids could be crucial membrane players that regulate the dynamics of the tubulovesicular ERGIC network and trafficking of cargos at the *cis*-side of the Golgi.

In plants, sphingolipids are involved in protein sorting of polar-targeted proteins at specific subdomains of the post-Golgi

Another tubulovesicular network is located at the *trans*-side of the Golgi and is called the *trans*-Golgi Network (TGN). The idea that the TGN of plants is composed from different subdomains fulfilling distinct functions is now widely accepted within the community [4,8,85–92]. One subdomain is labeled by the Rab-GTPase RAB-A2a and is strongly labelled by clathrin (Figure 4A) [8,86]. Another subdomain of TGN is labeled by the syntaxin SYP61 and the V-ATPase VHA-a1 (Figure 4B). This subdomain can either be completely uncoated and enriched in the adaptor protein complex AP-4 or be associated with clathrin with an enrichment of AP-1 (Figure 4A, B) [8,91,93,94]. Interestingly, beside the protein composition it was previously shown that the lipid composition of TGN subdomains differ. The SYP61/VHA-a1 subdomain is enriched in VLCFA-sphingolipid as compared to either the RAB-A2a subdomain of TGN or the Golgi apparatus [8]. Consistently, the IPCS2 enzyme that grafts an inositolphosphate group on ceramide to produce IPCs (Figure 1) is specifically localized to the SYP61 subdomain of TGN [3]. IPCs are further glycosylated to produce GIPCs, the final form of the most abundant sphingolipids in plants (Figure 1). This localization of IPCS enzymes at the SYP61 subdomain correlates well with the sphingolipid enrichment observed at this subdomain [3,8]. Interestingly, the morphology of the Golgi apparatus and the SYP61/VHA-a1 subdomain of TGN were not altered upon FB1, instead some endosomal RAB-A2a-positive aggregates were detected [5]. FB1 decreases the amount of C24-ceramides to the profit of C16-ceramides without altering the GIPC composition [5]. Thus, we foresee that RAB-A2a/clathrin subdomain would be enriched C24-ceramides while the SYP61/VHA-a1 subdomain would be enriched in h24-GIPC (due to IPCS localization) [4]. In the future it will be essential to precisely characterize all the sphingolipid species present at the different subdomains of TGN using immuno-purification coupled to lipidomic analyses, as done previously [3,8]. At the functional level, the endocytic sorting of the auxin carriers PIN1 (polar localization at the basal membrane of root stele cells) and AUX1 (polar localization at the basal membrane of root epidermal cells), but not PIN2 (polar localization at apical membrane of root epidermal cells) depends on the acyl-chain length of ceramides at RAB-A2a/TGN (Figure 4A) [5]. Conversely, the secretory sorting of PIN2, but neither of PIN1 nor

AUX1, depends on the acyl-chain length of GIPC and/or GlcCer at SYP61/TGN (Figure 4B) [3,8]. Thus, TGN subdomains are functionally diversified to fulfil different trafficking routes to distinct polar domains of the cell. A substantial amount of work remains to be done to fully address the sphingolipid segregation in different subdomains of TGN, identify the underlying mechanisms and how those impact the selectivity of protein sorting and selectivity. However, it is now clear that the TGN, which is a tubulo-vesicular network and a membrane continuum, is defined by distinct subdomains where different sphingolipid species segregate polar-targeted cargos into different trafficking pathways.

Sphingolipid-mediated protein sorting through PI4P consumption by PLC at TGN in plant cells

In plants, sphingolipids have been shown to act through the consumption of PI4P to correctly sorts the auxin efflux carrier PIN2 at TGN [3]. Sphingolipid-mediated PI4P consumption at TGN is achieved by the PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASES C (PI-PLC or PLC) that hydrolyses PI4P to produce PI and a DAG molecule [3,95]. In Arabidopsis, 9 PLCs are present in the genome, all related to the PI-PLC- ζ subfamily of mammals [96,97]. The functional redundancy between PLCs complicates the identification of the PLCs targeted by sphingolipids, moreover certain PLCs mutants have gametophytic defects which complicate event further the genetic approach [3,98]. Nonetheless, PLC2 was genetically identified as an actor of sphingolipid-mediated consumption of PI4P at TGN, but probably PLC2 is not the only PLC targeted by sphingolipids (Figure 6) [3]. Similarly, in animal cells, the TGN-localized PLC γ 1 is activated upon cargo arrival and is involved in TGN-trafficking [99,100]. In the future, it would be highly valuable to explore whether PLCs act at ER/TGN membrane contact sites both in plants and animal cells. Interestingly, the hydrolysis of PI4P by PLCs produces a DAG molecule as well as does the grafting of the phosphocholine head on ceramide by the sphingomyelin synthase at TGN in animal cells or the grafting of the inositolphosphate on ceramide by the IPCS enzymes at TGN in plant cells (Figure 6). Given the role of DAG in the activation of several downstream proteins that act in lipid exchange at membrane contact sites, and the role of DAG in Golgi vesicles and tubule formation, it will be very interesting to address the function of DAG at TGN in plants in the future [4,101].

The coupling mechanism described above between sphingolipids and PI4P is raising an important issue. Actually, sphingolipids are expected to localize at the luminal leaflet of TGN (where the glycosylation occurs), while PI4P and PLC locate in the cytosolic leaflet (Figure 6A). Hence, how sphingolipids could possibly mediate PI4P homeostasis across leaflets? The answer might reside in the acyl-chain of sphingolipids. In mammalian cells, it is known that the PM lipid composition is highly asymmetric between the inner and outer leaflet with sphingolipids being localized at the outer leaflet and PS and PE being localized at the inner leaflet [102]. Interestingly, molecular dynamics allowed to identify that

VLCFAs of the animal sphingomyelin (d18:1/24:0) can extend particularly deep in the opposite leaflet and interact preferentially with PS in the opposite leaflet in a cholesterol-dependent manner [103]. This process involves the potency of the VLCFA of sphingolipids to cross-digitate the opposite leaflet, a property known as “lipid interdigitation” [104]. In plants, there is a variety of GIPCs molecular species but the most abundant GIPCs contain VLCFA of 24 atoms of carbons that is alpha-hydroxylated (h24:0 and h24:1), h26:0 are also found in GIPCs although less abundant than h24 [8,43,105]. These VLCFA-GIPCs constitute up to 85% of the pool of GIPC, the remaining pool is mostly composed from h16:0-GIPCs [8,106]. Moreover, in contrast to animals or yeasts, plants contain up to 25% of VLCFAs in the global PS pool [107–109]. Thus, it is possible that outer-leaflet GIPCs interact with PS at the opposite leaflet. However, the GIPC/PI4P coupling mechanism that is acting in PIN2 sorting at TGN occurs independently from PS [3]. Indeed, sphingolipids could interact with another lipid than PS at the cytosolic leaflet of TGN or interact with yet to identified proteins that could mediate the effect of sphingolipids across the two membrane leaflets (Figure 6A). Alternatively, the acyl-chain composition of PS might not be the same between the TGN and the PM, therefore a GIPC/PS coupling could occur at the PM but not the TGN. This will remain to be explore in the future to further decipher the mechanisms through which sphingolipids are acting during protein sorting and trafficking.

Towards the identification of the mechanisms involved in sphingolipid-mediated protein sorting and selectivity in plant cells

A proteome-wide identification of sphingolipid-binding domains in animal cells revealed that GPCRs frequently contain potential sphingolipid-binding domains [110,111]. In animal cells, GPCR activate some PLC enzymes, mostly from the PLC- β subfamily [112–114]. GPCR could be the missing link between sphingolipids and PLC in plants. However, Arabidopsis’ PLCs are all from the PLC- ζ subfamily that rely on other mechanisms than GPCR for their activation in mammals [114]. Moreover, in plants, the potential coupling between GPCRs and PLC is not clear, experimental evidence for that coupling is scarce and might be dependent on the type of organ or developmental stage analyzed [115,116]. Indeed, both in mammals and plants, the mechanism of regulation of the PI-PLC- ζ subfamily is not well understood. While an alternative mechanism of activation could be through the binding of PLC to their substrate lipids (PI4P and PI4,5P₂), the PI-PLC- ζ subfamily lacks the Pleckstrin Homology (PH) domain which allows the binding of PLC to PIPs [117–119]. PI-PLC- ζ proteins do not have transmembrane domains, but they are known to associate with membrane through the Pleckstrin Homology (PH) domain or the C-terminal C2 domains which are sensitive to Ca²⁺ [117,120]. Ca²⁺ is actually required for the catalytic activity of Arabidopsis’ PLCs and their membrane targeting activity through the C2 domain

[121–123]. Interestingly, an important discovery was the identification of a new sphingolipid mutant allele that displays an increase in cytosolic free Ca^{2+} concentration in response to a NaCl salt stress [11]. This mutant was called *mocal* for *monocation-induced [Ca^{2+}]_i increases 1 (mocal1)* and is defective in the gene coding the IPC glucuronosyltransferase 1 (IPUT1) [11,45]. IPUT1 is the enzyme acting downstream of IPCS to add a glucuronic acid on IPC to produce GlucA-IPC (Figure 5) [42,45]. Strikingly, Na^+ ions are able to directly bind the polar head of GIPC, probably through electrostatic interaction with the negative charge of the phosphate and glucuronic acid [11]. At the PM, the binding of Na^+ to GIPC is gating the Ca^{2+} influx channels and thus regulates the level of cytosolic Ca^{2+} concentration which consequently activates Ca^{2+} -binding proteins [11]. GIPC-mediated calcium signaling at the outer surface of the PM might follow the same rule at TGN (Figure 6B). Indeed, Na^+ , K^+/H^+ exchangers of the NHX family have been identified at TGN [124,125]. Hypothetically, it is possible that the Na^+ ions bind the phosphate group of the polar head of GIPC inside the lumen of the TGN and positively induce the gating of Ca^{2+} ions which will further recruit and activate PLC at TGN (Figure 6B). This mechanism would ultimately control the level of PI4P at TGN. The NHX5 and NHX6 proteins have been shown to be involved in endosomal recycling of the brassinoid receptor BRI1 at TGN, but not in polar trafficking of PIN2 or PIN1 proteins, although the abundance of PIN2 and PIN1 at the PM was reduced in the *nhx5;nhx6* double mutant suggesting a potential involvement in secretion [124,125]. These results suggest that either some other Na^+ exchangers than NHX5 and NHX6 are involved in polar trafficking at TGN, actually a TGN-localized Ca^{2+} channel would also need to be identified in that case, or that this mechanism is selective of certain specific cargos.

Sphingolipid function in ER/PM membrane contact sites of plant cells

The field of membrane contact sites is rapidly expanding in plants, mostly for the ER/PM interface. More particularly, the role of sphingolipids in plasmodesmata, a specialized ER/PM membrane contact site, has recently been evidenced by several studies [10,126–128]. Plasmodesmata are intercellular PM-lined nanopores that connect neighboring plant cells to each other and allow the exchange of molecules, such as RNAs, hormones, metabolites or small proteins, and additionally support local signaling events [129]. Embedded into the pore, a central constricted ER is encircled by a fine cytoplasmic space delimited by a cell-to-cell continuous PM (Figure 7A). Please note that another review on sphingolipid function at plasmodesmata nicely summarized the recent findings in this field, thus we will only elaborate relevant points related to trafficking and signaling in our review [130]. Sphingolipid-involvement in plasmodesmata-mediated cell-to-cell connectivity has been evidenced in a mutant allele of the *PHLOEM UNLOADING MODULATOR (PLM)* gene that encodes an enzyme involved in sphingolipid biosynthesis,

resulting in altered levels of VLCFA-ceramides [10]. In the *plm* mutant, the symplastic transport at the phloem pole pericycle-endodermis interface is enhanced and the narrow cytoplasmic space between the PM and the constricted ER was absent at plasmodesmata [10]. Plasmodesmata purification revealed that they are enriched in sphingolipids, especially FA h24-containing sphingolipids, and sterols [126]. Interestingly, both sterols and sphingolipids have been shown to play a role in cell-to-cell connectivity and the targeting at plasmodesmata of GPI-AP such as PLASMODESMATA-LOCATED- β -1,3-GLUCANASE 2 (PdBG2) (Figure 7A) [10,126,128]. Given that PdBG2 is involved in the degradation of callose, a major regulator of plasmodesmata aperture, the function of sphingolipids in GPI-AP sorting at plasmodesmata is an important matter for cell-to-cell connectivity (Figure 7A) [128]. In yeast, as described in the first parts of this review, VLCFA-ceramides are crucial in GPI-AP sorting in ceramide-enriched ERES which further traffic independently from transmembrane cargos [68]. In plants, it has been suggested that the GPI-AP PdBG2 and the non-GPI-AP PLASMODESMATA LOCALIZED PROTEIN 1 (PDLP) traffic through different secretory pathway (Figure 7A) [128]. However, this assumption needs to be further tested. Indeed, the role of sphingolipids, and more particularly (h)VLCFA-ceramides, in plasmodesmata-localized GPI-AP and non-GPI-AP sorting and targeting requires further investigation to be fully demonstrated.

Plasmodesmata are not only gating-structures but are also a preferential place for signaling. The best example is the two receptor kinases CLAVATA1 (CLV1) and Arabidopsis CRINKLY4 (ACR4) that can perceive signals from mobile peptide to control stem cell number in plant's meristems. ACR4 and CLV1 are both located at the PM but ACR4 accumulates at plasmodesmata and induces the oligomerization of ACR4 and CLV1 (Figure 7A) [131]. The function of this signaling at plasmodesmata is not clear yet but the maintenance of stem cells is regulated by mobile signals that travel through plasmodesmata. Thus, the targeting of cell signaling at the precise place where it operates might be a cost-efficient and focus way of transmitting a signal without interfering with the whole cell status. However, the role of sphingolipids in this process is still to be addressed. As a note, the relocation of the receptor like kinase QIĀN SHŌU KINASE 1 (QSK1) from the PM to plasmodesmata upon osmotic stress is independent from either sterols or sphingolipids [132].

Sphingolipids involvement in plasma membrane-mediated signaling in plant cells

In animal cells, it has been suggested that the interaction between gangliosides and the extracellular juxtamembrane domain of synaptotagmins 1 and 2 constitutes a pre-assembled receptor platform for high-affinity binding of the Botulinum neurotoxin type B [133]. In plants, some toxin secreted by Fungi or bacteria during pathogen attack are also recognized by complex glycosylated sphingolipids and constitute

a pore-forming platform that could kill the cell [12,134,135]. For example, the NECROSIS AND ETHYLENE-INDUCING PEPTIDE 1-LIKE (NLP), produced by pathogenic bacteria, oomycetes or fungi, actually directly bind GIPC polar head at the PM [12,134,135]. Nevertheless, NLPs cause the necrosis only in the case of 2 sugars-GIPC, but not with 3 sugars-GIPC likely because the distance of NLP to the PM is too high in the latter case to induce deadly forming pores into the PM [12,134]. This explains why NLP kill dicots plant cells, but not monocots, unless a given monocots possess a high proportion of 2 sugars GIPC [135]. Sphingolipids are also involved in the hypersensitive-like programmed cell death (HR-like) immune-response activated by the deposition of insect eggs on the leaves surface [136]. Interestingly, the catalog of cell-surface pattern recognition receptors is so wide that plants can specifically recognize sphingolipids coming from fungi and trigger defense responses [137]. Indeed, the degradation in the plant apoplast of the ceramide D from *Phytophthora infestans* by the plant NEUTRAL CERAMIDASE 2 (NCER2) produces a sphingoid base that is recognized by the receptor-like kinase RESISTANT TO DFPM-INHIBITION OF ABSCISIC ACID SIGNALING 2 (RDA2) [137]. Many publications report a role of sphingolipid in plant defense responses; thus we apologize to our colleagues for which work we did not cite. At the molecular level, virtually all forms of sphingolipids are involved in immune-response. For example, the role of the GIPC in plant immunity is supported by the constitutive immune-response observed in several mutants defective in either GMT1, that grafts a mannose on GlucA-IPC, or GONST1, that transports mannose from the cytoplasm to the lumen of the Golgi apparatus to produce GIPC (Figure 5) [14,48,138]. Moreover, ceramide, GlcCer and the sphingoid bases have all been shown to be involved in pathogen response as well [139–143]. At the mechanistic level, sphingolipid-mediated signaling is most probably transmitted through several pathways but we do not know yet if these pathways eventually lead to some specificity in the response. Some sphingolipid-mediated response pathways trigger reactive oxygen species (ROS) by directly binding to or indirectly inducing the NADPH oxidase RbohD (Figure 7B) [144–147]. For the latter, hydroxylation of fatty acids on the second carbon (2-hydroxy fatty acids, specific signature of plant' sphingolipids [148]) by the ER-localized 2-hydroxylases FAH1 and FAH2 has been shown to be essential for the production of ROS through RbohD upon elicitation (Figure 7B) [146,149]. Interestingly, the production of ROS upon elicitation requires the PLC2 [150]. Given that the catalytic activity of PLCs requires Ca^{2+} and that GIPC gate the influx of Ca^{2+} inside the cell, it is very likely that sphingolipids activate PLCs and the production of ROS upon elicitation (Figure 7B) [11,122].

Another sphingolipid-mediated immune-response pathway, not necessarily completely uncoupled from the ROS, is the production of hormones and mainly salicylic acid that is involved in programmed cell death (PCD) [46,48,141,151,152]. Indeed, abscisic acid, jasmonic acid and salicylic acid trigger ROS production and ROS themselves activate Ca^{2+} channels [153–157]. Probably, hormones, sphingolipids,

ROS and calcium waves work in a self-reinforcement mechanism or amplification loop to induce a cell-to-cell systemic response (Figure 7B). Recently, a leucine-rich-repeat receptor-like kinase H₂O₂-INDUCED CA²⁺ INCREASES 1 (HPCA1) was shown to act as a central ROS receptor that is required for the propagation of cell-to-cell ROS signals and systemic signaling in response to different biotic and abiotic stresses, therefore making a link between calcium and ROS [158]. The formation of specific liquid-ordered nano-domains within the membrane could be an additional key to support this amplification loop. Indeed, we know that the sphingolipid FAH hydrolases are essential for both ROS production and the formation of liquid-ordered domains in rice [146]. Moreover, salicylic acid promotes high liquid order phases in a REMORIN (REM)-dependent manner, a protein embedded in the inner leaflet of the PM by mechanisms involving PI4P and sterols [159,160].

Sphingolipid-mediated targeted secretion during plant immunity signaling

In mammalian cells, the transmembrane proteins of the TETRASPANIN (TET) family are other proteins that localized in nano-domains and have been shown to interact with lipids such as gangliosides and sterols, induce membrane curvature and form TET-enriched domains [161–164]. Interestingly, TET-enriched domains are preferential sites for the release of Extracellular Vesicles (EVs), the latter being membrane-enclosed nanoparticles entirely secreted outside the cell and containing proteins, RNAs and other metabolites that play diverse roles in development and defense response against pathogens [165–167]. Importantly, plant EVs were shown to be strongly enriched in GIPCs and a knockout mutant of the *Arabidopsis* TETRASPANIN 8 (TET8) has a lower amount of cellular GIPCs and produces less EVs [168]. Accordingly, the TET8 protein contains a putative sphingolipid-binding motif in the fourth transmembrane domain that was identified based on sequence homology with the sphingolipid-binding domain of the p24 protein of animal cells [54,56,168]. However, the binding of TET8 to GIPC remains to be confirmed and characterized. Nonetheless, exogenous supplementation of GIPC could partially rescue the formation of EVs in the *tet8* mutant and this mutant is impaired in the ROS burst after flagellin treatment [168]. Together, these results suggest that GIPCs play an important role in EV membrane trafficking to the apoplastic region during plant immunity signaling [168].

Conclusion

The plant' lipid field is facing a number of important conceptual challenges that could only be resolved with the development of new technical tools and methodologies. One conceptual advance we should further consider in the plant field is the molecular interaction network that sphingolipids entertain with

targeted proteins and other lipids, either directly or indirectly through homeostatic or signaling circuits. For example, sphingolipids impact the homeostasis of another lipid, PI4P, during protein sorting of PIN2 at TGN [3]. As for the sphingolipid-protein interaction, in contrast to the animal model where a direct interaction has been demonstrated between sphingolipids and a domain present in transmembrane regions of some integral membrane proteins, this needs to be fully demonstrated in plants [54,56]. The potential binding of the TET8 protein to GIPC is however a good candidate to be confirmed in the future [168]. Liposomal Förster Resonance Energy Transfer (FRET) analysis between fluorescent sphingolipid analogs and tagged proteins, Surface Plasmon Resonance (SPR) or Langmuir monolayer are some of the methods that helped in characterizing the sphingomyelin/p24 or gangliosides/synaptotagmin interactions in animal cells and that could be adapted to plant tissues [54,133]. It is worth to note that sphingolipids can not only target proteins and lipids but also fluxes of ions such as in the case of the calcium channels upon salt stress in plant cells [11]. Calcium is regulating the activity of many proteins such as proteins containing C2-domains, thus it could be a real challenge to decipher the primary to secondary effect of sphingolipids on a specific cellular process.

An important issue to consider to dissect sphingolipid function in cellular processes is their actual subcellular localization. Direct biochemical measurement on purified plant PM and Golgi/TGN revealed their presence in these compartments but the development of new tools that would allow to visualize them or, even better, track them in living cells is missing in plants. Sphingolipid biosensors developed in animal cells can unfortunately not be directly transferred to plants as the polar head of sphingolipids greatly differs from animal to plants. To develop a collection of sphingolipid biosensors as it was done recently for plant' anionic lipids, we would first need to characterize the interactome of plant' sphingolipids. An alternative is the production of fluorescent-tagged sphingolipids, such as bodipy-sphingolipids but this remains a challenge as the production of purification of GIPC is not an easy task, although a protocol for GIPC extraction and purification using plant material has been recently published [169]. Moreover, the polar head of GIPC binds to components of the plant cell wall, such as the RhamnoGalacturonan-II (RG-II), thus labeled-GIPC could be stacked in the cell wall if externally applied [170]. The best hope remains the translation of click-chemistry tools to plant cells. Click-chemistry would in theory allowed the absorption of an LCB chain containing either an azide or alkyne group, by plant cells and its integration in endogenous sphingolipid pools. This methodology needs however to be fully developed in plants and used with click reagents compatible with the plant cell metabolism or auto-fluorescence.

Based on these technical developments we foresee several conceptual questionings that could be addressed in a near future: 1/ How do sphingolipids mediate the transport and sorting of cargos? In plants,

we already know that sphingolipids are enriched at specific subdomains of TGN and are involved in polar sorting of auxin carriers but we do not know if specific sphingolipid molecular species are differentially enriched at TGN, Golgi or pre-Golgi subdomains, or elsewhere in the endomembrane system, and if these molecular species are involved in the sorting of other targeted cargos. 2/ How do sphingolipids get and keep concentrated at specific areas of the endomembrane system? The identification of sphingolipid-binding proteins as well as the visualization of sphingolipids will be necessary to address this question, a big challenge for the future is to characterize sphingolipid transport at potential membrane contact sites, identify the lipid transfer proteins involved in this process and decipher the role of lipid-exchange mechanisms in protein sorting and targeting. 3/ What are the mechanisms through which sphingolipids transmit a signal from the outside to the inside of the cell? The role of sphingolipids in building membrane nano-domains should be further explore in the future, the interaction of sphingolipids with other lipids, and/or with proteins, could create pre-existing platforms in which intracellular signaling-proteins could be stabilized together with transmembrane receptors that perceive the signals outside the cell. In animal cells, sphingolipids are mainly distributed at the outer layer of the PM, although this remains to be shown in plants the role of the lipid asymmetry in PM-signaling and how sphingolipid-mediated signaling function across PM leaflets will be a main issue to solve in the future.

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Figure legends

Figure 1: Molecular structures and biosynthesis pathways of sphingolipids in animal, yeast and plant cells. In animal cells, sphingolipid biosynthesis starts by the condensation of the di-hydroxylated Long Chain Base (LCB) sphingosine with a non-hydroxylated fatty acid, up to 24 atoms of carbons (24:0), to produce a ceramide molecule. Ceramide is then modified in sphingomyelin (addition of a phosphocholine group by the sphingomyelin synthase SMS), or in glucosylceramide (GlcCer, addition of a glucose residue by the glucosylceramide synthase GCS). GlcCer is the precursor for both globosides (addition of two galactose residues for Gb3) or gangliosides (addition of a galactose, a N-acetyl galactosamine and a neuraminic acid for GM1). In yeasts and plants, sphingolipid biosynthesis starts by the condensation of the tri-hydroxylated LCB phytosphingosine with an alpha-hydroxylated Very-Long Chain Fatty Acid (hVLCFA), up to 24 atoms of carbons (h24:0), to produce a ceramide molecule. hVLCFA constitutes up to 85% of the pool of sphingolipids. In yeasts and plants, the inositolphosphorylceramide (IPC) synthase enzyme (IPCS) graft an inositolphosphate group to ceramide producing an IPC molecule. In yeast, the mannosyl phosphorylinositol ceramide (MIPC) synthase SUR1 adds a mannose residue on IPC to produce MIPC. A phosphorylinositol group is then added to MIPC by the inositolphosphotransferase1 (IPT1) to form mannosyl diphosphorylinositol ceramide (M(IP)₂C). In plants, IPC is modified by the addition of a glucuronic acid (GlucA), by the inositol phosphorylceramide glucuronosyltransferase1 (IPUT1) to produce GlucA-IPC. The Glycosylinositol phosphorylceramide Mannosyl Transferase1 (GMT1) adds a mannose residue on GlucA-IPC to produce mannose-GlucA-IPC, the final major form of glycosylinositol phosphorylceramide (GIPC) in *Arabidopsis thaliana*. Mannose-GlucA-IPC is part of the series A of GIPC but in other eudicots or in monocots the sugar structure can be more complex.

Figure 2: Schema of the ER/Golgi interface organisation in animal cells. In animal cells, the Golgi ribbon is an assembly of several Golgi units which remains in close association with the endoplasmic reticulum (ER) with the noticeable presence of the ER-Golgi intermediate compartment (ERGIC). The *trans*-Golgi Network (TGN) is differentiated at the trans-most cisternae of the Golgi and remains associated with the Golgi. (A) At ER/TGN membrane contact sites, ceramide is transferred from the ER to the TGN by the CERT protein, the synthesis of sphingomyelin (SM) at TGN from ceramide and PC produces a di-acylglycerol (DAG) molecule that acts both on CERT and OSBP. Given that OSBP exchanges PI4P from the TGN against sterols from the ER, SM biosynthesis impact PI4P homeostasis and PI4P-mediated sorting mechanisms at TGN. (B) At the *cis*-Golgi/TGN membrane contact sites, GlcCer is transferred via the intermediate protein (PLEKHA8)/FAPP2 that contains a PH domain interacting with TGN-enriched PI4P. GlcCer transferred through *cis*-Golgi/TGN membrane contact sites

fuels the synthesis of globosides (Gb) at TGN through galactosyltransferase (GalT-I) and N-acetylgalactosaminyltransferase (GalNAcT) enzymes. (C) ER-derived membrane pearled protusions from the ER-Exit Sites (ERES), COPII accumulates at the constricted neck region of the ERES where it acts in protein sorting. The protusions extend towards the Golgi where COPI is localized, more distally than COPII. In COPI regions, sphingomyelin with 18 atoms of carbon (SM18:0) induces the homodimerization of the p24 protein complex.

Figure 3: Schema of the ER/Golgi interface organisation in yeasts. In budding yeast, the Golgi apparatus cisternae are not bound into a stack but are dispersed in the cytosol. At the ER (magnification box), C26-ceramides (cer-26) co-cluster with and are further integrated into GPI-anchored proteins (GPI-APs) at ceramide-enriched ERES while transmembrane proteins (TM-proteins) are sorted into distinct ERES-derived COPII-vesicles.

Figure 4: Schema of the ER/Golgi interface organisation in plants. In plant cells, the Golgi stacks are numerous, mobile and dispersed in the cytoplasm. The Golgi stacks are often found close to the endoplasmic reticulum (ER) and ER-Exit Sites (ERES) where it might establish membrane contact sites, alternatively but not necessarily exclusively, the Golgi Entry Core Compartment (GECCO) could play a role as a putative ER-Golgi Intermediate Compartment (ERGIC) in plant cells. However, the existence and nature of the putative ERGIC of plant cells remains to be fully addressed and characterized. Similarly, the existence and characteristics of a putative ER/*trans*-Golgi Network (TGN) membrane contact sites (MCS) in plants remains to be determined. In plants, the TGN can either be associated with or independent from the Golgi stack. Plant' TGN is both a secretory and endocytic compartment that merges a myriad of distinct subdomains. (A) Clathrin-Coated Vesicles (CCVs) are around 30 nm in diameter and are marked by the Rab-GTPase RAB-A2a and clathrin that co-localizes with the adaptor protein complex AP-1. At this site of TGN ceramides were shown to function in the endocytic recycling of the basal auxin carriers PIN1 and AUX1. (B) Uncoated Vesicles (UVs) are between 70-150 nm in diameter, sphingolipid-enriched and marked by the V-ATPase VHA-a1 and the syntaxin SYP61 that co-localizes with adaptor protein complex AP-4. At this site of TGN, GIPCs have been shown to be involved in the secretory sorting of the apical auxin carrier PIN2.

Figure 5: Subcellular compartmentalization of sphingolipid biosynthesis in plant cells. Ceramide (Cer) and Glucosylceramide (GlcCer) are synthesized in the endoplasmic reticulum (ER) by LOH and

GCS enzymes, respectively. Ceramide might either be transferred to *trans*-Golgi Network (TGN) directly through putative ER/TGN membrane contact sites (MCS) or transferred to TGN through the Golgi stack, these two hypotheses are not necessarily exclusive. At uncoated vesicles (UVs), where IPCS enzymes locate, an inositolphosphate (IP) group will be added to ceramide to produce IPC. IPC needs to be transferred back to the Golgi where IPUT1 will add a glucuronic acid (GlucA) residue on IPC and GMT1 will add a mannose residue on IPC-GlucA to produce a GIPC. GIPC might then cross the TGN on their way to the PM. These hypotheses need to be further tested in the future.

Figure 6: Hypothetical models of the mechanisms involved in the secretory sorting of PIN2 at TGN.

The secretory sorting of PIN2 is dependent on the acyl-chain length of GIPC (located in the outer leaflet of the plasma membrane (PM)) by impacting the consumption of PI4P (located in the inner leaflet of the PM) through phospholipase C (PLC). (A) First hypothesis: the hydroxylated Very Long Chain Fatty Acid (hVLCFA) of GIPC allows the recruitment of PLC by a yet to be identified molecular intermediate (X) that would interact with both the acyl-chain of GIPC and PLC. (B) Second hypothesis: the arrival of PIN2 cargo activates a sodium (Na^{2+}) transporter that would increase the binding of Na^{2+} to GIPC in the lumen of the TGN, this would in turn favour the gating of calcium (Ca^{2+}) from the TGN lumen to the cytoplasm and activate PLC. In both cases, the hydrolysis of PI4P by PLC release a DAG molecule that could be involved, through yet to be identified mechanisms, in PIN2 sorting or vesicle budding or fission.

Figure 7: Sphingolipid function at plasmodesmata ER/PM contact sites and in PM-mediated cell signaling.

(A) Targeting of the transmembrane (TM) plasmodesmata-localized protein PDLP and the GPI-anchored protein (GPI-AP) PDBG2 at plasmodesmata endoplasmic reticulum (ER)/plasma membrane (PM) membrane contact site depends on the sphingolipid membrane composition. As these proteins regulate the deposition of callose at the plasmodesmata neck region, sphingolipids play a central role in modulating plasmodesmata aperture and cell-to-cell signaling. Dimerization of the receptor kinases ACR4 and CLV1 reinforce cell signaling at plasmodesmata. However, sphingolipid function in this process remains to be addressed. (B) Upon pathogen elicitation, a burst of Reactive Oxygen Species (ROS) is produced, partly through the activity of the RbohD enzyme. Fatty acid alpha-hydroxylation by the Fatty Acid Hydroxylases FAHs is a specific signature of sphingolipids. Alpha-hydroxylated sphingolipids (SL-OH) are required for the ROS burst upon elicitation that subsequently triggers several hormone-mediated pathways, such as the salicylic acid responses, and activation of calcium (Ca^{2+}) channels. Phospholipase C (PLC) are probably induced by the influx of calcium, although this remains to be directly demonstrated upon elicitation. PLC hydrolyze equally well PI4P and PI4,5P2 to produce a di-

acylglycerol (DAG) molecule, that quickly converts into phosphatidic acid (PA), and polyphosphorylated forms of inositol (PPIs), all serving as secondary messengers.

Figure 1

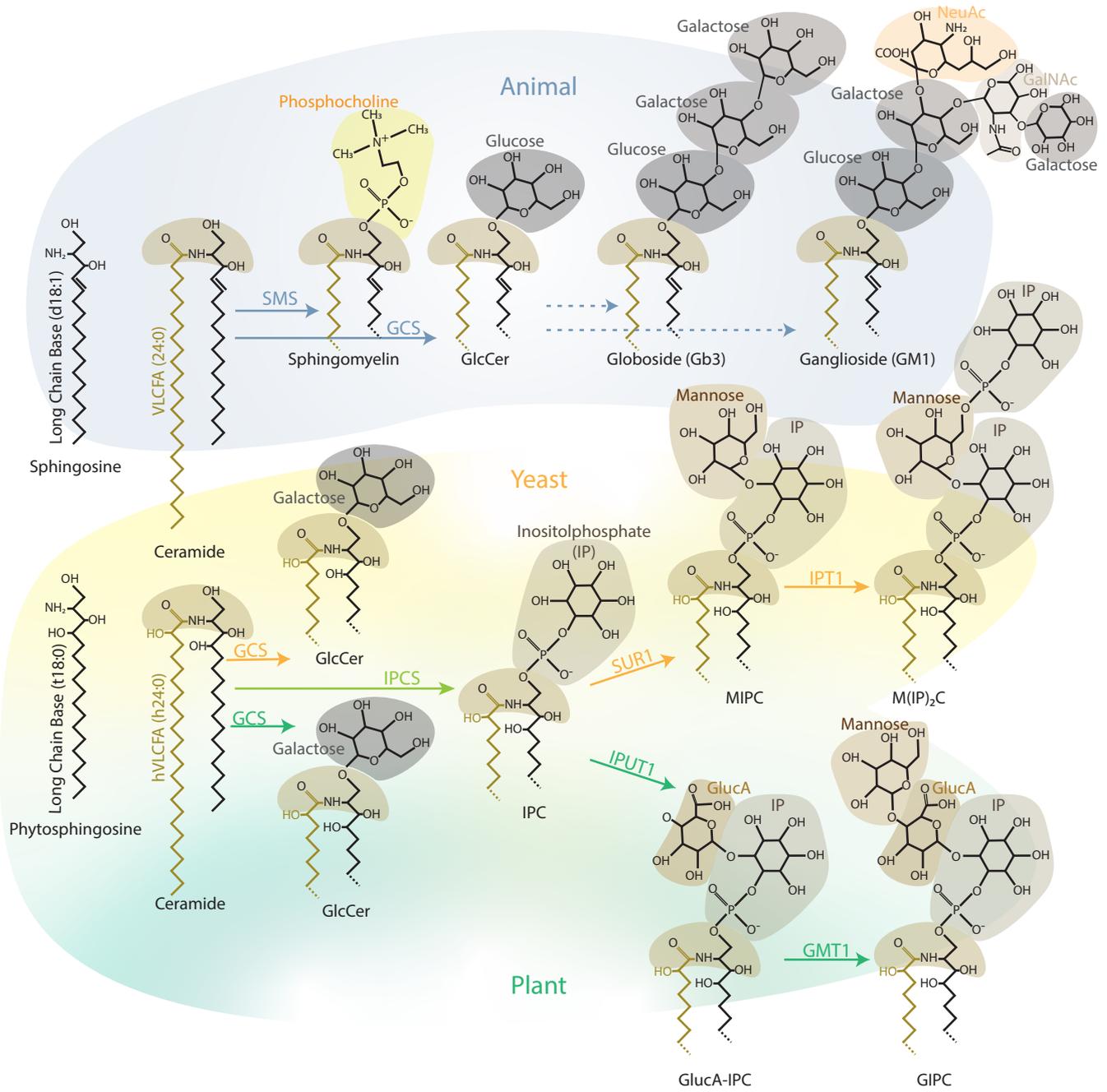


Figure 2

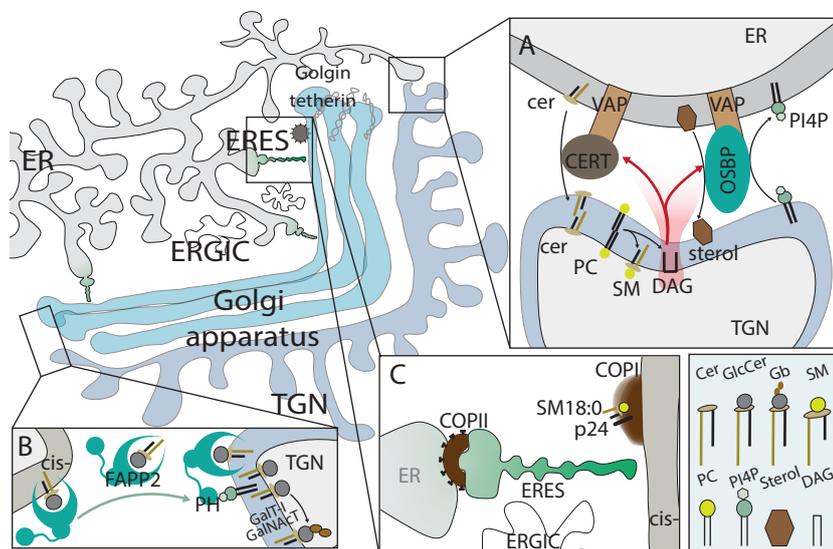


Figure 3

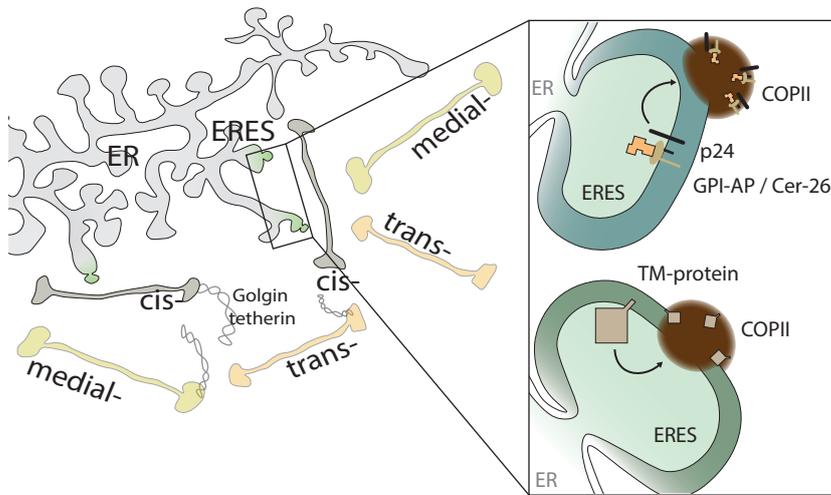


Figure 4

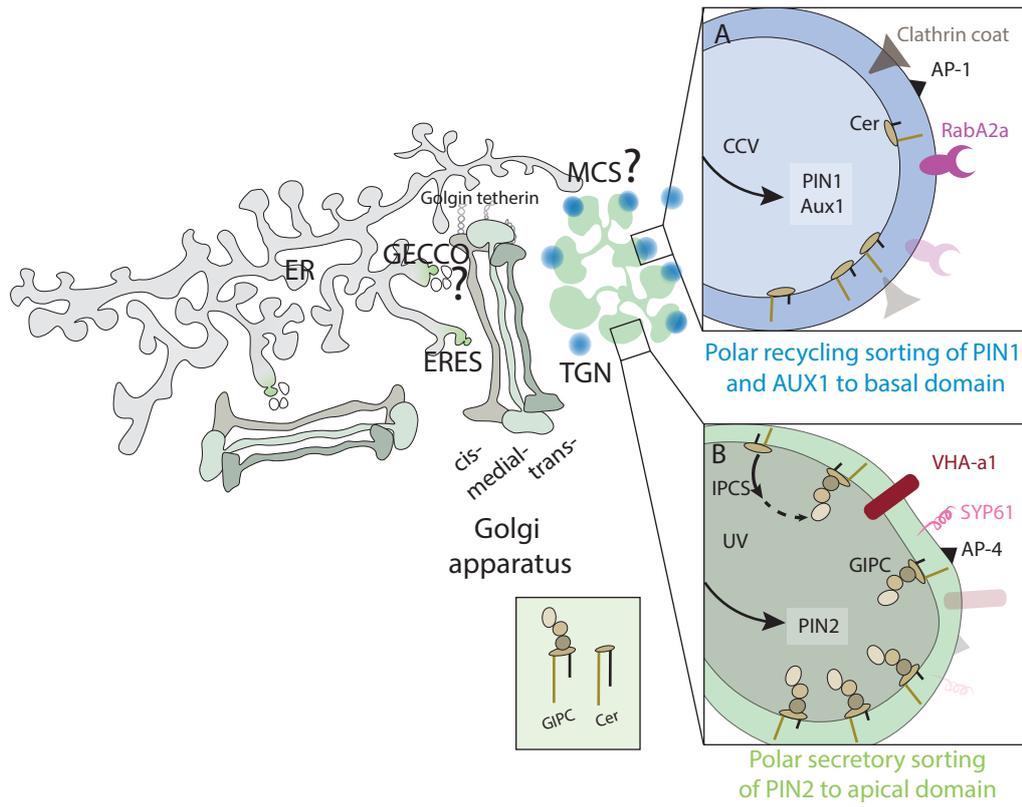


Figure 5

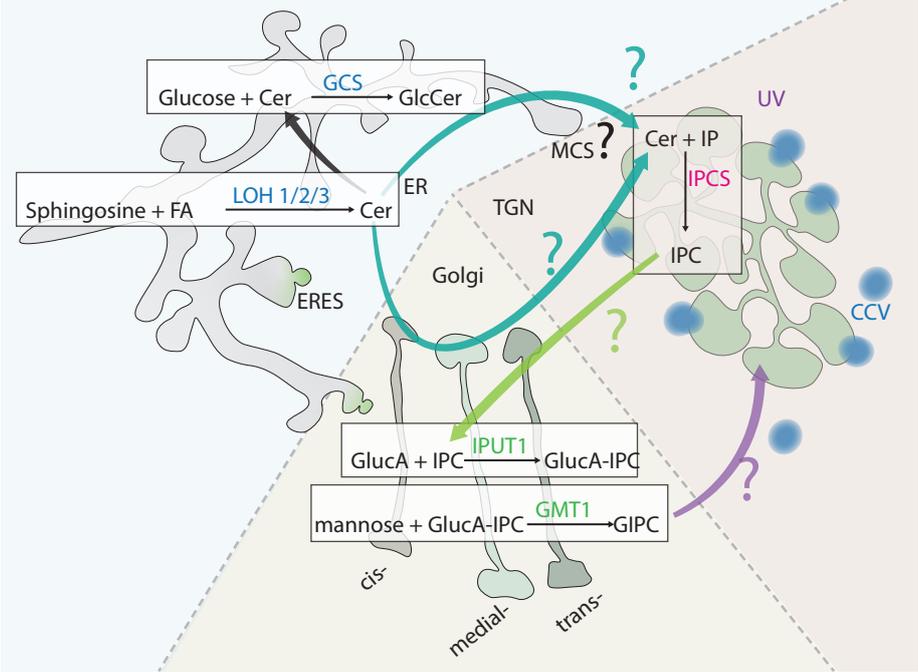


Figure 6

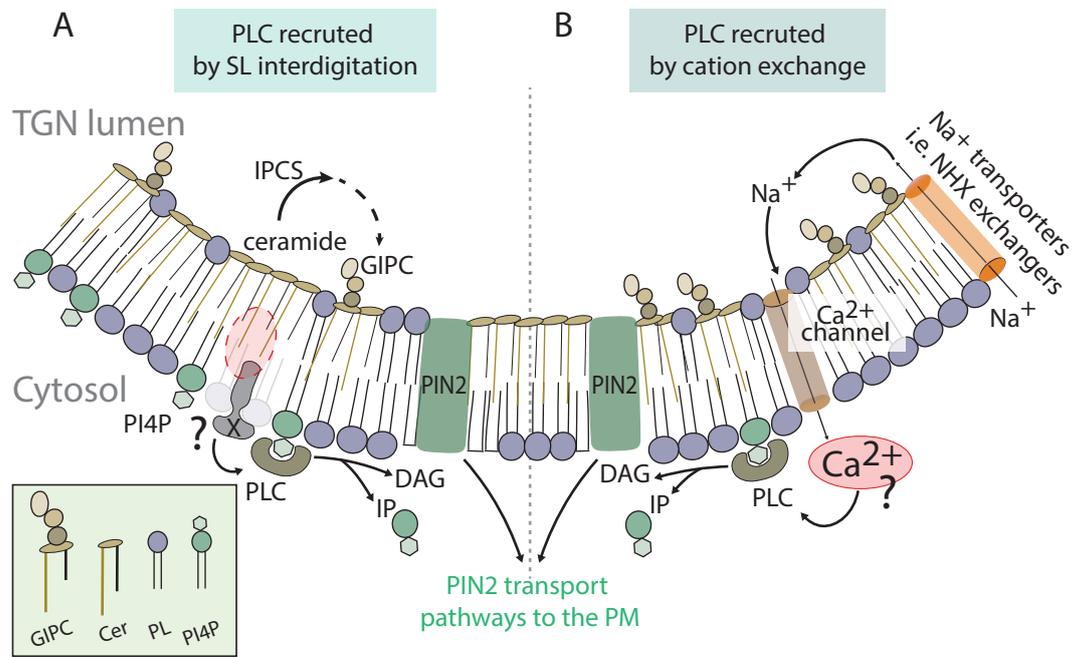


Figure 7

