Contribution of galactoglycerolipids to the 3-dimensional architecture of thylakoids

Bruno Demé,* Céline Cataye,[†,‡,§,‖] Maryse A. Block,[†,‡,§,‖] Eric Maréchal,[†,‡,§,‖] and Juliette Jouhet[†,‡,§,‖,1]

*Institut Laue-Langevin, Grenoble, France; [†]Centre National de la Recherche Scientifique (CNRS), Unité Mixte de Recherche (UMR) 5168, [‡]Univ. Grenoble Alpes, [§]Commissariat à l'Énergie Atomique et aux Énergies Alternatives (CEA), Direction des Sciences du Vivant (DSV), Institut de Recherches en Technologies et Sciences pour le Vivant (iRTSV), and [‖]Institut National de Recherche Agronomique (INRA), USC 1359, Laboratoire de Physiologie Cellulaire et Végétale (LPCV), Grenoble, France

ABSTRACT **Thylakoid membranes, the universal structure where photosynthesis takes place in all oxygenic photosynthetic organisms from cyanobacteria to higher plants, have a unique lipid composition. They contain a high fraction of 2 uncharged glycolipids, the galactoglycerolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), and an anionic sulfolipid, sulfoquinovosediacylglycerol (SQDG). A remarkable feature of the evolution from cyanobacteria to higher plants is the conservation of MGDG, DGDG, SQDG, and phosphatidylglycerol (PG), the major phospholipid of thylakoids. Using neutron diffraction on reconstituted thylakoid lipid extracts, we observed that the thylakoid lipid mixture self-organizes as a regular stack of bilayers. This natural lipid mixture was shown to switch from hexagonal II toward lamellar phase on hydration. This transition and the observed phase coexistence are modulated by the fine-tuning of the lipid profile, in particular the MGDG/DGDG ratio, and by the hydration. Our analysis highlights the critical role of DGDG as a contributing component to the membrane stacking *via* hydrogen bonds between polar heads of adjacent bilayers. DGDG interactions balance the repulsive electrostatic contribution of the charged lipids PG and SQDG and allow the persistence of regularly stacked membranes at high hydration. In developmental contexts or in response to environmental variations, these properties can contribute to the highly dynamic flexibility of plastid structure.—Demé, B., Cataye, C., Block, M. A., Maréchal, E., Jouhet, J. Contribution of galactoglycerolipids to the 3-dimensional architecture of thylakoids. *FASEB J.* 28, 3373–3383 (2014). www.fasebj.org**

Key Words: glycolipid · neutron diffraction · hexagonal II phase · lamellar phase · membrane stacking

INSIDE THE CHLOROPLASTS of plant and algal cells, the photosystems that collect solar energy are inserted into photosynthetic membranes, also called thylakoids. Our understanding of the complex architecture of thylakoids is based on the analysis of electron micrograph of thin cell sections (1). The most striking morphological feature is the capacity of thylakoid membranes to form flattened cisternae that can pile up in stacked domains. The form of thylakoid stacks is essential to ensure enough density of photosystems to capture solar energy. Stacked thylakoids typically account for 50–70% of the membrane surface in plants. In 1 m^2 of leaves, we estimate that the total surface of photosynthetic membrane can be as high as ~2.5 ha. Taking together cyanobacteria, algae, and plants, thylakoids represent the most abundant biological membrane system on earth (2).

In plants and algae, photosynthetic membranes have a unique lipid composition. They differ from all other cellular membranes by their very low amount of phospholipids, besides some phosphatidylglycerol (PG), and a high fraction of glycolipids. These glycolipids are the uncharged galactolipids, mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), and an anionic sulfolipid, sulfoquinovosyldiacylglycerol (SQDG). In all photosynthetic membranes analyzed to date, from cyanobacteria to algae, protists, and plants, the lipid quartet constituted by MGDG, DGDG, SQDG, and PG has been highly conserved (for recent review, see ref. 3). Why were these lipids conserved? Based on the evolution of chloroplasts, characterized by the loss of some prokaryotic components (proteins, RNAs, metabolites), replaced by components of eukaryotic origin, could some of these thylakoid lipids be substituted by others, biosynthesized in the endoplasmic reticulum?

The different thylakoid lipid classes have diverse phase behaviors, essential for membrane organization. By contrast with DGDG, PG, and SQDG that form

Abbreviations: DGDG, digalactosyldiacylglycerol; HII, hexagonal II; L_α, lamellar; LHCII, light-harvesting complex II; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol

[1] Correspondence: Laboratoire de Physiologie Cellulaire et Végétale, 17 rue des Martyrs, 38054 Grenoble cedex 9, France. E-mail: juliette.jouhet@cea.fr

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bilayers from low to high hydrations, the most abundant thylakoid lipid, MGDG, does not form bilayers. When dispersed pure, MGDG forms an inverted hexagonal structure in aqueous medium, also known as hexagonal II (HII) phase (4).

Lipids may contribute to the architecture of thylakoids at multiple scales. Thylakoids form flattened cisternae, around a single aqueous space called lumen, that pile up in higher plants into stacked domains called grana (for a review see refs. 5, 6). The intermolecular forces displayed in the persistence of flat membranes and their stacking have been investigated (7). Possible forces have been inventoried by Ladygin and Semenova (8) and could involve external osmotic or colloidal pressure, lateral intramembrane interaction, and eventually attraction between membrane pairs on the inner side facing the lumen. This latter attractive force was well analyzed: it is enhanced during light treatment by acidification of the lumen, provoking the lumen shrinking, and, in the dark, it is counterbalanced by Mg^{2+} ions that mask the negative charges of the membranes. Other actors might participate to membrane flattening like protein maturation (9) or the content in polar carotenoids that were shown to have sterol-like properties, *e.g.*, ordering the membrane and increasing its rigidity (10). Nevertheless, some force components are still missing.

The hypothesis of a specific contribution of galactolipids to thylakoid morphogenesis was brought to light by the analysis of plants deficient or impaired in MGDG synthesis (11–13). Indeed, in these plants, the biogenesis of thylakoids is strongly altered, and formation of subspherical structures budding from the inner envelope membranes is triggered inside chloroplasts. Furthermore, in DGDG-deficient mutants (knockout of DGDG synthase genes), the thylakoid structure is also strongly affected showing highly curved thylakoid membranes (14, 15). Complementation of the DGDG synthase double mutant by a glucosyltransferase synthesizing glucosylgalactosyldiacylglycerol restores the flat thylakoid architecture, indicating that sugar heads are important for membrane flattening (15). This hypothesis is supported by freeze fracture micrographs showing that *in vitro* aggregated DGDG vesicles are flattened (16).

The architecture of flattened sacs implies the existence of highly curved edges. A recent study demonstrated the involvement of CURT1 in the formation of the grana margin (17). Galactolipids, especially MGDG that forms elongated inverse micelles, have also been proposed to take part in the formation of these marginal membrane structures (6, 18) forming the edges of stacked double bilayers. Such organization would imply a lateral and transmembrane nonhomogeneous distribution of thylakoid lipid classes. This phenomenon has also been described in mixtures of oppositely charged surfactants known as catanionics (19, 20). In thylakoids, *in vivo* studies support an uneven distribution of lipid classes but with contradictory patterns (21, 22). *In vitro* studies also support nonmonophasic thylakoid lipid distribution by showing a coexistence of HII and lamellar (L_α) phases in aqueous dispersions of chloroplast lipid extracts (23) and in intact thylakoids (24). Up to now, no indubitable evidence has been provided for MGDG involvement into thylakoid edge formation.

In addition to a structuration into flattened sacs, thylakoids can pile up into grana, which are not essential for photosynthesis, but ubiquitous in higher plants (for a review, see ref. 25). Forces that sustain thylakoid stacking were listed by Chow and Anderson (7). The light-harvesting complex II (LHCII) of photosystem II is supposed to drive the structural dynamics of grana stacking in higher plants; phosphorylations of LHCII and photosystem II are involved in state transition implying stacking and destacking of thylakoids (for a review see 26). Indeed, *in vitro* and in presence of cations, LHCII can promote the formation of stacked regions in liposomes made of pure phosphatidylcholine (PC) and of thylakoid lipid extracts (27, 28). *In vivo*, the absence of LHCII in chlorophyll b mutants or a defect in LHCII trimerisation produces plants with highly reduced grana stacks (29, 30). However, LHCII is apparently not the only factor driving thylakoid stacking. MGDG and DGDG were recently shown to support LHCII aggregation, whereas PG and SQDG exert a strong disaggregating effect (31). Furthermore, DGDG regulates the formation of LHCII macroarrays, showing that DGDG plays an important role in the overall organization of thylakoid membranes (32). Important evidence comes also from cyanobacteria, where thylakoids are not stacked and no grana can be seen, the distance between thylakoid membranes being correlated with the size of the phycobilisome antenna, the equivalent of LHC in higher plants (33). Interestingly, thylakoids from cyanobacteria depleted in phycobilisomes are stacked (34), indicating that galactolipids might play a role in stacking as well.

Indication of the role of galactoglycerolipids in thylakoid stacking relies on *in vitro* experiments using either synthetic or natural lipids. First, liposomes prepared from spinach chloroplast glycerolipids are multilamellar (28). Second, whereas no aggregation is observed for PC (16, 35), DGDG vesicles were shown to aggregate regardless of the presence or absence of MGDG, PG, or SQDG. In the presence of MGDG, aggregation is irreversible (35). In a recent study, Schneck *et al.* (36) have shown that the interacting forces measured in stacks of membranes made with synthetic glycolipids are significantly higher than those reported for PC membranes, indicating that these synthetic glycolipid membranes are coupled more strongly than PC membranes, due to an additional attractive force component between carbohydrates that competes with the repulsive hydration force. DGDG aggregation requires a decrease of the hydration repulsive force between neighboring bilayers and an interaction between DGDG head groups. This interaction could be mediated by the formation of multiple hydrogen bonds with the galactolipid polar heads (35, 37). Pieces of arguments are therefore dispersed in the literature, and our current understanding on the role

of lipids relies partly on speculations. It is therefore of critical importance to investigate the precise role of MGDG and DGDG in the establishment of attractive interactions between neighboring membranes.

To dissect the influence of thylakoid lipids on membrane architecture, we analyze here the phase and swelling properties of reconstituted membrane stacks using mixed proportions of MGDG, DGDG, SQDG, and PG. Since the fatty acid composition of lipids likely affects their biophysical properties, we used natural thylakoid lipids extracted from spinach leaves. These lipids were purified and finally reconstituted pure and into relevant binary, ternary, and quaternary lipid mixtures. We confirm that pure MGDG and DGDG molecules self-organize into HII and L_α phases, respectively, and we demonstrate that the MGDG/DGDG ratio influences the transition from HII to L_α on hydration. We show that the thylakoid natural lipid composition contributes to attractive forces involved in membrane interactions, reducing the water uptake in membrane stacking. We propose that these properties, which are clearly distinct from those of phospholipids like PC, are at the heart of the remarkable conservation of thylakoid lipids throughout evolution.

MATERIALS AND METHODS

Purification of thylakoid glycerolipids

Thylakoids were prepared from intact spinach leaves, as described previously (38, 39), and resuspended at 21 mg protein/ml in washing buffer (10 mM MOPS, pH 7.8; 1 mM PMSF; 1 mM benzamidine; and 5 mM caproic acid). Isolated thylakoids were frozen in liquid nitrogen and stored at −80°C until use. Thylakoids lipids were then extracted with chloroform/methanol (1/2; v/v) as described previously (40) and separated on a silica gel column (SIL-B-200, Sigma-Aldrich, Saint-Quentin Fallavier, France). The column was equilibrated in pure chloroform (700 mg in a Pasteur pipette). Around 120 mg of thylakoid lipids was charged on the column in 2 ml of chloroform. Pigments and a part of MGDG were eluted with 40 ml of pure chloroform; galactolipids (MGDG and DGDG) were eluted with 14 ml of pure acetone. Phospholipid and sulfolipid fractions were eluted with 4 ml of chloroform/methanol (50/50; v/v). MGDG, DGDG, SQDG, and PG were then purified on thin layer chromatography plates (silica gel 60, Merck, Darmstadt, Germany) with chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5; v/v) and eluted from silica according to Bligh and Dyer (40). Lipids were quantified as described previously (41).

Neutron diffraction

Neutron diffraction experiments were performed on the small-momentum transfer cold neutron diffractometer D16 at the Institut Laue-Langevin (ILL; Grenoble, France) as described previously (36, 42, 43). The instrument was operating at a wavelength $\lambda = 4.75$ Å ($\Delta\lambda/\lambda = 1\%$) and a sample-to-detector distance of 900 mm. The focusing option provided by the vertically focusing graphite monochromator was used to maximize the incident neutron flux at the sample. To unambiguously identify the lipid phases by proper indexation of Bragg peaks, complementary experiments were performed with the detector centered on the direct beam to obtain a symmetric diffraction pattern. In this pinhole small-angle diffraction configuration, the unfocused beam was collimated with 2 distinct pairs of motorized slits.

Samples consisted of oriented lipid multilayer films (10 mg) spread from 750 μl chloroform/methanol (1/2: v/v) on silicon wafers (2 inches diameter, 275 μm thick) from Si-Mat (Kaufering, Germany). After lipid deposition, the films were evaporated at room temperature and ambient pressure and then dried overnight under vacuum. The samples were then annealed at room temperature in saturated D_2O vapor before transfer into a humidity chamber for the diffraction experiments.

The samples were held vertically in a dedicated temperature-controlled humidity chamber and aligned on a manual 4-axis goniometer head (Huber, Rimsting, Germany) embedded in the humidity chamber. The chamber was mounted on the sample rotation stage, where the lipid multilayer stacks were scanned by rocking the wafers horizontally. The sample temperature in the chamber was maintained at 25°C during the measurements, and the humidity was varied by changing the temperature of the liquid reservoir generating the water vapor from 10°C to 24.5°C, leading to relative humidities ranging from to 40 to 98.5%. After annealing in a saturated vapor atmosphere overnight, each sample was transferred to the humidity chamber and then investigated from low to high hydration by increasing the humidity step by step without opening the chamber at any time during the humidity scan.

The intensity of the diffracted beam was recorded by the millimeter-resolution large-area neutron detector (MILAND) ^{3}He position-sensitive detector, which consists of 320×320 *xy* channels with a resolution of 1×1 mm^2. Data analysis was performed using the ILL in-house LAMP software (44). Intensities on the detector surface were corrected for solid angle and pixel efficiency by normalization to the flat incoherent signal of a 1 mm water cell and by subtraction of the empty chamber background.

The lamellar spacing d was obtained by a linear fitting of the peak positions *vs.* lamellar diffraction order h of the lamellar (*h00*) reflections, according to Bragg's law: $n\lambda = 2d \sin\theta$, where $n = h$ for a lamellar phase, and θ is half the scattering angle. The periodicity between (*100*) planes in the hexagonal phase (see **Fig. 4**) was calculated the same way. Indeed, unlike the 1-dimensional order L_α phase whose Miller indices (*hkl*) (45) simplify to a series of [*h00*] reflections perpendicular to the bilayer planes, the HII phase is a 2-dimensionnal phase whose reflections in reciprocal space sit on a hexagonal lattice with [*hk0*] indices. In that case, the distance extracted from the (*hk0*) reflections corresponds to the distance between [*hk0*] planes of the 2-dimensional hexagonal arrangement in real space. The linearity in the $\log(\Pi)$ *vs.* d_w plot through the HII to L_α transition, as calculated from the hexagonal (*hk0*) and lamellar (*h00*) indicates that despite its different organization, the periodicity of the [*100*] planes of both phases evolves continuously. Like bilayers in the L_α phase, cylinders in the HII phase are parallel to the solid substrate, while hexagonal domains can be randomly oriented in the vertical (*h00*) direction.

RESULTS

Structural organization of thylakoid lipid mixtures on hydration

To measure the role played by lipids in membrane-to-membrane interactions, we reconstituted biomembrane stacks from the extracted glycolipids at the

surface of silicon wafers (thin slice of semiconductor material), and we analyzed the molecular organization and periodicity as a function of the hydration as set by the controlled relative humidity in the chamber. Spinach thylakoid lipids are composed mainly of MGDG, DGDG, PG, and SQDG in a molar ratio 100/50/18/12 (46). We purified MGDG, DGDG, PG, and SQDG from spinach thylakoids. The fatty acid composition of these lipids (**Fig. 1**) is consistent with previous analyses (47). These lipids are highly unsaturated: MGDG is enriched in C16:3 and C18:3, DGDG is mainly composed of C18:3, PG contained C16:0 and C18:3 plus a unique C16:1 with an unsaturation in *trans* configuration, and SQDG is composed of C16:0 and C18:3.

In addition to pure MGDG and pure DGDG, a quaternary MGDG/DGDG/PG/SQDG mixture, with the respective proportions of 100/48/17/17, representing the closest composition to that found *in vivo*, was reconstituted by pooling each purified class of lipid extracted from spinach thylakoid lipids. Pure lipids, as well as the lipid mixture, were resuspended in chloroform/methanol 1:2, v/v, and dried on 2-inch silicon wafers at room temperature. The samples consisted of oriented lipid films that were investigated as a function of lipid composition and hydration. For neutron diffraction experiments, the wafers were held in a humidity chamber in which the relative humidity was tightly adjusted with D_2O vapor. Because hydrogen and deuterium have different neutron-scattering lengths and because thylakoid lipids are naturally hydrogenated, D_2O enhances the contrast between the lipids and the water layers and therefore the diffracted intensity (48).

Dispersions of MGDG in aqueous phase are known to self-organize in HII phase (ref. 4 and **Fig. 2*A***). Indeed, whatever the sample hydration, neutron diffraction patterns of pure MGDG (Fig. 2*C*) were characteristic of

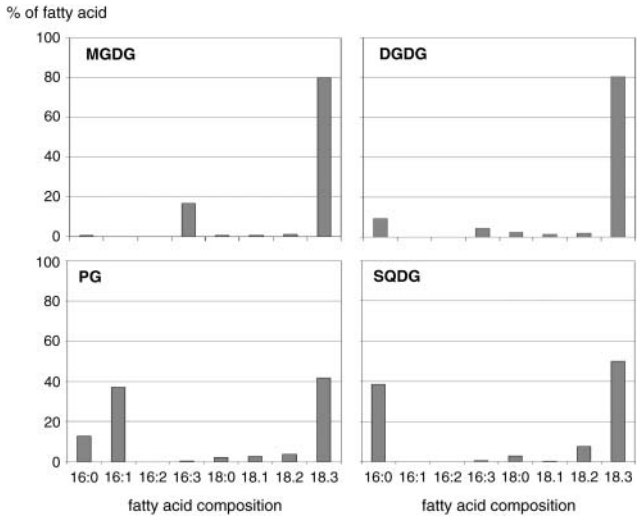

Figure 1. Fatty acid composition of single polar lipids MGDG, DGDG, PG, and SQDG purified from spinach thylakoids. Results are expressed in percentage of total fatty acids in a single lipid.

an HII phase (49), while dispersions of DGDG in aqueous phase are organized in the L_α phase (Fig. 2B and ref. 4), as observed on neutron diffraction patterns (Fig. 2*C* and refs. 37, 50). This L_α phase was observed in the entire range of hydrations investigated, from bilayer close apposition, at very low hydration, to maximum swelling at full hydration. For the reconstituted quaternary thylakoid-like lipid mixture (Fig. 2*C*), at low hydration, the pattern was similar to that of pure MGDG, indicating that the lipid mixture was organized in HII phase, whereas at high hydration it formed an L_α phase similar to that of DGDG. This result shows that the mixture of thylakoid lipids can switch from HII toward L_α on hydration.

Influence of the lipid composition on the organization of thylakoid lipid mixtures

To understand which lipids are involved in the lamellar phase formation at high hydration, different lipid mixtures were investigated (**Table 1** and **Fig. 3**). Binary (MGDG/DGDG) mixture at the natural ratio of these two lipids, as well as several ternary (MGDG/DGDG/PG) mixtures, were reconstituted by pooling each purified class of lipid extracted from spinach thylakoid lipids. As for the binary mixture, one of the ternary mixtures was matching the natural MGDG/DGDG/PG ratio. Finally, the quaternary MGDG/DGDG/PG/SQDG mixture, described above, represented the closest composition to that found *in vivo* investigated in this study. Whatever the hydration of the lipid, MGDG was always forming the HII phase, whereas DGDG was always forming a lamellar phase. At low hydration, all tested lipid mixtures containing MGDG as the main lipid were organized in HII phases. However, at high hydration, the organization depends on the proportion of DGDG, PG and SQDG relative to MGDG.

At high hydration, the quaternary thylakoid-like mixture was organized in L_α phase (Fig. 2*C*). In the ternary thylakoid-like mixture corresponding to MGDG/DGDG/PG 100/48/17, where SQDG was removed, a coexistence of hexagonal and lamellar domains was observed even at high hydration (Fig. 3). In this mixture, the ratio of hexagonal forming lipids *vs.* bilayer forming lipids was shifted from 100/82 to 100/65. DGDG was then increased to obtain a HII/L_α ratio of 100/72 in the ternary mixture composed of MGDG/DGDG/PG 100/60/12. In this mixture and at high hydration, only an L_α phase was observed, and no HII phase could be detected (Fig. 3), showing that SQDG is not essential for the formation of lamellar stacks. To test the influence of PG, this lipid was removed from the mixture, and the same MGDG/DGDG ratio (100/60) was used. At high hydration, the lamellar phase was preserved (Fig. 3), indicating that, like SQDG, PG is not essential for the bilayer formation, whereas the DGDG presence is fundamental. To confirm this hypothesis, the MGDG/DGDG/PG mixtures at ratios 100/48/17 and 100/37/25, corresponding, respectively, to an HII-

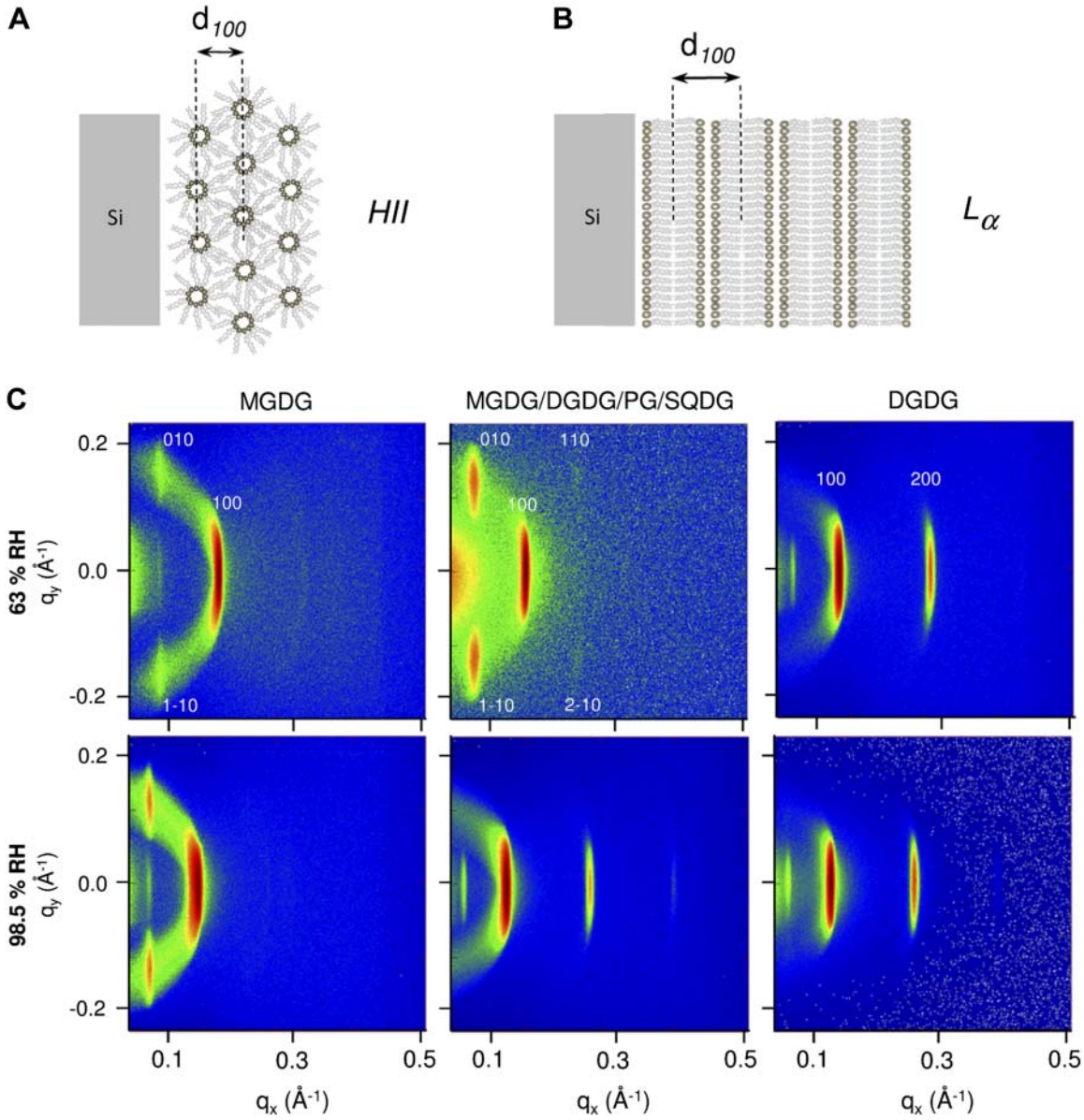

Figure 2. Organization and diffraction patterns of MGDG, DGDG, and the thylakoid-like lipid mixture. *A*) Scheme of lipid structure in hexagonal HII phase. Lipids organize in tubes (elongated reversed micelles) arranged on a 2-dimensional hexagonal array. *B*) Scheme of lipids organized in lamellar phase. *C*) Neutron diffraction images were obtained by integrating the intensity of a sample rocking scan in the horizontal plane. The broadening of the Bragg peaks along the q_y direction is due to the vertical slit collimation combined to the focusing of the beam to the sample in the vertical direction. From left to right: pure MGDG, the quaternary thylakoid-like lipid mixture, and pure DGDG at low humidity (top images at 63% RH) and high hydration (bottom images at 98.5% RH).

forming/L_α-forming lipid ratio of 100/65 and 100/62, were organized at high hydration as a 2-phase mixture of HII and L_α domains, consistent with freeze-fracture observations using total polar lipid extracts of chloroplast (23) or mixtures of MGDG and DGDG (51, 52), whereas the binary mixture MGDG/DGDG with an HII/L_α ratio of 100/60 was organized as a single L_α phase (Fig. 3).

These results show that the proportion of DGDG at a HII/L_α ratio around 100/60 is the main factor contributing to the lamellar organization of membranes made of thylakoid lipids at high hydration level, rather than the ratio of HII-forming *vs.* L_α-forming lipids. However, the results indicate that DGDG does not prevent the formation of an HII phase at low hydration.

Influence of lipid composition on membrane stacking

Within a stack of bilayers, the strength of the interactions between neighboring membranes was analyzed according to the evolution of membrane-to-membrane distances as a function of hydration. Membranes that are prone to be cohesive should stay close, whereas those having neutral or repulsive

TABLE 1. *Lipid composition of reconstituted lipid mixtures in reference to MGDG*

Parameter	Sample						
	MGDG	DGDG	Quaternary thylakoid-like	Ternary thylakoid-like	Ternary +25% DGDG −25% PG	Binary +25% DGDG	Ternary −25% DGDG +50% PG
Proportion of lipid (mol/mol/mol/mol)							
MGDG	100	0	100	100	100	100	100
DGDG	0	100	48	48	60	60	37
PG	0	0	17	17	12	0	25
SQDG	0	0	17	0	0	0	0
Ratio hexagonal/bilayer	100/0	0/100	100/82	100/65	100/72	100/60	100/62
Organization at low hydration	HII	L_α	HII	HII	HII	HII	HII
Organization at high hydration	HII	L_α	L_α	HII/L_α	L_α	L_α	HII/L_α

Each lipid class was quantified before pooling and after neutron experiment.

interactions should drift apart, resulting in an increased swelling of the stack. Neutron diffraction patterns were recorded at different hydrations in the following lipid mixtures, described in Table 1: pure DGDG; the binary + 25% DGDG mixture; and the ternary +25% DGDG −5% PG mixture and the quaternary thylakoid-like mixture; which will be called hereafter DGDG, MGDG/DGDG, MGDG/DGDG/PG, and MGDG/DGDG/PG/SQDG, respectively. To evaluate the interaction between lipid polar heads, we first measured the periodicity (d_{100}) of the HII and L_α phases (Fig. 4) as a function of hydration. The periodicities were then converted into bilayer separation distances (equivalent to water layer thickness) by subtracting the bilayer thickness. The thickness of the DGDG bilayer is known and relatively constant over a wide range of hydrations (4). To obtain the water layer thickness from the experimental lamellar periodicities, we used a DGDG bilayer thickness as calculated from a linear regression of the data reported previously (4) at nonzero hydration. The water layer thickness was then calculated by subtracting the bilayer thickness to the periodicity point by point. For polar lipid extracts from maize chloroplasts it was evaluated to be 38.7 Å (53). From these bilayer thicknesses and from the periodicity of the L_α phase, we determined the thickness of the water layers (d_w) as a function of the applied pressure to the bilayers (Π), as calculated from the relative humidity (36). Π represents a measure of the force per surface unit to remove hydration water from the bilayer surface. Thus, when the hydration decreases, Π increases. The same method, described in more detail in Materials and Methods, was applied to the HII phases observed with MGDG/DGDG, MGDG/DGDG/PG, and MGDG/DGDG/PG/SQDG mixtures at low hydration.

Once periodicities are extracted for both HII and L_α phases, the pressure-distance curves can be plotted (**Fig. 5**). We observed for DGDG the typical exponential decay of the pressure *vs.* water layer thickness as commonly observed with phospholipids, and known as the regime dominated by the hydration pressure (54). The thickness of the water layer

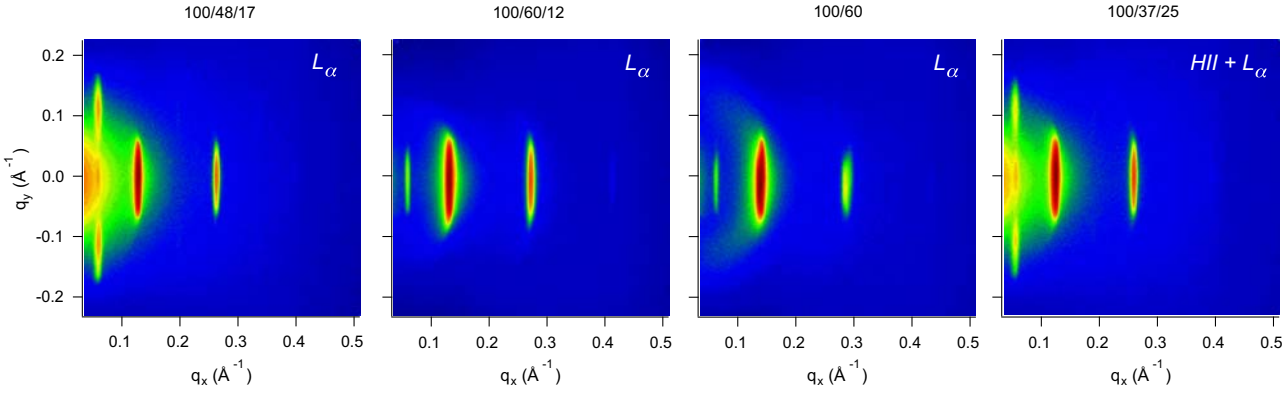

Figure 3. Organization and diffraction patterns at high hydration of different thylakoid lipid mixtures described in Table 1. Neutron diffraction images were obtained by integrating the intensity of a sample rocking scan in the horizontal plane. The broadening of the Bragg peaks along the q_y direction is due to the vertical focusing of the beam combined with the horizontal slit collimation. From left to right, lipid mixtures at RH 98.5% correspond to MGDG/DGDG/PG mixtures at ratios 100/48/17, 100/60/12, 100/60/0, and 100/37/25.

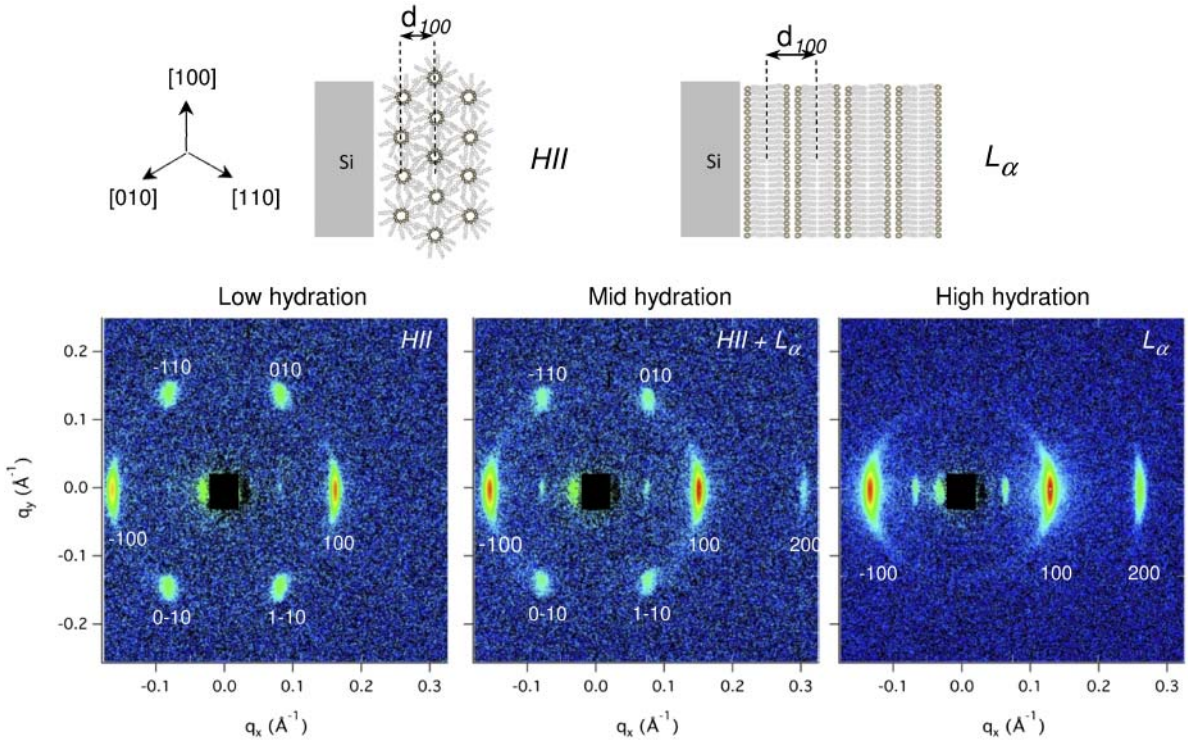

Figure 4. Small-angle diffraction patterns collected in pinhole geometry for the ternary +25% DGDG −5% PG mixture at different hydrations. Neutron diffraction images were obtained by integrating the intensity of a sample rocking scan in the horizontal plane. Bragg peaks are referenced with their (*hkl*) Miller indices. The lamellar spacing d_{100} is measured by a linear fitting of the (*h00*) peak positions as described in Materials and Methods.

remains rather small (<12 Å) for this uncharged glycolipid, compared to typical values of 25 Å observed for PC, known to behave as an uncharged phospholipid (55) despite its zwitterionic head group. This observation is consistent with a higher cohesive interaction between pure-DGDG membranes compared to pure-PC membranes. The water layer thickness is compatible with hydrogen bonding between DGDG galactose heads if some are extended toward bulk water, unlike those remaining oriented parallel to the plane of the bilayer, as described by McDaniel (37). The MGDG/DGDG, MGDG/DGDG/PG, and MGDG/DGDG/PG/SQDG mixtures also present the typical exponential decay of the pressure-distance curves. However, at high hydration, the increase of the water layer thickness vanishes and remains limited, as in the case of DGDG (<15 Å), contrasting with bilayers of charged lipid mixtures, where a water layer thickness of up to 150 Å was observed for plant phospholipids (53) and up to 600 Å for the anionic phospholipid dioleoyl phosphatidylserine in salt-free solution (56). At constant pressure, the presence of PG and SQDG is clearly responsible for the increase of the thickness of the water layers due to the presence of the electrostatic repulsion between these negatively charged polar heads. However, these negatively charged lipids do not seem to affect the swelling limit, indicating that the attractive interaction between DGDG polar heads dominates electrostatic repulsions between membranes.

Taken together, these results show that hydrogen bonding between DGDG polar heads dominate PG and SQDG electrostatic repulsions and represent a strong cohesive force.

DISCUSSION

Self-organization of thylakoid lipids into membrane structures capable of dramatic phase transitions

In this work, we show that the lipid composition of membranes reconstituted from thylakoid lipids can, on one hand, modulate the organization of the membranes by tuning the transition between bilayer and nonbilayer structures and, on the other hand, contribute to membrane stacking by exerting cohesive forces between distant polar headgroups when bilayers are formed. Our results indicate that the MGDG/DGDG ratio plays a determinant role in stabilizing lamellar thylakoid structures and in the reversible formation of HII domains inside lamellar structures as hypothesized previously (18).

What is the biological relevance of a reversible L_α-to-HII phase transition and on the coexistence of L_α and HII structure? We observed that this phase transition could be tuned by variations of the MGDG/DGDG balance. In addition, we observed that this phase transition was dependent on hydration, a condition that can simulate various biological

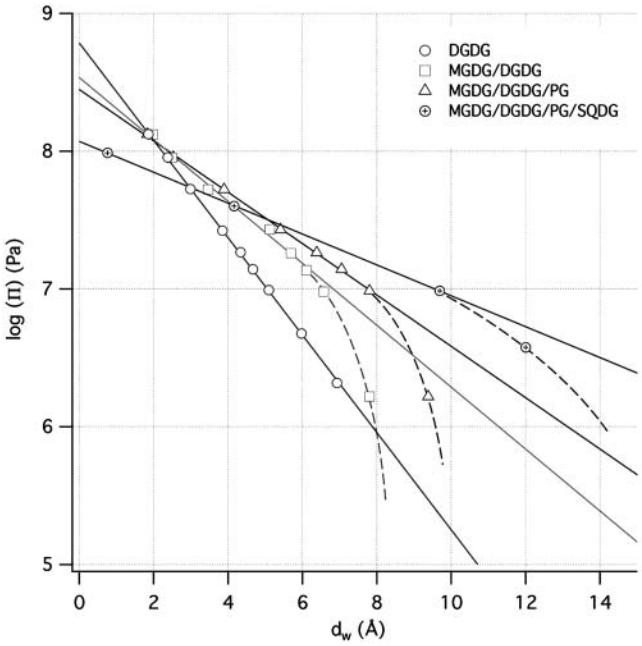

Figure 5. Pressure-distance curves obtained with 4 different lipid compositions: pure DGDG, a MGDG/DGDG (100/60) binary mixture, the MGDG/DGDG/PG (100/48/17) ternary mixture, and the quaternary MGDG/DGDG/PG (100/48/17/17) thylakoid-like lipid mixture. The solid lines are linear fits to the data in the linear range of the decay.

contexts, such as direct hydration changes, but also temperature changes or osmotic variations related to the local concentrations of macromolecules, colloids, proteins, ions, *etc.* The presence of an HII phase has been speculated in various physiological contexts or as rationales for the observation of suborganellar structures in plastids, although not demonstrated. First, because aqueous dispersions of chloroplast lipid extracts are able to form mixed L_α and HII phases (23), thylakoids are likely to contain domains of L_α and HII phase coexisting in the same membrane, with the HII MGDG-rich domains coexisting with the lamellar DGDG-rich domains. This HII/L_α coexistence is consistent with molecular processes occurring in photosynthetic membranes and would for instance explain the influence of MGDG on the violaxanthin de-epoxidase activity (57), involved in the nonphotochemical quenching of the photosynthesis (58), because this enzyme requires inverted hexagonal lipid structures for its activity (58).

HII phase-forming lipids are also possibly important for the prolamellar body formation in etioplasts. When plants are grown in the dark, plastids differentiate in etioplasts that contain a cubic membrane structure highly enriched in chlorophyll precursors (59, 60). Cubic structures are dependent of nonbilayer-forming lipids (61) and can be formed in an equimolar mixture of MGDG and DGDG (62). Prolamellar bodies contain a higher MGDG/DGDG ratio than thylakoids and envelope membranes (63) that might explain the transition from cubic to lamellar. The dynamic conversion of etioplasts into chloroplasts could operate, at least partly, *via* an HII-to-L_α lipid-phase transition, triggered by a MGDG/DGDG variation, and use the energy of this spontaneous phase transition as a driving force for this spectacular conversion of the plastid inner architecture. The appearance of HII phases might also be deleterious by disorganizing chloroplast membranes if HII phases are formed or stabilized inside the bilayer. Consistently with our results supporting that HII should appear on removal of water, when plants are exposed to dehydration, directly in drought stresses or indirectly on freezing, the MGDG/DGDG ratio is decreased either by increasing DGDG synthesis (64) or by consumption of MGDG by activating SFR2 (65, 66). This readjustment of the lipid composition of chloroplasts might therefore prevent the formation of HII phases and the bilayer rupture in these conditions, thus preserving the chloroplast integrity.

Our results comfort the theory (67) that cells adjust their membrane lipid composition in response to perturbations in order to maintain bilayer stability, but keeping the bilayer close to a point of instability, where a confined transformation to some nonbilayer structure would tend to occur (for a review, see ref. 68). HII/L_α coexistence in natural membrane and the regulation of MGDG/DGDG ratio might explain a lot of biological phenomena like the regulation of the membrane lipid/protein ratio (69), the stabilization of membrane proteins by increasing the membrane lateral pressure (70), or the adaptation to environmental stresses (frost, drought, or salt, as described above).

Contribution of a galactolipid zipper to membrane stacking and neutralization of repulsive forces

Spontaneous stacking of lipid membranes is observed in two major biological structures found, on one hand in animals, in myelin sheaths, and on the other hand, in plants in thylakoid grana. These two membrane systems contain glycolipids and are highly enriched in charged proteins, the myelin basic protein (71) and LHCII (7), that have been shown to be involved in membrane stacking. In both cases glycolipids are playing a determinant role in membrane stacking: in the myelin sheath around nerve cells, membrane stacking is in part mediated by Ca^{2+}-induced interactions between glycosylceramide and cerebroside sulfate (72–75); and in plant thylakoid model, membranes involve DGDG in such interactions (see refs. 16, 35, 76, 77 and the present work).

It is known that LHCII in plants, and phycobilisomes in cyanobacteria, control the thylakoid stacking, but they are not the only forces involved in this process (7). In this work, we tried to evaluate the contribution of glycolipids to the interactions between adjacent membranes, with a specific focus on thylakoid membrane stacking. We show that DGDG polar heads do interact together probably *via* hydrogen bonds. In the absence of bridging between membranes, additivity of the attractive van der Waals contribution and of the repulsive hydration force leads to an equilibrium distance be-

tween membranes at maximum swelling. The introduction of an additional attractive molecular interaction logically modifies this equilibrium to reach a smaller bilayer separation. In the present study, the hydration pressure is counterbalanced at much shorter membrane separation distances than with phospholipids, due to the additional attractive term brought by the hydrogen bonding. The thickness of the water layer remained <12 Å for DGDG, whereas a value of 25 Å is typically observed for PCs, known to behave as uncharged phospholipids (55). Cohesive interaction forces between pure DGDG membranes are therefore higher than those between pure PC membranes. This difference of behavior between PC and DGDG has some important biological consequences. Indeed, following phosphate shortage, it has been shown that DGDG is exported to nonplastid membranes to replace PC (78). This natural DGDG-to-PC substitution suggests an equivalence of both lipids for their bulk biophysical properties, including their role as bilayer-forming lipids. Nevertheless, PC does not cover the full range of the biophysical properties of DGDG, in particular as a membrane-to-membrane cohesive agent.

Furthermore, the electrostatic repulsion force brought by the addition of negatively charged lipids like PG or SQDG is not strong enough to overcome the attraction between bilayers because the swelling limit of these charged membranes (15 Å) remains below that of uncharged phospholipids (25 Å) (55). This prevention of electrostatic repulsion by DGDG is likely determined by the presence of a hexosyl polar head and adds to the remarkable properties of this thylakoid lipid.

The bilayer separation in the grana is ≥4.5 nm at the lumen side, due to the protrusion of photosystem II, and lies between 2 and 4 nm at the stroma side (79). Interestingly, the distance at the stroma side, but not at the lumen side, is consistent with DGDG polar head interactions as long as the digalactosyl heads are organized perpendicularly to the bilayer, because the fully extended DGDG polar head measures 1.8 nm (37). In addition, LHCII is not the only factor responsible for the stacking of thylakoids: cyanobacteria lacking phycobilisome present stacked thylakoids (34, 80), and the absence of trimeric LHCII does not suppress all grana (29, 30, 81). Thylakoid formation as stacked flattened cisternae is most likely due to protein contributions but, based on the present work, is also favored by the glycolipid environment. Taken together, our results show that DGDG can contribute to the formation of membrane-to-membrane cohesive interaction, acting as a galactolipid zipper.

CONCLUSIONS

In this work, we addressed the question of the biophysical features that could justify the conservation of thylakoid lipids throughout evolution, from cyanobacteria to chloroplasts. By reconstituting biomembranes made of natural lipids, we first observed that the thylakoid lipid mixture self-organized as a membrane bilayer, but that this structure was close to the reversible transition from the HII to the L_α phase. This shift and the coexistence of HII and L_α phases can be modulated by the fine tuning of the lipid profile, in particular the MGDG/DGDG ratio, or by hydration changes. This property can contribute to the highly dynamic flexibility of structure of the plastids, in some developmental contexts, like the etioplast-to-chloroplast transition, or in response to environmental variations. Rapid conversions between nonmembranous and membranous architectures could be driven by the energy generated during spontaneous phase changes.

Furthermore, our analyses highlight a critical role for galactolipids, and most importantly DGDG, as contributing components to the membrane stacking *via* hydrogen bonds between galactose polar heads of adjacent bilayers. This role is emphasized by the fact that DGDG cohesive interactions indirectly neutralize the electrostatic contribution of charged lipids like PG and SQDG and allow the persistence of regularly stacked membranes at high hydration despite negative charges harbored by a significant fraction of constituting lipids. This remarkable property of chloroplast galactolipids might be determinant in other membrane-ordered systems, such as glycolipid-rich myelin sheath.

In summary, we confirmed the properties of individual thylakoid galactoglycerolipid (or glycolipid) classes previously reported as HII- or L_α-forming lipids, but brought to light how these properties are subtly orchestrated in the matrix in which proteins are embedded, as contributing components for the elaboration of the architecture of photosynthetic membranes and its dynamics. The dissection of the roles of MGDG, DGDG, SQDG, and PG will be helpful to examine (and reexamine) chloroplast ultrastructure observations in various developmental, physiological, genomic, and environmental contexts. FJ

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