Revisiting Plant Plasma Membrane Lipids in Tobacco: A Focus on Sphingolipids

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The lipid composition of plasma membrane (PM) and the corresponding detergent-insoluble membrane (DIM) fraction were analyzed with a specific focus on highly polar sphingolipids, so-called glycosyl inositol phosphorylceramides (GIPCs). Using tobacco (Nicotiana tabacum) Bright Yellow 2 cell suspension and leaves, evidence is provided that GIPCs represent up to 40 mol % of the PM lipids. Comparative analysis of DIMs with the PM showed an enrichment of 2-hydroxylated very-long-chain fatty acid-containing GIPCs and polyglycosylated GIPCs in the DIMs. Purified antibodies raised against these GIPCs were further used for immunogold-electron microscopy strategy, revealing the distribution of polyglycosylated GIPCs in domains of 35 ± 7 nm in the plane of the PM. Biophysical studies also showed strong interactions between GIPCs and sterols and suggested a role for very-long-chain fatty acids in the interdigitation between the two PM-composing monolayers. The ins and outs of lipid asymmetry, raft formation, and interdigitation in plant membrane biology are finally discussed.

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Eukaryotic plasma membranes (PMs) are composed of three main classes of lipids, glycerolipids, sphingolipids, and sterols, which may account for up to 100,000 different molecular species (Yetukuri et al., 2008; Shevchenko and Simons, 2010). Overall, all glycerolipids share the same molecular moieties in plants, animals, and fungi. By contrast, sterols and sphingolipids are different and specific to each kingdom. For instance, the plant PM contains an important number of sterols, among which β-sitosterol, stigmasterol, and campesterol predominate (Furt et al., 2011). In addition to free sterols, phytosterols can be conjugated to form steryl glycosides (SG) and acyl steryl glycosides (ASG) that represent up to approximately 15% of the tobacco (Nicotiana tabacum) PM (Furt et al., 2010). As for sphingolipids, sphyngomyelin, the major phosphosphingolipid in animals, which harbors a phosphocholine as a polar head, is not detected in plants. Glycosyl
inositol phosphorylceramides (GIPCs) are the major class of sphingolipids in plants, but they are absent in animals (Sperling and Heinz, 2003; Pata et al., 2010). Sphingolipidomic approaches identified up to 200 plant sphingolipids (for review, see Pata et al., 2010; Cacas et al., 2013).

Although GIPCs belong to one of the earliest classes of plant sphingolipids that were identified in the late 1950s (Carter et al., 1958), only a few GIPCs have been structurally characterized to date because of their high polarity and a limited solubility in typical lipid extraction solvents. For these reasons, they were systematically omitted from published plant PM lipid composition. GIPCs are formed by the addition of an inositol phosphate to the ceramide moiety, the inositol headgroup of which can then undergo several glycosylation steps. The dominant glycan structure, composed of a hexose-GlcA linked to the inositol, is called series A. Polar heads containing three to seven sugars, so-called series B to F, have been identified and appeared to be species specific (Buré et al., 2011; Cacas et al., 2013; Mortimer et al., 2013). The ceramide moiety of GIPCs consists of a long-chain base (LCB), mainly t18:0 (called phytosphingosine) or t18:1 compounds (for review, see Pata et al., 2010), to which is amidi-fied a very-long-chain fatty acid (VLCFA), the latter of which is mostly 2-hydroxylated (hVLCFA) with an odd or even number of carbon atoms. In plants, little is known about the subcellular localization of GIPCs. It is assumed, however, that they would be highly represented in the PM (Worrall et al., 2003; Sperling et al., 2005), even if this remains to be experimentally proven. The main argument supporting such an assumption is the strong enrichment of trihydroxylated LCB (t18:n) in detergent-insoluble membrane (DIM) fractions (Borner et al., 2005; Lefebvre et al., 2007), LCB being known to be predominant in GIPC’s core structure as aforementioned.

In addition to this chemical complexity, lipids are not evenly distributed within the PM. Sphingolipids and sterols can preferentially interact with each other and segregate to form microdomains dubbed the membrane raft (Simons and Toomre, 2000). The membrane raft hypothesis suggests that lipids play a regulatory role in mediating protein clustering within the bilayer under going phase separation into liquid-disordered and liquid-ordered phases. The liquid-ordered phase, termed the membrane raft, was described as enriched in sterol and saturated sphingolipids and is characterized by tight lipid packing. Proteins, which have differential affinities for each phase, may become enriched in, or excluded from, the liquid-ordered phase domains to optimize the rate of protein-protein interactions and maximize signaling processes. In animals, rafts have been implicated in a huge range of cellular processes, such as hormone signaling, membrane trafficking in polarized epithelial cells, T cell activation, cell migration, and the life cycle of influenza and human immunodeficiency viruses (Simons and Ikonen, 1997; Simons and Gert, 2010). In plants, evidence is increasing that rafts are also involved in signal transduction processes and membrane trafficking (for review, see Mongrand et al., 2010; Simon-Plas et al., 2011; Cacas et al., 2012a).

Moreover, lipids are not evenly distributed between the two leaflets of the PM. Within the PM of eukaryotic cells, sphingolipids are primarily located in the outer monolayer, whereas unsaturated phospholipids are predominantly exposed on the cytosolic leaflet. This asymmetrical distribution has been well established in human red blood cells, in which the outer leaflet contains sphingomyelin, phosphatidylycerine, and a variety of glycolipids like gangliosides. By contrast, the cytoplasmic leaflet is composed mostly of phosphati-dylethanolamine, phosphatidylserine, phosphatidylinositol, and their phosphorylated derivatives (Devaux and Morris, 2004). With regard to sphingolipids and glycerolipids, the asymmetry of the former is established during their biosynthesis and that of the latter requires ATPases such as the aminophospholipid translocase that transports lipids from the outer to the inner leaflet as well as multiple drug resistance proteins that transport phosphatidylycerine in the opposite direction (Devaux and Morris, 2004). This ubiquitous scheme encountered in animal cells could apply in plant cells as proposed (Tjellstrom et al., 2010). Indeed, the authors showed that there is a pronounced transverse lipid asymmetry in root at the PM. Phospholipids and galactolipids dominate the cytosolic leaflet, whereas the apoplastic leaflet is enriched in sphingolipids and sterols.

From such a high diversity of the plant PM thus arises the question of the respective contribution of lipids to membrane suborganization. Our group recently tackled this aspect by characterizing the order level of liposomes prepared from various plant lipids and labeled with the environment-sensitive probe di-4-ANEPPDHQ (Grosjean et al., 2015). Fluorescence spectroscopy experiments showed that, among phytosterols, campesterol exhibits the strongest ability to order model membranes. In agreement with these data, spatial analysis of the membrane organization through multispectral confocal microscopy pointed to the strong ability of campesterol to promote liquid-ordered domain formation and organize their spatial distribution at the membrane surface. Conjugated sterols also exhibit a striking ability to order membranes. In addition, GIPCs enhance the sterol-induced ordering effect by emphasizing the formation and increasing the size of sterol-dependent ordered domains.

The aim of this study was to reinvestigate the lipid composition and organization of the PM with a particular focus on GIPCs using tobacco leaves and cv Bright Yellow 2 (BY-2) cell cultures as models. Analyzing all membrane lipid classes at once, including sphingolipids, is challenging because they all display dramatically different chemical polarity, from very apolar (like free sterols) to highly polar (like polyglycosylated GIPCs) molecules. Most lipid extraction techniques published thus far use a chloroform/methanol mixture and phase partition to remove contaminants, resulting in the loss GIPCs, which remain in the aqueous...
RESULTS

GIPCs Are Enriched in PM Microdomains

Because fatty acid-containing lipids (i.e. glycerolipids, acylated steryl glucosides, and GIPCs) exhibit markedly different fatty acid compositions, the total fatty acid distribution of microsomal, PM, and DIM fractions isolated from BY-2 cells and tobacco leaves was determined to test for the assumption that GIPCs mainly reside in PM. Samples were transesterified in hot methanol/sulfuric acid solution to fully release both fatty acid-esterified glycerolipids and steryl glucoside and fatty acid-amidified sphingolipids. Total fatty acid content was then quantified by GC-MS (for a typical GC-MS spectrum, see Supplemental Fig. S1).

Figure 1A shows that, in both tobacco leaves and BY-2 cells, the combined percentage of VLCFA and hVLCFA increases from approximately 3% in total tissue to approximately 55% in DIM at the expense of long-chain fatty acids (i.e. 16- and 18-carbon atom-long fatty acids; Fig. 1A; for detailed fatty acid content, see Supplemental Fig. S2). Comparison of the fatty acid contents of DIM fractions floating in the Suc gradient and detergent-soluble membrane fractions in the bottom of the gradient revealed a higher level of long-chain fatty acids in detergent-soluble membrane, which correlates with a lower percentage of VLCA and hVLCA in this fraction (Supplemental Fig. S3).

In order to understand the origin of VLCA and hVLCA, we analyzed the structures of the different families of plant PM lipids. We previously showed that VLCA and hVLCA are absent from tobacco glycerolipids, except for a few percent of 20:0/22:0 in phosphatidylserine (Mongrand et al., 2004; Lefebvre et al., 2007). Further extraction and structural analyses revealed that tobacco ASG consist of saturated fatty acid with mainly 16 and 18 carbon atoms and common sterols of the PM (Supplemental Fig. S4). Glucosylceramide (gluCER), which accounts for only 5 to 10 mol % of PM (Furt et al., 2010), is acylated by h16:0 as a major hydroxylated fatty acid (Supplemental Fig. S5). Therefore, we hypothesized that VLCA and hVLCA, present in high amounts in PM and DIM, likely originate from GIPCs.

To test this hypothesis, we purified total GIPCs from tobacco leaf and BY-2 cells as described (Büré et al., 2011) and determined their fatty acid and LCB contents by GC-MS.

hVLCA and VLCFA contents are highly comparable in DIMs and pure GIPCs (Fig. 1B), with an even higher proportion of hVLCA in DIMs purified from BY-2 cells, suggesting that hVLCA-containing GIPCs are most likely present in membrane microdomains. Besides, levels of the two LCBs t18:0 and t18:1, which are mostly present in GIPCs (Büré et al., 2011), strongly increase in DIMs when compared with PM, reaching 80% of total LCBS in DIMs (Supplemental Fig. S6; Borner et al., 2005). This is also in good agreement with a strong enrichment of GIPCs in DIMs. Note that h16:0 is enriched in DIMs (Fig. 1B). Logically, this fatty acid is found in tobacco gluCER (Supplemental Fig. S5), a sphingolipid enriched in tobacco DIMs (Mongrand et al., 2004; Lefebvre et al., 2007).

To further characterize the enrichment of GIPCs in DIMs, we first compared the total GIPCs extracted from leaf and BY-2 cells by matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectrometry (Büré et al., 2011). Leaf GIPCs mostly contain GIPCs of series A (with two sugars), and BY-2 GIPCs contain, in addition to series A, a vast array of polyglycosylated GIPCs of the B to E series (Fig. 2), as described previously (Büré et al., 2011). The detailed molecular species of the polar head and LCB/fatty acid combination are provided in Supplemental Figure S7.
with the nomenclature described by Buré et al. (2011). We next used HP-TLC to separate the different series (Kaul and Lester, 1975; Fig. 2B), scratched the corresponding silica bands, and quantified the fatty acid methyl esters by GC-MS after transmethylation/silylation (Figs. 3 and 4). Note that the series A partitioned into two bands called phytosphingolipid: PSL1 (GlcNAc-GlcA-inositol phosphorylceramide) and PSL2 (GlcN-GlcA-inositol phosphorylceramide), as established previously (Kaul and Lester, 1975).

GIPCs of series A were found in both PM and DIM fractions of tobacco leaves (Supplemental Fig. S8), but the fact that BY-2 cells contain different series of GIPCs prompted us to determine whether polyglycosylated GIPCs were enriched in DIMs of BY-2. We performed HP-TLC coupled to GC-MS, as described above. GIPCs of series B were enriched three times in DIMs when compared with PM, reaching 17% of total GIPCs in BY-2 DIM (Fig. 3).

The lipid composition of tobacco plant PM based on the latter set of data was combined with previous findings (Furt et al., 2010), and the global lipid composition of PM and DIM fractions was recalculated taking into account GIPC concentrations (Fig. 3). As expected, glycerolipids were depleted in DIM fractions when compared with PM, whereas the exact opposite trend was observed for sphingolipids, whether these fractions were prepared from tobacco leaves (Fig. 4A) or BY-2 cells (Fig. 4B). Remarkably, GIPCs that have long been omitted for technical reasons in PM composition represent up to 45 and 30 mol % of total PM lipids isolated from leaves and BY-2 cell suspensions, respectively. Furthermore, DIM fractions purified from both BY-2 cells and photosynthetic tissues display a huge proportion of GIPCs that reaches 60 mol %, suggesting that the contribution to sphingolipid enrichment in PM microdomains is mainly due to GIPCs. It is also worth noting that the sum of sterols and sphingolipids averages 90 and 88 mol % in DIM of tobacco leaves (Fig. 3A, right) and BY-2 cells (Fig. 3B, right), respectively. We reasoned that if GIPCs are located exclusively in the outer leaflet of PM (see “Discussion”), the presence of more than 50% of GIPCs in DIMs suggests a higher solubilization of the inner leaflet by Triton X-100.
Figure 2. Analysis of GIPCs extracted from tobacco leaf and BY-2 cells by MALDI-TOF mass spectrometry and high-performance thin-layer chromatography (HP-TLC). Polyglycosylated GIPCs are enriched in DIMs. A, MALDI-TOF mass spectrometry analysis of GIPC extracts from BY-2 cells and tobacco leaf. Spectra were acquired in the negative ion mode using 2,6-dihydroxyacetophenone as a matrix. GIPCs are grouped in series according to their number of saccharide units, from two (series A) to six (series E);
Hence, these data arise the question of the lipid-to-protein ratio, commonly thought to be close to 1 for plant PM. This ratio was experimentally reinvestigated using BY-2 PM samples. One hundred micrograms of PM vesicles was extracted by the gold-standard Folch protocol (i.e. chloroform:methanol, 2:1 [v/v] extraction). As described previously (Markham et al., 2006), almost half of the VLCFA- and hVLCFA-containing GIPCs were lost in the lower aqueous phase (Supplemental Fig. S9). Therefore, this phase was evaporated to remove solvents and resuspended in pure water, and GIPCs were reextracted with butanol-1 (which extracts 98% of plant GIPC from water, as shown previously [Buré et al., 2011]). Importantly, no fatty acid was recovered in water after this double extraction (Supplemental Fig. S9). In addition, when comparing direct transesterification with the Folch protocol followed by butanol-1 extraction, the estimated lipid recovery yield using fatty acid levels as a proxy was close to 100%, indicating full extraction of lipids irrespective of their polarity. Based on these results, the lipid-to-protein ratio was calculated to be 1.3 ± 0.07 for BY-2 PM.

We further decided to test other solvents for lipid extraction to get a simple, quantitative, and unbiased recovery of lipid species from plants. MTBE extraction was tested because it has been shown to allow faster and cleaner lipid recovery (Matyash et al., 2008). Its low density forms the upper organic phase during phase separation, which simplifies its recovery (Supplemental Fig. S10A). We thus compared the Folch protocol (extraction 1 in “Materials and Methods”), MTBE extraction (extraction 2), and the Markham protocol (extraction 3) developed to fully extract plant sphingolipids (Markham et al., 2006). Rigorous testing demonstrated that the extraction in hot isopropanol followed by one of the three extractions was suitable to extract total polar lipids of plant samples (see “Materials and Methods”). Nevertheless, Markham’s extraction (extraction 3) displays the disadvantage of containing a large amount of water, hardly evaporated, and protein contamination in the organic phase because of the absence of liquid-liquid phase separation. In the Folch extraction (extraction 1), inconvenience resides in the fact that the higher density of chloroform forms the lower phase in the two-phase partitioning system, and a glass pipette or a needle must cross the aqueous phase to collect the lipid-containing one. By contrast, lipid extraction by upper phase MTBE/methanol/water (extraction 2) greatly simplifies sample handling. Therefore, we propose the MTBE method as the method of choice to extract total plant polar lipids.

**Figure 3.** Quantification of polyglycosylated GIPCs found in PM and DIMs of BY-2 cells. Quantification was by HP-TLC coupled to GC-MS of polyglycosylated GIPCs found in PM and DIMs of BY-2 cells. The data are expressed as means of three independent experiments (percentage of total GIPCs found in PM and DIMs, respectively) ± sd.

**Purification of GIPC Series from BY-2 Cells and Production of Antibodies against Polyglycosylated GIPCs**

Pure GIPCs are not commercially available, nor are the corresponding molecular tools dedicated to their study, like fluorescently labeled lipids or specific antibodies. We thus purified several milligrams of GIPCs in order to immunize rabbits and raise antibodies to be used in immunolabeling experiments. Because of their anionic phosphate groups, GIPCs can be purified by anion-exchange chromatography on DEAE cellulose. This approach has the double advantage of allowing sample cleanup and concentration.

The preparative purification procedure was carried out according to Kaul and Lester (1975) with slight modifications described in “Materials and Methods.” A home-packed DEAE cellulose chromatographic column was used for that purpose, and GIPCs were eluted with increasing concentrations of ammonium acetate dissolved in chloroform:methanol:water (30:60:8, v/v/v). Under our experimental conditions, GIPCs of series A (namely PSL1 and PSL2) were successfully separated from polyglycosylated GIPCs of series B to F; the former eluted in fractions 41 to 45, whereas the latter eluted in fractions 46 to 49 (Supplemental Fig. S11A). Glycerolipid contaminations were discarded from these fractions by methylene treatment, which hydrolyzes ester bonds (Markham et al., 2006). Fractions were then dialyzed to remove ammonium acetate, and their concentration and purity were estimated by GC-MS and MALDI-TOF mass spectrometry (Supplemental Fig. S11B). They were subsequently used for preparing liposomes supplemented with bacterial lipid A known to boost rabbit immunity (Richards et al., 1998). The two immune sera obtained following rabbit injection with

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**Figure 2.** (Continued.) for detailed analysis of the peaks, see Supplemental Figure S7. B, HP-TLC was used to separate the different series of GIPCs. Note that series A shows two bands called by Kaul and Lester (1975) phytosphingolipid: PSL1 and PSL2, corresponding to GlcNAc-GlcA-inositol phosphorylceramide and GlcN-GlcA-inositol phosphorylceramide, respectively. GIPCs extracted from Arabidopsis (Arabidopsis thaliana; At) and leek (Allium porrum; Ap.) were used as HP-TLC standards for series A and B, respectively, according to Cacas et al. (2013).
GIPC of series A did not react with the DEAE-purified fractions used for the immunization protocol (data not shown). By contrast, one immune serum (rabbit 46) raised against polyglycosylated GIPCs of series B to F clearly reacted with the chromatographic fractions used for the immunization protocol, whereas no reaction was observed with the corresponding preimmune serum (Fig. 5A). ELISA performed on PM showed a strong signal increase between negative controls (preimmune serum) and final serum or purified IgG antibodies (Supplemental Fig. S12). No specific signal was detected with antibodies against GIPCs on hydrophobic membranes that had been spotted with all eight phosphoinositides and seven other biological important lipids (PIP strip; Supplemental Fig. S13). Finally, to further test the specificity of the antibodies on the different GIPC series purified from BY-2 cells, eastern blots were directly performed on HP-TLC plates containing PM lipids extracted with the MTBE protocol described above. Rabbit polyclonal antibodies were able to recognize polyglycosylated GIPCs of series B to F but not those of series A (Fig. 5B).

Polyglycosylated GIPCs Cluster within Nanodomains in Tobacco PM

Since polyglycosylated GIPCs are enriched in BY-2 DIM fractions (Fig. 4B), the possibility of visualizing GIPC-enriched clusters was challenged by transmission electron microscopy using anti-GIPC antibody-based immunogold labeling experiments. PM vesicles were purified from BY-2 cells and directly deposited onto microscope grids, allowing for the exposure of large membrane sheets. The grids were then pretreated to prevent nonspecific binding, incubated first with primary antibodies against polyglycosylated GIPCs and then with secondary IgG conjugated to colloidal gold particles. Preparations were negatively stained with ammonium molybdate to reveal the vesicle morphology and observed by transmission electron microscopy.

Statistical analysis was performed on 49 independent gold-labeled PM vesicles to analyze the putative clustering of gold particles. The mean and SD were calculated for different parameters: diameter of PM vesicles, area of PM vesicles, number of gold particles per PM vesicle, number of gold particles per cluster, size of gold particle clusters, and distance between two neighboring clusters. The mean labeling density was quantified to be seven gold particles per vesicle. Groups of particles were composed of an average of four gold particles (three to seven particles were clustered). We calculated that 88% ($n = 3$) of the gold particles showed a clustered distribution throughout the vesicle surface, with an average of four gold particles and with an average cluster diameter of 35 ± 7 nm (Fig. 6, A and B). Only 12% of the gold particles exhibited a random distribution on the PM surface. Ripley’s K-function analysis
indicated that the gold pattern was aggregated, since $K(r)$ values of experimental data were clearly above the Poisson simulation curve corresponding to a completely random pattern (Fig. 6C). Negative controls including omission of the primary antibody or use of preimmune serum exhibited very weak labeling. Positive controls carried out using antibodies raised against the proton pump ATPase showed heavy labeling of the PM (Raffaele et al., 2009; Supplemental Fig. S14). Therefore, GIPCs (series B–F) exhibit an aggregated pattern within the PM of BY-2 cells with a mean size of 35 nm.

Order Level of Model Membranes Prepared with GIPCs Isolated from Leaves or Cell Cultures

The ability of GIPCs to change membrane order and organize a liquid-ordered domain was investigated using the environment-sensitive probe di-4-ANEPPDHQ, as described (Grosjean et al., 2015). Briefly, membrane organization alters the fluorescence emission spectrum of the probe that emits in both red (635–655 nm) and green (545–565 nm) spectral regions. Increase in green fluorescence emission correlates with a higher average order level of the membrane. Conversely, an emission shift toward red wavelengths indicates a decrease in the relative amount of ordered domains in the lipid bilayer. The red-to-green fluorescence ratio of the membrane (RGM) thus reflects the relative proportions of liquid-disordered/liquid-ordered phases within membranes (Gerbeau-Pissot et al., 2013).

Previously, we showed that plant sphingolipids, especially GIPCs, enhanced the sterol-induced ordering effect by increasing the size of sterol-dependent ordered domains (Grosjean et al., 2015). In order to test for the impact of GIPC composition on global membrane organization, large unilamellar vesicles (LUVs) were prepared using a distinct combination of lipids and incubated in the presence of di-4-ANEPPDHQ, and the RGM was calculated upon spectral acquisition. The phospholipids dioleoylphosphatidylcholine and dipalmitoylphosphatidylcholine, phytosterol (campesterol or a sterol mix isolated from BY-2 cells [tobacco mix]), and GIPCs were used to produce LUVs. We compared GIPCs of series A isolated from tobacco leaves and GIPCs of series B to F, which include polyglycosylated molecules purified from BY-2 cell suspensions. Importantly, the ceramide moiety of tobacco leaves mostly contains hVLCFA, whereas that of the BY-2 suspension harbors an equimolar mix of VLCFA and hVLCFA.

The results in Figure 7 confirmed that GIPCs have no particular ability to modify the order level of model membranes containing only phospholipids and suggest that the size of their sugar head and the hydroxylation of VLCFA do not change this capacity. When 33% of campesterol or a sterol mixture mimicking the one found in tobacco BY-2 PM (tobacco mix) was added to phospholipids and GIPCs, a significant and similar decrease of the RGM was observed (Fig. 7). Such a decrease of RGM confirms the major involvement of sterol in increasing membrane order level and suggests that 17% polyglycosylated GIPCs (Fig. 3) or the presence of 50% VLCFA in GIPCs (Fig. 1B) does not drastically modify the ability of sphingolipids to order membrane with sterols.

Molecular Simulation Modeling and Biophysical Analysis Reveal GIPC-Sterol Interaction and the Interdigitation of GIPC’s VLCFA between the Two Leaflets of the PM

Based on the literature (see “Discussion”) and immunolabeling experiments (Fig. 6), we reasonably hypothesized that GIPCs may preferentially reside in the apoplastic leaflet. Therefore, we conducted biophysical experiments and energetic calculation to characterize the outer leaflet organization of the plant PM (i.e. structure, organization, and behavior).
The Langmuir trough technique applied on a monolayer model at the air-water interface has been used extensively to characterize the interfacial organization of lipids and lipid-lipid interactions at the micrometric level (Deleu et al., 2014). The GIPC compression isotherm (Fig. 8A) showed a low and relatively constant surface pressure in large molecular areas, corresponding to a gaseous state. Compression of a pure GIPC monolayer induced a progressive increase in surface pressure, indicating the appearance of a liquid-expanded state, which is characterized by a certain degree of cooperative interaction between the molecules at the interface (Fig. 8A). This was confirmed by the value of the two-dimensional compressibility modulus (Cs$^{-1}$ = 31.9 mN m$^{-1}$ in the 180- to 70-Å$^2$ per molecule region), which is lower than the highest value (100 mN m$^{-1}$) for a liquid-expanded film (Davis and Rideal, 1963). At the onset of the liquid-expanded state, corresponding to the more expanded configuration, the molecule occupies a mean interfacial area of 209.9 ± 3.6 Å$^2$ per molecule. This increase in surface pressure was followed by a small plateau of quite constant surface pressure and by a sharp increase in surface pressure at low areas per molecule. This indicated that GIPC monolayers can adopt a more condensed state (Cs$^{-1}$ > 80 mN m$^{-1}$) under high compression. In this state, the lipids occupy a mean molecular area of 66 ± 11.3 Å$^2$ per molecule and probably adopt a vertical orientation at the interface.

We next assessed the interaction between GIPCs and sitosterol; information can be obtained by a thermodynamic analysis of the compression isotherms of the mixed GIPC-sitosterol monolayers. Within a mixed monolayer, if the two components are immiscible (or ideally miscible), the area occupied by the mixed film will be the sum of the areas of the separate components (obeying the additivity rule; Maget-Dana, 1999). Any deviation from the additivity rule can be attributed to a specific interaction between the two components (Maget-Dana, 1999; Fang et al., 2003). Whatever the surface pressure considered (10, 20, or 30 mN m$^{-1}$), the
The monolayer is thermodynamically stable. The negative value of the free energy of mixing (Gaines, 1966; Maget-Dana, 1999; Eeman et al., 2005). Moreover, the negative value of the free energy of mixing (Fig. 8C) suggests a strong attractive interaction between the two components. The negative deviation of the area together with the negative excess free energy of mixing (Fig. 8C) indicates that the mixed GIPC/sitosterol monolayers was lower than the area of individual molecules (Fig. 9C), again in very good agreement with the experimental assays on monolayers (Fig. 8B).

To analyze the behavior of GIPCs with t18:0/h24:0 in a lipid membrane, we calculated its insertion into a simplified implicit bilayer (IMPALA method; Ducarme et al., 1998) and compared it with gluCER with d18:2/h16:0. Figure 9B clearly shows significant differences between gluCER and GIPC: (1) the size of the polar heads and the positioning of acyl chains are strikingly different; and (2) the saturated VLCFA of GIPCs runs out of the middle of the bilayer and interdigitates by at least six to seven carbon atoms within the second leaflet.

**DISCUSSION**

**GIPCs Are by Far the Major Lipids of the Plant PM**

In this work, we reinvestigated the lipid composition of PM and ordered domain isolated as DIMs, with a particular focus on GIPCs. The latter class of sphingolipids has long been neglected because it is not extracted by conventional lipid extraction procedures (Supplemental Fig. S9; Sperling et al., 2005; Markham et al., 2006). Here, we showed that GIPCs represent up to 30 to 40 mol% of the total PM lipids of tobacco plants and, therefore, represent the bulk of PM outer leaflet lipids, with 60% to 80% of total outer leaflet lipids (Figs. 3 and 10). Taking into account this striking result, we recalculated the lipid-to-protein ratio of plant PM and found a ratio of 1.3. Hence, bearing in mind that PM contains a high protein density, it is tempting to propose that plant PM should not be considered as a system where proteins are floating in a sea of lipids but as a lipid-protein composite in which a very high density of transmembrane and anchored proteins may modify the order of nearby lipids (Jacobson et al., 2007). A recent publication in plants showed that PMs are subcompartmentalized into a plethora of coexisting and diverse microdomains labeled by the different isoforms of the inner leaflet plant raft protein REMORIN (Jarsch et al., 2014). The respective roles of lipids and proteins in this segregation of membrane compounds remain to be elucidated.

**GIPC’s Polar Headgroups Are Much Bulkier Than Phospholipid Ones**

The volume occupied by the glycosyl-phosphoinositol headgroup of GIPCs increases with the complexity of the oligosaccharide chain. Our experimental data obtained by the Langmuir monolayer technique indicates that the molecular area occupied by tobacco GIPCs of series A was comparable to that of the GIPC monomolecular layer, with a slight increase in Van der Waals interactions (Fig. 9C). This suggests a good steric fit between the two molecules, as shown in Figure 9A. This molecular fitting can also be correlated to the fact that the mean area calculated in mixed GIPC/sitosterol monolayers was lower than the area of individual molecules (Fig. 9C), again in very good agreement with the experimental assays on monolayers (Fig. 8B).

mean molecular area of mixed monolayer GIPC:sitosterol (85:15) was significantly lower than the theoretical value calculated from the additivity rule (Fig. 8B). The negative deviation of the area together with the negative excess energy of mixing (Fig. 8C) suggests a strong attractive interaction between the two components (Gaines, 1966; Maget-Dana, 1999; Eeman et al., 2005). Moreover, the negative value of the free energy of mixing (Fig. 8C) indicated that the mixed GIPC/sitosterol monolayer is thermodynamically stable.

**Molecular Modeling of the GIPC Monolayer and Membrane Insertion**

We finally used a simple theoretical docking method called Hypermatrix. This method is particularly useful to compare the specific interaction of a molecule of interest with lipids and with itself and, hence, helps to understand its organization according to the different interacting forces. The analysis of the assembly of GIPC molecules with t18:0/h24:0 in a monolayer showed that the calculated interaction is mainly driven by hydrophobic energy (Fig. 9C). The mean interfacial area occupied by one molecule in the monolayer is $69 \pm 10$ Å$^2$ (Fig. 9C). This is in very good agreement with the area measured experimentally using the Langmuir technique in high-compression conditions, suggesting that the calculated structure of GIPC corresponds to this configuration. When the interaction between GIPCs and sitosterol was analyzed, the energy of the interaction was comparable to that of the GIPC monomolecular layer, with a slight increase in Van der Waals interactions (Fig. 9C). This suggests a good steric fit between the two molecules, as shown in Figure 9A. This molecular fitting can also be correlated to the fact that the mean area calculated in mixed GIPC/sitosterol monolayers was lower than the area of individual molecules (Fig. 9C), again in very good agreement with the experimental assays on monolayers (Fig. 8B).

Figure 7. Effect on membrane order level of tobacco leaf or BY-2 cell purified GIPCs, in combination with phospholipids and free sterols. The RGM of 1 μm diameter of LUVs of different composition labeled with di-4-ANEPPDHQ (3 μM) was measured by spectrofluorimetry in the presence of GIPCs isolated from tobacco BY-2 cells or tobacco leaves. Data shown are means ± SD (n = 5 or more independent repetitions). The different letters indicate significantly different values (P < 0.05). DOPC, Dioloeylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; TM, tobacco mix.
varies from 66 ± 11.3 to 209.9 ± 3.6 Å² per molecule from a condensed to an expanded state (Fig. 8A). This was further corroborated by our computational calculations, indicating a value of 69 ± 10 Å² per molecule (Fig. 9C). In good agreement with the values of the interfacial area either calculated or measured by the Langmuir monolayer technique, as reviewed (Deleu et al., 2014). By contrast, phospholipids occupy 95 to 110 Å² per molecule in an expanded state and 45 to 55 Å² per molecule (Eeman et al., 2005; Scheffer et al., 2005). Predictions based on the geometrical properties of glycosphingolipid molecules indicate that the separation of a glycosphingolipid-rich phase in a phospholipid bilayer would imply a minimization of the interfacial free energy required to accommodate the amphipathic glycosphingolipid in the bilayer. Therefore, the geometrical properties inherent in the bulky headgroup of glycosphingolipids strongly favor phase separation and spontaneous membrane curvature (Sonnino and Prinetti, 2010). In animals, the extent of ganglioside phase separation in glycerophospholipid bilayers depends on the surface area occupied by the oligosaccharide group that is usually directly correlated with the number of sugar residues (Masserini et al., 1989). Nevertheless, one must note that gangliosides are present in very low amounts in animal membrane (less than a few percent), whereas plant GIPCs represent the major sphingolipids of the PM. In that context, the biophysical properties of the plant PM must be fully reinvestigated.

Figure 8. Surface pressure-area (Π-A) isotherms, at the air-aqueous phase interface, of pure GIPC (circles) and sitosterol (squares) monolayers and of mixed GIPC/sitosterol monolayer (triangles) prepared at a molar ratio of 0.85. A, The isotherms were recorded at 22°C ± 1°C with an aqueous subphase composed by 10 mM Tris buffer at pH 7. Duplicate experiments using independent preparations yielded similar results. B, Comparison of the experimental (white bars) and theoretical (black bars) mean molecular areas at a surface pressure of 10, 20, and 30 mN m⁻¹ for a GIPC/sitosterol molar ratio of 0.85. The theoretical value is obtained according to the additivity rule: Π₁₂ = Π₁X₁ + Π₂X₂, where Π₁₂ is the mean molecular area for ideal mixing of the two components at a given Π, Π₁ and Π₂ are the molecular areas of the respective components in their pure monolayers at the same Π, and X₁ and X₂ are the molar ratios of components 1 and 2 in the mixed monolayers. C, Excess free energy of mixing (ΔGₓ) and free energy of mixing (ΔGₓ; black bars) of the pure monolayer GIPC/sitosterol at a molar ratio of 0.85 for various surface pressures. ΔGₓ and ΔGₓ were calculated according to the following equations (Maget-Dana, 1999; Eeman et al., 2005):

\[ \Delta G_x = \int_0^{A_{12}} A_{12}(\Pi - X_1 \int_0^{A_1} d\Pi - X_2 \int_0^{A_2} d\Pi), \]

where A is the mean molecular area, X is the molar fraction, subscripts 1 and 2 refer to pure components 1 and 2, respectively, and subscript refers to their mixtures; and \( \Delta G^M = \Delta G_x + \Delta G^d \), where \( \Delta G^d \) is the free energy for ideal mixing and can be calculated from the following equation: \( \Delta G^d = RT(X_1 \ln X_1 + X_2 \ln X_2) \), where R is the universal gas constant and T is the absolute temperature.
Role for GIPCs and Sterols in Coupling the Inner and Outer Leaflets of the PM

The Asymmetry of Lipids in the Tobacco PM

A common feature of eukaryotic PMs is the nonrandom distribution of lipids in the two leaflets of the membrane, called lipid asymmetry. Lipid asymmetry within the two PM monolayers is responsible for different biophysical properties and influences numerous cellular functions. The lipid asymmetry lies in the facts that glycerolipids are primarily synthesized on the cytosolic side of the membrane whereas the production of complex sphingolipids is completed in the endoplasmic reticulum/Golgi, rendering the latter exposed to the outer surface. In addition, sterols have higher affinity for sphingolipids than glycerolipids. This out-of-equilibrium situation is maintained by the activity of lipid translocases, which compensate for the slow spontaneous transverse diffusion of lipids (Devaux and Morris, 2004).

To build a model of the plant PM, we used the results of this study and those obtained by Tjellstrom et al. (2010), who showed that there is a transverse lipid asymmetry in root plant PM. They calculated that the distribution of cytosolic to apoplastic leaflet was 65:35 for phospholipids, 30:70 for total sterols, and 30:70 for gluCER. Digalactosyldiacylglycerol is located exclusively in the inner leaflet (Tjellstrom et al., 2010). Here, we considered that the glycerophospholipid-rich inner leaflet is unsaturated and that plant phosphatidylserine and polyphosphoinositides (e.g. phosphatidylinositol-4,5-bisphosphate [PIP2]) were present exclusively in the inner leaflet, as described in animal models (Di Paolo and De Camilli, 2006). As filipin III labeling used to assess sterol distribution could
not discriminate between free sterols and sterol derivatives (ASG and SG; Tjellstrom et al., 2010), we drew free sterols, SG, and ASG equivalently distributed between the two leaflets, with a molar ratio for sterols of 30:70 in:out.

We tried to experimentally access the distribution of GIPCs in the PM. We treated right side out and inside out vesicles with sphingolipid ceramide N-deacylase enzyme able to hydrolyze GIPCs (Blaas and Humpf, 2013), as described with phospholipase A2 for the phospholipid asymmetrical distribution (Tjellstrom et al., 2010). In our hands, no GIPC hydrolysis occurred (data not shown). Nevertheless, we reasonably hypothesized that GIPCs are located exclusively in the apoplastic face for different independent reasons: (1) the two first steps of GIPC synthesis (inositol phosphorylceramide synthase and glucuronic transferase) occur in the Golgi apparatus (Wang et al., 2008; Rennie et al., 2014); (2) the mannosylation of series A of GIPC to build series B is dependent on the GDP-Man transporter GOLGI-LOCALIZED NUCLEOTIDE SUGAR TRANSPORTER1, suggesting a luminal glycosylation of GIPCs so that the polar heads are exposed in the outer leaflet of the PM after vesicular fusion (Mortimer et al., 2013); (3) GIPCs are structural homologs to gangliosides, present exclusively in the outer leaflet of the PM in animal cells (Sonnino and Prinetti, 2010); (4) it is very unlikely that GIPCs may spontaneously flip-flop in the PM because of the size and polarity of their heads; and (5) immunogold labeling of GIPCs (series B–F) on PM vesicles seems to be unilaterally distributed outside of the PM vesicles (Fig. 6).

**Saturated VLCFAs of GIPCs Interdigitate the Two Leaflets**

A key question in understanding the functional role of the PM is whether lipids of the outer leaflet are coupled to those of the inner leaflet. Plant GIPCs exhibit a high content of VLCFAs that can be hydroxylated on carbon 2. The presence of VLCFAs in DIMs was recently observed in bean (*Phaseolus vulgaris*) and maize (*Zea mays*) DIMs (Carmona-Salazar et al., 2015). Here, the modeling approach suggests a strong and stable interdigitation of VLCFA and hVLCFA of GIPCs from the outer leaflet into the inner leaflet by six to seven carbon atoms (Fig. 9B). VLCFAs are also abundant in sphingolipids in animal cells. It has been proposed that the lipid bilayer organization of the stratum corneum could be stabilized by a partial interdigitation between the two leaflets (Ruettinger et al., 2008). Interdigitation of long-chain fatty acid residues between complex lipids might thus represent a common feature in plants and animals that allows a higher thermal stability of the outer leaflet, as described in artificial asymmetrical liposomes prepared with animal lipids (Cheng et al., 2009).

**GIPCs Are Able to Organize in Liquid-Ordered Domains with Sterols**

Tobacco cells produce several hundred GIPCs of different structures (Fig. 2; Cacas et al., 2013) with the same ceramide moiety and a variable glycan part. A publication from the 1970s suggest that up to 20 sugars can be added to the GIPC core structure, but little is
known about such molecules (Kaul and Lester, 1975). Here, we demonstrate that GIPCs with more than two sugars are enriched in DIMs (Fig. 2C) and that these complex sphingolipids cluster in domains of 35 nm (Fig. 6). In addition, we show that 2-hydroxylated GIPCs were enriched in DIMs (Fig. 1B). This result is consistent with biophysical studies where raft phase separation is favored by the fact that sphingolipids, as ceramide-based amphipathic lipids, can create a network of hydrogen bonds due to the presence of the amide nitrogen, the carbonyl oxygen, and the hydroxyl group positioned in proximity to the water-lipid interface of the bilayer (Pascher, 1976). In addition, GIPC’s LCB being dominated by trihydroxylated LCBs (Supplemental Fig. S6), the presence of two additional hydroxyl groups at the interface may be of importance for sphingolipid-phytosterol interactions. The contribution of hydrogen bonds between lipids stabilizing a more rigid segregated phase in the bilayer is energetically remarkable (Quinn and Wolf, 2009). We recently showed that GIPCs enhance the sterol-induced ordering effect by stimulating the formation and increasing the size of sterol-dependent ordered membrane domains (Grosjean et al., 2015), suggesting a strong interaction between phytosterols and GIPCs, leading to a well-defined liquid-ordered phase separation. Docking calculation between phytosterols and GIPCs showed that the interaction is mostly of hydrophobic and Van der Waals types (Fig. 9C). Hence, the closer the molecules are, the stronger the interaction is. This aspect is also pointed out by Langmuir monolayer experiments, where we measured an attractive interaction between phytosterols and GIPCs, leading to a molar ratio of 85:15 (Fig. 8).

Finally, interdigitated hydrocarbon chains may play a role in the stabilization of lipid domains, as reviewed by Sonnino and Prinetti (2010).

A model summarizing these data is presented in Figure 10. To build this model, the molar composition of lipids from BY-2 PM (Fig. 4) and the information presented above were considered. This model emphasizes the strong enrichment of GIPCs in the apoplastic phase of the PM. In accordance with this assumption, only a little space would be left in the outer leaflet for phospholipids, which consequently are concentrated on the cytoplasmic leaflet. Polyglycosylated GIPCs cluster with sterols in domains of approximately 35 nm in the outer leaflet, and polyphosphoinositide-enriched domains are present in the inner leaflet according to our previous work (Furt et al., 2010).

Are Rafts in the Two Leaflets Coupled?

Plant GIPCs are clearly involved in raft formation, and rafts exist in both external and internal leaflets of the plant PM (Raffaele et al., 2009; Furt et al., 2010; Mongrand et al., 2010). Biological rafts are likely of nanometer scale and certainly differ in size and stability in the two monolayers. It is not known whether they overlap so that they are coupled functionally and structurally (Subczynski and Kusumi, 2003; Eisenberg et al., 2006). By exploring this possibility, one could shed light on how cues are transmitted through the bilayer. Does the clustering of proteins or lipids in the outer leaflet trigger the rearrangement of downstream proteins or lipids in the inner leaflet (kinases, phosphatases, small G proteins, and PIP2), leading to signal transduction and amplification? Can rafts in the outer leaflet be enriched in GIPCs and sterols mirrored by PIP2-enriched cytoplasmic leaflet rafts, as represented in Figure 10? What could be the role of fatty acid interdigitation and lipid asymmetry in plants, and how is this process regulated? Proteins, omitted in our model, will certainly influence raft composition, size, shape, and overall physical properties, independently of thermodynamic considerations of the pure lipid phases (Devaux and Morris, 2004). This last aspect remains to be fully elucidated in plant PM.

CONCLUSION

In plants, GIPCs have been shown to be involved in early stages of symbiosis (Hernandez et al., 1995), in Golgi and ER integrity (Chen et al., 2008), in growth, and in the hypersensitive response through salicylic acid production (Mortimer et al., 2013). A recent study on cell wall rhamnogalacturonan II showed that GIPCs are able to bind rhamnogalacturonan II, possibly via a boron bridge, and that they can favor the boron-dependent dimerization of rhamnogalacturonan II (Voxeur and Fry, 2014). The cell wall is an important feature in regulating protein lateral mobility. In plant cells, turgor pressure tightly pressed PM against the cell wall. Martiniere et al. (2012) showed that this intimate connection affects protein lateral mobility, including that in the inner leaflet. This suggests that the plant cell wall, and by extension the continuum between the PM and the cell wall, influences protein lateral mobility (Martinière et al., 2012). This regulation of protein lateral mobility by the cell wall certainly plays a role in plant cellular processes. GIPCs may also be important determinants in cell signaling, cell-to-cell communication, plant defense, and the sorting of proteins, as also described for complex sphingolipids in animal development (Worrall et al., 2003). The link between outer leaflet lipids and the cell wall also deserves to be fully investigated. Finally, the apoplastic leaflet that contains high order-forming lipids (GIPC/phytosterols) likely represents a physical barrier involved in the maintenance of thermal tolerance (Cheng et al., 2009), cell integrity, and responses to pathogens. The preparation of asymmetric vesicles that mimic the plant PM will be of great interest to study this coupling, the effect of lipid raft formation, and the distribution of transmembrane protein helices (Cheng et al., 2009). In animals, alteration of lipid asymmetry plays a prominent role during cell fusion, activation of the coagulation cascade, and the recognition and removal of apoptotic cells. Our
work should pave the way to address such questions in plants.

MATERIALS AND METHODS

Materials

HP-TLC plates were Silicagel 60 F254 (Merck).

Plant Materials

Leaves were obtained from 8-week-old tobacco (Nicotiana tabacum ‘Xanthi’) plants grown in a growth chamber at 25°C under 16/8-h day/night conditions. Wild-type tobacco (cv Bright Yellow 2) and grapevine (Vitis vinifera ‘Cabernet Sauvignon’) cells were grown as described by Morel et al. (2006) and Cacas et al. (2013).

Preparation and Purity of Tobacco PM

All steps were performed at 4°C. PMs were obtained after cell fractionation according to Mongrand et al. (2004) by partitioning in an aqueous polymer two-phase system with polyethylene glycol/dextran.

Fatty Acid Analysis

Each sample was transmethylated at 110°C overnight in methanol containing 5% (v/v) sulfuric acid and spiked with 10 μg of heptadecaenoic acid (c17:0) and 10 μg of 2-hydroxy-tetradecaenoic acid (b14:0) as internal standards. After cooling, 3 mL of NaCl (2.5%, w/v) was added, and the released fatty acyl chains were extracted in hexane. Extracts were washed with 3 mL of saline solution (200 mM NaCl and 200 mM Tris, pH 8), dried under a gentle stream of nitrogen, and dissolved in 150 μL of BSTFA and trimethylchlorosilane. Free hydroxyl groups were derivatized at 110°C for 30 min, surplus BSTFA-trimethylchlorosilane was removed under nitrogen, and samples were dissolved in hexane for analysis using GC-MS under the same conditions as described (Bür et al., 2011). Quantification of fatty acids and hydroxyl acids was based on peak areas, which were derived from total ion current (Rehman et al., 2008), and using the respective internal standards.

Sphingoid Base (LCB) Analysis

Samples were heated at 110°C for 24 h with 4 mL of dioxane (Sigma) plus 3.5 mL of 10% (w/v) aqueous Ba(OH)2 (Sigma). The sphingoid bases were oxidized to their corresponding aldehydes by stirring the sample with 100 μL of 0.2 M sodium periodate (Sigma) at room temperature for 1 h in the dark. The aldehydes were recovered by hexane extraction and used directly for gas chromatography analysis as described (Cacas et al., 2012b).

Extraction of Total Polar Lipids: Setup of the Lipid Extraction Protocol for Total Polar Lipids in Plants

Membrane fractions (100–200 μg) or grape cell culture (approximately 20 mg of lyophilized material) were extracted according to three independent methods. For extraction 1, 3.5 mL of chloroform:methanol:HC1 (200:100:1, v/v/v) supplemented with 0.01% (w/v) butylated hydroxytoluene (BHT) was incubated with the sample. Then, 2 mL of 0.9% (w/v) NaCl was added, vortexed for 5 min, and centrifuged. The lower organic phase was collected, and the upper phase was reextracted once with 4 mL of pure chloroform. For extraction 2, 3.5 mL of MTBE:methanol:water (100:50:25, v/v/v) supplemented with 0.01% (w/v) BHT was incubated with the sample. Then, 2 mL of 0.9% (w/v) NaCl was added, vortexed for 5 min, and centrifuged. The upper organic phase was collected, and the lower phase was reextracted once with 4 mL of pure MTBE. In both extractions, the organic phases were combined and dried. The aqueous phases were dried to remove any trace of organic solvent and resuspended in 1 mL of pure water, and GIPCs were back extracted twice by 1 mL of butanol-1. For extraction 3 (adapted from Markham et al., 2006), 3.5 mL of the lower phase of propan-2-ol:hexane:water (55:20:25, v/v/v) was incubated with the sample. The sample was incubated at 60°C for 15 min with occasional shaking. The extract was spun at 500g while still warm, and the supernatant was transferred to a fresh tube. The pellet was extracted once more, each time with 3.5 mL of extraction solvent, and the supernatants were combined and dried. The pellet was dried to remove any trace of organic solvent and resuspended in 1 mL of pure water, and GIPCs were back extracted twice by 1 mL of butanol-1. Extracted lipids were dissolved in chloroform:methanol:water (30:60:8, v/v/v) for storage. Alternatively, before lipid extraction, biological samples were transferred to isopropanol (3 mL) with 0.01% (w/v) BHT at 75°C and incubated for 15 min to inhibit lipase activity.

The rationale for the three lipid extraction protocols is presented in Supplemental Figure S10A. We used lyophilized grape cell culture shown previously to contain similar VLFCAs, hVLFCAs, and GIPCs to tobacco cells (Cacas et al., 2013; Supplemental Fig. S10B). We first compared the Folch protocol (extraction 1) and MTBE extraction (extraction 2) followed by butanol-1 extraction of the aqueous phase with the Markham protocol (extraction 3) shown to fully extract plant sphingolipids (Fig. 4B; Markham et al., 2006). The results in Supplemental Figure S10C, middle, show that all classes of lipids were extracted with no significant differences between the three different protocols. We performed HP-TLC to test the integrity of polar lipids, and we observed, as often described during plant lipid extraction, a major activation of phospholipase D leading to the conversion of phospholipids into phosphatic acid (Supplemental Fig. S10D). To circumvent this problem, we boiled the lyophilized grape cells in hot isopropanol and further extracted with the three protocols. To our surprise, all the polar lipids including VLFCA-containing GIPCs were extracted without the need of the second extraction step by butanol-1 of the aqueous phase (Supplemental Fig. S10C, right). As expected, no degradation of phospholipids was observed on thin-layer chromatography plates (Supplemental Fig. S10D).

Extraction and Purification of GIPCs by DEAE Chromatography

GIPCs were purified according to Bür et al. (2011) and Grosjean et al. (2015) to obtain milligram amounts. Alternatively, GIPCs were purified by DEAE chromatography. DEAE Sephadex DE-52 (Whatman preswollen, ρgranular) was suspended in chloroform:methanol:water (30:60:8, v/v/v) supplemented with 1 M ammonium acetate. A glass column (40 cm high, 2.2 cm diameter) was plugged by defatted cotton, filled with DEAE Sephadex, and washed with 600 mL of chloroform:methanol:water (30:60:8, v/v/v). GIPC-whisk pelleted extract from BY-2 cell culture was dissolved in 10 mL of chloroform:methanol:water (30:60:8, v/v/v) and loaded on the column. The column was washed with 800 mL of chloroform:methanol:water (30:60:8, v/v/v) for the removal of neutral compounds: fractions 1 to 8. The fractions were sequentially eluted with 500 mL of chloroform:methanol:water (30:60:8, v/v/v) supplemented with 5 mM ammonium acetate (fractions 9–14), 10 mM ammonium acetate (fractions 15–20), 25 mM ammonium acetate (fractions 21–28), 50 mM ammonium acetate (fractions 29–35), 100 mM ammonium acetate (fractions 36–43), and 250 mM ammonium acetate (fractions 44–49; Supplemental Fig. S11). The purification process was monitored by HP-TLC on plates impregnated with freshly prepared 0.2% ammonium acetate dissolved in methanol and chromatographed in chloroform:methanol:NH4OH (4:4:1 in water; 9:7:2, v/v/v). Lipids were visualized by spraying plates with primuline. GIPC-containing fractions were dissolved in water and dialyzed against water at 4°C for 2 d to remove ammonium acetate (Spectra/Por Dialysis Membrane; molecular weight cutoff, 3,500). The water was changed every 6 h. The desalted fractions were dried and dissolved in a volume of 3 mL of chloroform:methanol:water (30:60:8, v/v/v) and stored at 4°C.

Generation of Rabbit Polyclonal Antibodies to GIPC

Preparation of Liposomes

Liposomes were prepared essentially as described previously (Richards et al., 1998). Liposomes for the primary immunization were composed of purified BY-2 cell GIPCs (series A or a mix of series B and E) plus phosphatidylcholine, phosphatidylethanolamine, and cholesterol in a ratio of 0.9:0.1:0.75. Lipid A was included in the liposomes at 20 nmol μmol−1 phospholipid. Lipids were dried from a chloroform:methanol:water (30:60:8) solution. The liposomes were swollen in 1 mL of Tris-buffered saline (TBS) by vigorous shaking in a vortex mixer and sonicated at room temperature for 30 min.

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Plasma Membrane Lipids in Tobacco
Immunization of Rabbits

Rabbits were immunized four times at 0, 21, and 42 d (COVAXALB; France Biotechnologies). Preimmune serum was analyzed compared with serum at 53 d post injection.

Immunogold Labeling of Purified Plant PMs

Labeling was performed on purified BY-2 cell PM vesicles according to Noirot et al. (2014). Immunological reaction on grids was performed for 1 h with rabbit polyclonal antibody against polyglycosylated GIPCs (rabbit no. 46) diluted 1:40, which was revealed with a goat anti-rabbit IgG conjugate (Aurion) labeled to 6-nm colloidal gold particles. Three independent experiments using three independent biochemical PM purifications from BY-2 cells were recorded. For each experiment, three replicates of immunolabeling and two replicates of each control sample (omission of the primary antibody and use of the pre-immune serum) were observed with a Hitecchi H7500 transmission electron microscope equipped with an AMT camera driven by AMT software. In order to characterize the distribution of the detected antigen on the PM vesicle surface, the density of labeling was evaluated by counting the number of colloidal gold particles per labeled vesicle. Groups of gold label were visualized, and the sizes of the clusters were measured on each labeled vesicle with the AMT software. Proportions of gold particles in groups and of isolated gold particles were evaluated. Counting and measurement were performed on 49 images of PM vesicles from the three independent experiments. The spatial distribution was determined as described (Noirot et al., 2014) using the Ripley function (Ripley, 1976).

Order Level Measurement of Artificial Membranes

The preparation of LUVs, fluorescence spectroscopy, and membrane order level measurement were as described (Grojans et al., 2015).

Home-Made Lipid Blot

Immun-Blot PVDF membranes (Bio-Rad) were activated with methanol for 30 s. GIPC DEAE fractions (5 μL) were deposited and allowed to dry. Membranes were further reactivated in methanol for 2 s and blocked in TBS supplemented with 5% (w/v) defatted bovine serum albumin for 1 h. Antibodies to GIPC (dilution, 1:100) were incubated for 1 h at room temperature; membranes were then rinsed three times with TBS supplemented with 0.1% (v/v) Tween 20 and revealed with anti-rabbit secondary antibodies coupled to horseradish peroxidase (1:100).

ELISA

All steps were performed at 37°C. ELISA plates were filled with or without 500 ng of BY-2 cell PM vesicles in 0.1 mL of phosphate-buffered saline for 2 h, then blocked for 2 h with 1% (w/v) bovine serum albumin in phosphate-buffered saline. Anti-GIPC antibodies (dilution, 1:50 for sera or 1:100 for purified IgG) were incubated for 1 h and revealed with anti-rabbit secondary antibodies coupled to alkaline phosphatase (1:5,000). Reaction with p-nitrophenyl phosphate (0.01% in 10% [w/v] diethanolamine buffer) was read at 405 nm after 1 h.

Langmuir Trough

Total BY-2 GIPC (approximately 1.260 g mol⁻¹) was used in this study. A solution at 0.39 mM in chloroform:methanol:water (30:60:8) was prepared. Sitosterol was purchased from Avanti Polar Lipids. It was dissolved at 0.39 mM in chloroform:methanol (2:1). The surface pressure-area (H-A) isotherms were recorded on a Langmuir trough (KSV Minitrough [width, 75 mm; area, 24.225 mm²]; KSV Instruments) equipped with a platinum plate attached to a Wilhelmy-type balance. The GIPC sample was heated to 60°C for 15 min for a better solubilization. Pure solutions and 0.15:0.85 molar mixtures of sitosterol:GIPC were spread (fixed volume of 30 μL) as tiny droplets to produce a uniform monolayer on a Tris-NaCl 10:150 mM (Millipore) subphase adjusted to pH 7 with HCl. After evaporation of the solvent (15 min), monolayers were compressed at a rate of 0.05 min⁻¹ and at a temperature of 22°C ± 1°C. Before each experiment, the cleanliness of the system was confirmed by checking the surface pressure over the surface compression of the pure subphase. The reproducibility of the H-A isotherms was checked by repeated recordings, and the relative so in surface pressure and area was found to be 3% or less.

Molecular Modeling Approaches

The conformation of GIPC sitosterol and gluCER [d18:2(Δ9, Δ16:0)] was calculated using the structure tree procedure, as described elsewhere (Lins et al., 1996). The Hypermatrix docking procedure was used to study the monolayer formed by GIPC and its interaction with sitosterol, as already described (Lins et al., 1999; Fa et al., 2007; Benskaiddour et al., 2008) and reviewed recently (Delieu et al., 2014). Briefly, one GIPC molecule is positioned and fixed for the whole calculation at the center of the system, oriented at the hydrophobic (Haimi et al., 2006)/hydrophilic interface (Brasseur, 1990). The interacting GIPC (for GIPC monolayer) or sitosterol (for mixed monolayer) is also oriented at the hydrophobic/hydrophilic interface, and by rotations and translations, more than 10° positions of the interacting molecule around the central molecule are calculated. The energy values together with the coordinates of all assemblies are stored in a matrix and classified according to decreasing values. The most stable matching is used to decide the position of the first interacting molecule. The position of the second one is then defined as the next most energetically favorable orientation stored in the matrix, taking steric and energetic constraints due to the presence of the first molecule into account. The process ends when the central molecule is completely surrounded with the other molecule. In this method, the lipid/water interface was taken into account by linearly varying the dielectric constant ε from 3 (above the interface) and 30 (below the interface), and an empirical equation for the hydrophobic energy is added in the force field, as described (Lins and Brasseur, 1995). The mean area occupied by one molecule in the complex was estimated by projection on the x-y plane using a grid of 1 A².

To calculate the insertion of GIPCs or soybean (Glycine max) gluCER into an implicit simplified bilayer, we used the IMPALA method described previously (Ducarme et al., 1998). Briefly, this method simulates the insertion of any molecule into a bilayer by adding energy restraint functions to the usual energy description of molecules. The lipid bilayer is defined by C(z), which represents an empirical function describing membrane properties. This function is constant in the membrane plane (z and y axes) but varies along the bilayer thickness (z axis). Two restraints simulate the membrane, one the bilayer hydrophobicity (Ez) and the other the lipid perturbation (Eφ). All the equations are described elsewhere (Ducarme et al., 1998). The method was notably successful when applied to small helical peptides of known configurations (Lins et al., 2001). It provides insights into the behavior of peptide dynamics that cannot be obtained with statistical approaches. All calculations were performed on a Linux station bixeon quad core using the home-designed Z-ultimate software.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Typical GC-MS spectrogram of total FAMES and sterols extracted from BY-2 cell PMs.

Supplemental Figure S2. Fatty acid content of tissue, microsomal, PM, and DIM fractions from tobacco leaves or BY-2 cell culture.

Supplemental Figure S3. Fatty acid content of DIM vs. detergent-soluble membranes from tobacco leaf purified PMs.

Supplemental Figure S4. Fatty acid and sterol content of purified ASGs extracted from tobacco leaves or BY-2 cell culture.

Supplemental Figure S5. Fatty acid content of purified gluCER extracted from tobacco leaves or BY-2 cell culture and purified by TLC.

Supplemental Figure S6. LCBI content of GIPCs, PMs, and DIMs from tobacco leaves or BY-2 cell culture.

Supplemental Figure S7. MALDI-MS analysis of GIPC extracts from BY-2 cells.

Supplemental Figure S8. MALDI-MS analysis of GIPC extracts purified from PMs and DIMs extracted from tobacco leaves.

Supplemental Figure S9. Determination of lipid-to-protein ratio in plant PMs.

Supplemental Figure S10. Fatty acid content of total lipids from grape cell culture.

Supplemental Figure S11. Purification of GIPCs from BY-2 cells by DEAE chromatography.
Supplemental Figure S12. Test by ELISA of the specificity of antibodies against polyglycosylated GIPCs.

Supplemental Figure S13. Cross-reactivity of antibodies against polyglycosylated GIPCs.

Supplemental Figure S14. Immunogold labeling controls of PM vesicles.

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