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Chloroplast membrane lipid remodeling protects against dehydration by limiting membrane fusion and distortion

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One sentence summary:

Galactolipid remodeling in chloroplast membranes alters both the extent and pattern of membrane fusion stalks to protect against cellular damage in response to dehydration.

W.M., K.J.H. and C.H. conceived the study. C.H. designed the study. C-P.C. performed the computer simulations and analyzed the data. C.-P.C. and C.H. wrote the initial draft of the manuscript. K.W. critically reviewed the manuscript and provided valuable inputs. All authors commented on the manuscript. C.H. agrees to serve as the author responsible for contact and ensures communication.

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Abstract

Dehydration damages the structural integrity of the chloroplast membrane and, consequently, the normal photosynthetic function of this organelle. Remodeling of galactolipids by converting monogalactosyl-diacylglycerol (MGDG) to digalactosyl-diacylglycerol (DGDG) and oligo-galactolipids is an effective adaptation strategy for protecting against dehydration damage to the chloroplast membrane. However, detailed molecular mechanisms are missing. In this study, by performing molecular-level simulations of bi-lamellar membranes under various dehydration conditions, we find that MGDG-to-DGDG remodeling protects the chloroplast membrane in a unique manner by simultaneously dictating both the extent and the pattern of fusion stalks formed with the apposed membrane. Specifically, MGDG-rich membranes form elongated stalks at a moderate dehydration level, whereas DGDG-rich membranes form smaller, rounded stalks. Simulations of wild-type and mutant Arabidopsis (*Arabidopsis thaliana*) outer chloroplast membranes further confirm that the mutant membrane without galactolipid remodeling is more susceptible to membrane fusion due to its higher MGDG content. Our work reveals the underlying physical mechanisms that govern the pattern and extent of membrane fusion structures, paving the way for rational genetic engineering of crops with improved dehydration tolerance.

Introduction

Plants encounter limited water resources during seasonal changes or under harsh environmental conditions, such as drought, high salt concentrations, and freezing conditions. Dehydration tolerance is a key determinant of plant natural habitats, and the yield and distribution of agricultural crops. This is of vital economic importance given the growing global population and environmental stresses caused by climate change. At the cellular level, dehydration damages plant cells by primarily causing the lipid membranes that enclose the plant cell and its intracellular organelles to become leaky, resulting in further loss of soluble cell contents in addition to other mechanisms (Zhu, 2002; Blum and Tuberosa, 2018). A direct correlation has been observed between the dehydration-induced increase in membrane permeability and the lamellar-to-hexagonal structural transformation of the lipid membrane (Luzzati, 1968; Simon, 1974). In the lamellar phase, lipids are arranged as a planar bilayer whereas the lipids are arranged into a hexagonal array of inverted cylindrical micelles in the hexagonal phase (Luzzati and Husson, 1962). It has been postulated that the plasma membrane and tonoplast in dry seeds are in the relatively porous hexagonal phase and thus do not act as effective barriers to prevent diffusion of cellular contents when their water content drops below 20% (Simon, 1974). Freezing conditions lead to severe dehydration due to formation of extracellular ice which results in a lower water chemical potential outside the cell (Steponkus, 1984). Liposomes composed of the total lipid mixture extracted from the plasma membranes of non-cold acclimated rye leaves showed formation of hexagonal phase (Cudd and Steponkus, 1988). Such dehydration-induced formation of the hexagonal phase occurs when the plant plasma membrane are brought into close contact and fuse with organelle membranes

(Uemura et al., 1995), and has been successfully modelled using molecular dynamics (MD) simulations (Marrink and Mark, 2004; López et al., 2013).

Many plant species have evolved mechanisms to cope with dehydration caused by environmental stresses. Arabidopsis (*Arabidopsis thaliana*) leaf membranes were found to be very tolerant to water loss under drought conditions (Gigon et al., 2004). Dehydration was found to trigger the increase of galactolipid digalactosyl-diacylglycerol (DGDG) at the expense of monogalactosyl-diacylglycerol (MGDG), both of which are major constituents of chloroplasts. This seems to be a common strategy employed by various plants to tolerate drought conditions, such as maize (Chen et al., 2018) and cowpea cultivar (Torres-Franklin et al., 2007). As the cylindrical DGDG prefers the lamellar phase whereas the conical MGDG prefers the hexagonal phase (Graham Shipley et al., 1973), the increase in the ratio of DGDG to MGDG was suggested to help maintain the chloroplast envelope membrane in the lamellar phase necessary for normal biological functions (Williams and Quinn, 1987). A study on the effects of chloroplast lipid composition on the stability of liposomes during freezing and drying found that MGDG was much more effective in inducing leakage during freezing than DGDG, and that MGDG was more destabilizing than egg phosphatidylethanolamine (PE), a conical phospholipid (Hincha et al., 1998). The same galactolipid remodeling strategy was also suggested to allow Arabidopsis to tolerate freezing conditions (Li et al., 2008; Du et al., 2010). A galactolipid remodeling enzyme has been identified to be encoded by the *SENSITIVE TO FREEZING 2 (SFR2)* gene in the wild-type plant, which converts MGDG to DGDG and oligogalactolipids, e.g., tri-galactosyl-diacylglycerol and tetra-galactosyl-diacylglycerol, in a processive manner under freeze treatment (Moellering et al., 2010).

In contrast, the *SFR2* loss-of-function mutant showed clear evidence of damage to chloroplast upon freezing treatment (Fourrier et al., 2008). In fact, *SFR2* protection goes beyond freezing tolerance as SFR2-like proteins have been widely found in land plants such as tomato, maize and rice that show no freezing tolerance (Fourrier et al., 2008). *Craterostigma plantagineum*, known as a resurrection plant due to its strong tolerance to drying, was found to activate DGDG synthase to convert MGDG to DGDG and SFR2 to convert DGDG to oligogalactolipids in response to desiccation (Gasulla et al., 2013). It was revealed that the SFR2-ortholog in tomato confers protection against salinity and drought stresses also through the remodeling of MGDG, which led to the proposal that lipid remodeling mediated by SFR2 is the first line of defense against cellular dehydration in plants in which SFR2 is expressed (Wang et al., 2016). It has been uncovered that SFR2 resides in the chloroplast outer envelope, however, it remains a mystery how it is activated by different types of treatments that lead to cellular dehydration. Although experimental results have suggested dehydration tolerance involves reducing non-lamellar phase MGDG and increasing lamellar phase DGDG and oligogalactolipids, the molecular mechanisms underlying this protective scheme via galactolipid remodeling in chloroplast outer envelope membrane remain unclear.

To gain insights into the fusion of chloroplast outer membrane with other plant organelle or plasma membrane during dehydration, we have set up a bi-lamellar membrane model system using coarse-grained (CG) lipid representations for MD simulations. In our model, a phosphatidylcholine (PC) lipid bilayer (representing organelle or plasma membrane) is placed close to a mixed galactolipid-phospholipid bilayer (representing chloroplast outer envelope membrane). The amount of water

molecules in the inter-membrane region is varied to resemble different dehydration levels. Our simulation results show that the membrane mixed with MGDG (i.e., MGDG-PC bilayer) exhibits more extensive membrane stalk formation with the PC bilayer than the one with DGDG (i.e., DGDG-PC bilayer) across a wide range of dehydration levels. More importantly, the fused regions in the MGDG-PC system form elongated patterns as compared to rounded patterns in the DGDG-PC system. Hence, the galactolipid remodeling from MGDG to DGDG confers higher tolerance to dehydration by reducing the extent of membrane fusion and having more rounded stalks, which may facilitate easy membrane separation upon rehydration that avoids permanent damages to the chloroplast. In addition, the stalk formation in the MGDG-PC system tends to cause substantial local bending of the membrane in the non-stalk regions, which may serve as a possible pathway to activate SFR2.

Results

MGDG-to-DGDG remodeling modulates both the abundance and pattern of inter-membrane stalks

As demonstrated in Fig. 1A, cellular dehydration forces the chloroplasts in individual mesophyll cells to get close to the plasma membrane of the cell due to the loss of cytoplasm. The chloroplast is surrounded by two layers of lipid bilayers, namely outer and inner chloroplast membranes, so the outer one is expected to interact directly with the plasma membrane because of dehydration. While the plasma membrane is primarily composed of phospholipids, the outer chloroplast membrane is rich in MGDG and DGDG. MGDG and DGDG contain one or two galactose residues as the headgroup, respectively, and 16- or 18-carbon long unsaturated fatty acid tails (Fig. 1B). The single galactose headgroup and two unsaturated tails give MGDG a conical

shape, thus causing MGDG to prefer packing in inverted micelles or hexagonal H_{II} structures like other lipids with small headgroups (Jouhet, 2013). In contrast, DGDG is more cylindrical because of its larger headgroup and prefers the lamellar structure like other cylindrical lipids such as PC. Hence, MGDG and DGDG lipids are expected to drive different behaviors during fusion of chloroplast outer envelope membrane with extraplastidic, phospholipid-dominant membranes. To understand the contributions of MGDG and DGDG to the initiation of dehydration-induced damage on the chloroplast outer envelope membrane, we construct bi-lamellar membrane systems by placing a phospholipid bilayer above a mixed galactolipid-phospholipid bilayer with a water layer of various thicknesses in between (Fig. 1, C and D). The upper bilayer consists of PC lipids which are the dominant phospholipids in the plasma membrane of many plants, such as spinach (*Spinacia oleracea*) (Block et al., 1983), and represents the extraplastidic membrane, while the lower one is composed of equal amounts of PC lipids and galactolipids (either MGDG or DGDG; Table 1) to mimic the chloroplast outer envelope membrane (Simon, 1974). In spinach chloroplast, C18:3 is the dominant type in both MGDG and DGDG (Block et al., 1983). Although the fraction of C18:3 in MGDG and DGDG is not clear for Arabidopsis chloroplasts, C18:3 is much more abundant than C16:3 among all the Arabidopsis chloroplast membrane lipids (Moellering et al., 2010). In our simulations, all lipids have two fatty acid tails with 4 CG particles in each tail. Due to the 4-to-1 mapping of carbon atoms to CG particles, the CG tail can represent both C16:3 or C18:3 tails.

For the system with MGDG in the lower bilayer, the two bilayers remain separated from each other when the initial inter-membrane separation is sufficiently large. As shown in Supplemental Fig. S1, no membrane stalk is formed between the two

bilayers when they are separated by water at a distance of about 4 nm (equivalent to a hydration level of 54 waters/lipid). The number of water molecules per lipid is calculated as the number of all-atom water molecules in the inter-membrane region divided by the number of lipids in the two leaflets facing the inter-membrane region. However, when the initial inter-membrane separation is reduced to about 2 nm at a hydration level of 22 waters/lipid, stable membrane stalks that connect the two bilayers are formed after equilibration (Fig. 1C). We observe that the formation of membrane stalks results in kinks in membrane profile as the lipids wrap around the enclosed water molecules. MGDG lipids preferentially reside at highly curved concave regions, confirming that MGDG lipids prefer the non-lamellar phase. In contrast, at the same hydration level, the mixed DGDG-PC bilayer remains flat and separated from the upper PC bilayer with an average separation of 1.72 nm (Fig. 1D). Our results confirm that MGDG has a destabilizing effect on the lamellar phase of the bilayer and promotes membrane fusion by initiating stalk formation.

More importantly, the MGDG- and DGDG-containing systems exhibit sharply different stalk patterns. As demonstrated in Fig. 2, we show how the steady-state membrane fusion stalk patterns in the inter-membrane region are obtained at a specific hydration level. For each system, the inter-membrane stalk pattern (if any) is monitored with time as the MD simulation proceeds. For the MGDG-containing system at a hydration level of 13 waters/lipid (Fig. 2A), it requires a long simulation of 4 μs to observe stabilization of the elongated stalk patterns. Those elongated stalks are formed through the merging of small rounded or slightly elliptical stalks and feature a constant width (~4 nm). In contrast, the stalks formed in the DGDG-containing system exhibit rounded patterns (Fig. 2C), and a shorter time of 2 μs is sufficient for the stabilization of the

rounded stalk patterns. From the steady-state snapshots, it appears that MGDG lipids show a clear preference to the boundaries of the elongated patterns (see the zoomed-in regions in Fig. 2A). Interestingly, they seem to be excluded at the rounded end region of elongated stalks (see the dashed ellipse within the zoomed-in region). DGDG lipids do not show clear preference to the boundaries of the rounded patterns and are more dispersed (see the zoomed-in region in Fig. 2C), further confirming that DGDG prefers a laminar membrane structure.

The pattern of inter-membrane stalks is dictated by the minimization of the system bending energy

The emergence of elongated or rounded stalk patterns in MGDG- and DGDG-containing systems can be explained by comparing the associated bending energy of the two lipid leaflets facing the inter-membrane region after either elongated or rounded stalks are formed. Similar to a lipid bilayer, the bending energy density of a lipid monolayer can be written as (Deserno, 2015):

$$e = \frac{1}{2}\kappa(2H - c_0)^2 + \bar{\kappa}K_G \quad (1)$$

where κ and $\bar{\kappa}$ are the bending and Gaussian curvature moduli, respectively. $H = (K_1 + K_2)/2$, $K_G = K_1K_2$ and c_0 are the mean curvature, the Gaussian curvature and the spontaneous curvature of the monolayer, respectively, where K_1 and K_2 are two principal curvatures. According to the Gauss-Bonnet theorem (Seifert, 1997), integrating the Gaussian curvature term over the entire surface composed of the two monolayers facing the inter-membrane region should lead to the same result no matter whether the stalks are in elongated or rounded patterns, since these two systems are topologically identical. Therefore, the Gaussian curvature term is excluded in our analysis. When a lipid monolayer patch forms elongated stalks, $K_{1,E} < 0$ and $K_{2,E} = 0$

(Fig. 2, B and D), so the bending energy density can be simplified as: $e_{elongated} = \kappa\left(K_{1,E} - c_0\right)^2/2$. When the same membrane patch forms rounded stalks, $K_{1,R} < 0$ and $K_{2,R} > 0$, so the bending energy density can be rewritten as: $e_{rounded} = \kappa\left(K_{1,R} + K_{2,R} - c_0\right)^2/2$. Considering that the two bilayers are separated from each other by the same distance in these two different configurations, $K_{1,E} = K_{1,R} = K_1$, so the difference in the bending energy density between these two types of configurations is given by:

$$\Delta e = e_{rounded} - e_{elongated} = \kappa K_{2,R}\left(K_1 + \frac{1}{2}K_{2,R} - c_0\right) \quad (2)$$

The values of K_1 and $K_{2,R}$ can be estimated based on the stalk structures observed in our simulations.

To estimate the principal curvatures, we first need to determine the pivotal planes of the membrane monolayers in the rounded stalk. The pivotal plane is defined as the position inside the monolayer (at a distance z_0 from the bilayer mid-plane) where the area/lipid is the same as the value on a flat bilayer and hence the area strain is zero (Wang and Deserno, 2015). According to previous studies (Wang and Deserno, 2015), z_0 is taken to be $2l/3$, where l is the length of a lipid. The radius of curvature corresponding to K_1 can then be calculated by: $2R_1 = d + 2(l - z_0)$, where $K_1 = 1/R_1$ and d is the water layer thickness (Supplemental Fig. S2A). Similarly, the radius of curvature that corresponds to the other principal curvature is given by: $2R_2 = w - 2(l - z_0)$, where w is the width (narrowest cross-section) of a stalk and $K_2 = 1/R_2$. The bilayer thickness is determined to be about 3.6 nm based on head-group PO_4 particle positions (from PC lipids) from the last 300 ns of DGDG 12 waters/lipid simulation data. As we have a mixture of PC and MGDG or PC and DGDG lipids in our systems sharing the same fatty acid tails, we take l to be an averaged lipid length of 1.8 nm.

The average water thickness d is about 1.83 nm from the MGDG 13 waters/lipid simulation and about 1.45 nm from the DGDG 12 waters/lipid simulation. The rounded stalk width w is estimated to be about 4 nm (Supplemental Fig. S2B) from DGDG 12 waters/lipid simulation. The estimated K_1 and K_2 values for MGDG and DGDG lipids are shown in Table 2.

For MGDG lipids, the spontaneous curvature, c_0 is negative as the lipids have a conical shape due to its small head-group and hence prefers to occupy the concave leaflet of a bent lipid bilayer membrane. Although to our knowledge the spontaneous curvature of MGDG has not been determined, the area/lipid of MGDG (estimated as 0.63 nm^2 at 310 K in the lamellar phase from our simulation) is close to that of dioleoylphosphatidylethanolamine (DOPE), another conical shaped lipid with small head-group, determined by either experiments (0.65 nm^2 at 293 K) (Boden and Sixl, 1986) or CG-MD simulations (0.67 nm^2 at 303 K) (Orsi and Essex, 2012). The area/lipid of MGDG (with 86% C18:3 tails) in the hexagonal phase has been determined by experiments to be 0.49 nm^2 at 293 K (Graham Shipley et al., 1973), which is very close to that of DOPE also in the hexagonal phase (0.496 nm^2 at 293 K) (Leikin et al., 1996). Considering that the tails of MGDG contain more unsaturated bonds than those of DOPE, it is reasonable to assume that c_0 of MGDG is comparable to or more negative than that of DOPE ($c_0 = -0.35$ nm^{-1} taken from (Leikin et al., 1996)). Substituting our estimated values (Table 2) into Eqn. (2) leads to $\Delta e > 0$, which explains why MGDG lipids prefer to form elongated stalks and are excluded from the two ends of elongated stalks. On the other hand, the cylindrical shape of DGDG lipids suggests $c_0 \approx 0$, so Eqn. (2) simplifies to $\Delta e = \kappa K_{2,R}\left(K_1 + 0.5K_{2,R}\right)$. As $K_1 \approx -K_{2,R} < 0$ (Table 2), $\Delta e < 0$, indicating that it is more energetically favorable for DGDG lipids to

form rounded stalks. Our energetic analysis agrees with the observation from our simulations that MGDG prefers to populate along elongated stalks rather than rounded stalks, whereas DGDG prefers to populate the surface of rounded stalks.

The effect of dehydration level on inter-membrane stalk formation

To further investigate the effect of the dehydration level on the inter-membrane stalk formation, we have systematically reduced the amount of water molecules between the two bilayers and performed independent simulations at each hydration level. Figure 3A, B shows the steady-state simulation snapshots of the bi-lamellar systems at different hydration levels. At the highest hydration level of 24 waters/lipid, the stalks are continuous across the periodic boundaries, forming a single elongated stalk (Fig. 3A). This is the same for the hydration level of 17 waters/lipid though the stalk is “U”-shaped. However, at lower hydration levels, the single stalk is “broken” into a collection of elongated stalks of various lengths. The elongated stalk patterns enclose water channels, akin to the patterns observed in a bi-lamellar DOPE (conical phospholipid) system at low hydration levels (Marrink and Mark, 2004). The width of the elongated stalks seems to have a similar width (~4 nm) across the different hydration levels. On the other hand, only rounded stalks are observed in the steady-state patterns for the DGDG-containing systems and the number or density increases with the decrease in hydration level (Fig. 3B). The diameter of the rounded stalks is similar to the width of the elongated stalks in the MGDG-containing systems, as both are structures consisting of a single type of fatty acid tails (C18:3) that are packed against each other within the stalk.

The total area occupied by lipid stalks in the inter-membrane region increases with the decrease in the hydration level for both systems (Fig. 3C). However, the area fraction of the stalks in the MGDG-containing system is consistently higher than that in the DGDG-containing system under the same dehydration level (Supplemental Fig. S3 shows that values for lowest hydration levels of MGDG reached steady state). The area fraction of the stalks is around 30% for the MGDG-containing system at the hydration level of about 17 waters/lipid whereas that of the DGDG-containing system becomes close to zero. The sharp increase in the area fraction of stalks with the reduction in the hydration level seems to akin to a thermodynamic phase transition behavior, with the transition hydration level being about 26 waters/lipid for the MGDG-containing systems and 15 waters/lipid for the DGDG-containing systems. The much lower transition hydration level of the DGDG-containing systems further confirms that they are much more tolerant to stalk formation under dehydration.

For the DGDG-containing systems, although the stalks increase greatly in number as the dehydration level increases, the diameter of the rounded patterns remains roughly the same and is consistent with the width of the elongated patterns formed in the MGDG-containing systems (Fig. 3D). In contrast to the DGDG-containing systems which features stalks with small aspect ratios between 1.0 and 1.5, a mixture of short and long elongated stalks are formed in the MGDG-containing systems at low hydration levels, rendering a large variation in aspect ratios. At high hydration levels (17 and 24 waters/lipid), a single long stalk is observed in the MGDG-containing systems. The aspect ratio of the stalk decreases when the hydration level further increases, and the reason is because the higher water content limits the extent of membrane fusion.

The quantitative relationship between the hydration level and the amount of membrane stalks formed can be predicted based on a geometric consideration of the volume conservation of water molecules in the inter-membrane region before and after stalk formation (Fig. 4A). Prior to stalk formation, the two membranes are separated by a water layer of thickness d_0 which depends on the hydration level *h* (see Supplemental Note S1 for more details):

$$d_0 = \frac{2v_0 h}{a_0} \quad (3)$$

where a_0 and v_0 are the average area per lipid and the effective volume occupied by each water molecule, respectively, and *h* has a unit of number of water molecules per lipid. This linear relationship is consistent with our simulation data without stalks (dashed lines in Fig. 4B). At low hydration levels, the thin water layer allows contact between the membranes under thermal undulation, facilitating membrane fusion via stalk formation. Upon stalk formation, lipids migrate from the lamellar membrane into the stalk, which results in a shrinkage of effective membrane area (Fig. 4C) and facilitates the inter-membrane water layer to increase in thickness (Fig. 4B). For the DGDG-containing system, the number of rounded stalks gradually increases with the decrease in hydration level, leading to a gradual decrease in the membrane area. However, the MGDG-containing system undergoes a phase transition-like change when the hydration level is reduced from 28 to 24 waters/lipid. More importantly, the stalk formation in MGDG-containing system results in substantial membrane bending in the non-stalk regions, which further facilitates the increase in inter-membrane water layer thickness in non-stalk regions and the reduction in membrane area. As more stalks are formed at lower hydration levels (from 24 to 13 waters/lipid), although the membrane area tends to decrease as a result of stalk formation, the membrane bending level is also reduced, which plays a more dominant role and leads to a slight

increase in the system area. We postulate that when the thickness *d* is larger than a critical value d_c, no additional stalks would form, otherwise more stalks would form. Indeed, we observe that the thickness of the water layer in the inter-membrane region tends to increase and stabilize at similar heights after stalk formation regardless of the hydration level (Fig. 4B).

Assuming that water is incompressible, the area fraction occupied by stalks in the inter-membrane region in the steady state can be derived based on the volume conservation of water in the inter-membrane region (see derivations in Supplemental Note S1):

$$\frac{A_s}{A} = 1 - \frac{A_0 d_0}{A d_c} \tag{4}$$

where A_0, A and A_s are the initial membrane area prior to stalk formation, the membrane area after stalk formation and the area occupied by stalks, respectively. Combining Eqns. (3) and (4) leads to a prediction of the fractional area of stalks as a function of the hydration level and allows us to predict the critical water layer thickness by comparing the theoretical predictions with our simulation results. The predicted d_c values are 2.22 nm and 1.26 nm for MGDG-containing and DGDG-containing systems, respectively, which are close to the expected value from our simulation data (Fig. 4D). The values agree with our expectation of a larger value for MGDG-containing systems.

Molecular simulations of freezing tolerant wild-type and mutant Arabidopsis chloroplast envelope membranes

To further verify our mechanistic understanding under a more physiologically relevant condition, we have extended our bi-lamellar model systems to simulate the response

of the chloroplast outer envelope membrane of Arabidopsis under freezing condition. Moellering *et al*. reported that the freezing tolerant wild-type ("WT-FT") Arabidopsis has a reduced MGDG content and increased DGDG content after freeze treatment (Moellering et al., 2010). This is facilitated by the activity of galactolipid:galactolipid galactosyltransferase, which also converts DGDG to oligogalactolipids with three or more sugar residues in the head-group, including tri-galactosyl-diacylglycerol (TGDG) and tetra-galactosyl-diacylglycerol (TeDG). A *sfr2*-mutant strain ("mutant-FT") lacking this enzyme showed no change in the MGDG content but a similar increase in the DGDG content upon freeze treatment. The mutant plant is less freezing-tolerant and showed extensive damage with ruptured chloroplasts after freezing recovery (Moellering et al., 2010).

We have constructed models for the mutant-FT and WT-FT chloroplast outer envelope membranes as shown in Fig. 5, with the latter containing TGDG and TeDG. The coarse-grained lipid models for TGDG and TeDG are constructed by adding additional sugar groups to the DGDG template. Although the galactose linkage in DGDG produced by DGDG synthase DGD1 or DFD2 is in α- form while that produced by SFR2 (and further oligogalactolipids) is in β- form (Moellering and Benning, 2011), our coarse-grained model do not distinguish between the two forms as the two glucose units are linked with a single bond. The type of galactose linkage does not affect the cylindrical shape of DGDG (and higher oligogalactolipids). For TGDG, a sugar residual consisting of GC1, GC2 and GC3 particles (linearly connected) is linked to GA3 in DGDG via GC1 (Fig. 5A). Similarly, TeDG is constructed by linking another sugar residual consisting of GD1, GD2 and GD3 particles (linearly connected) to GC3 in TeDG via GD1. The mutant-FT system contains 7% more MGDG and 3% less PC

than the WT-FT system (Table 1), whereas the WT-FT system contains a minor fraction of TGDG and TeDG lipids with the mole percentage (mol %) taken from (Moellering et al., 2010). As the mol % for TGDG and TeDG is only about 0.1% for mutant-FT membrane, they are omitted in our model. For both WT-FT and mutant-FT models, minor lipids, including C16:0/18:3 and C16:1/18:3 phosphatidylglycerols (PG) and C16:0/18:3 sulfoquinovosyl diacylglycerols (SQDG), are also included (Block et al., Journal of Biological Chemistry 1983) (Table 1 and Fig. 5B).

Similar to the MGDG-containing and DGDG-containing systems, we have placed a PC bilayer membrane atop either the mutant-FT or WT-FT membrane with a water gap of a few nm (Fig. 5C and D). At the hydration level of 21-22 waters/lipid, elongated fusion stalks appear between the two bilayers in both the mutant-FT and WT-FT systems within 1 μs. The fraction of inter-membrane area occupied by stalks is similar between mutant-FT and WT-FT systems at about 22-24%. However, at the higher hydration level of 33-34 waters/lipid, the WT-FT system showed no stalk formation within extended simulation of 1.5 μs, whereas stalks formed within 1 μs in the mutant-FT system. To further investigate the effect of each minor lipid species in the dehydration stability of the model chloroplast outer envelope membrane, we have simulated several variants of the WT-FT membrane (see Table 3). As shown in Table 3 and Supplemental Figure S4, both Variant 1 (no TGDG/TeDG/SQDG/PG) and Variant 2 (with TGDG/TeDG) showed no fusion within 1 μs at the inter-membrane hydration level of 22 waters/lipid, whereas Variant 3 (with SQDG), Variant 4 (with PG) and Variant 5 (with SQDG/PG) showed fusion with similar elongated fusion stalks as WT-FT. This suggests that introducing PG and SQDG lipids in the WT-FT membrane model reduced membrane stability against dehydration, even in the presence of

TGDG and TeDG with large, polar head-groups. As shown in both Fig. 5C-D and Fig. S4, SQDG lipids showed strong tendency to colocalize with MGDG lipids along elongated stalks, while colocalization is observed for PG lipids, suggesting that SQDG lipids promote membrane stalk formation more substantially than PG lipids. In summary, our results confirm that galactolipid remodeling in the WT-FT Arabidopsis chloroplast envelope membrane hinders inter-membrane stalk formation due to the reduction in MGDG content, and the minor lipids can affect the critical hydration level that leads to stalk formation.

Discussion

Galactolipid remodeling strategy has been employed by Arabidopsis and many other plants of great value to confer tolerance to water loss due to freezing, drought or high salt content. This strategy involves conversion of non-lamellar-phase MGDG to lamellar-phase DGDG (and also oligo-galactolipids) in the chloroplast outer membrane by an enzyme encoded by the *SFR2* gene, which prevents formation of the non-lamellar phase (Moellering et al., 2010; Moellering and Benning, 2011). To understand the molecular mechanisms underlying galactolipid remodeling as a protection strategy against dehydration, in this study, we have performed systematic MD simulations to interrogate how the MGDG-to-DGDG conversion mediates the interaction of the chloroplast outer membrane with an apposed non-chloroplast phospholipid membrane. In our bi-lamellar membrane system, one bilayer is composed of PC phospholipids and mimics the plasma membrane or other non-plastid organelle membrane. For the other bilayer that represents the chloroplast outer membrane, we have considered both the simplified chloroplast membranes with a mixture of 50% MGDG or DGDG and 50% PC phospholipid, and a more sophisticated

model with mixed MGDG-DGDG-phospholipid membranes representing wild-type and mutant freeze-treated Arabidopsis chloroplast outer membranes. Our results show that, under dehydration, membranes with a higher MGDG content tend to form more extensive membrane stalks that form elongated patterns, whereas DGDG not only limits the stalk formation but also switches the stalk pattern from elongated to rounded. We understand that fusion between chloroplast membranes may also occur, where both membranes contain 50% or more MGDG/DGDG (80% galactolipid content in inner membrane vs 50% in outer membrane (Block et al., 1983)). Our results suggest that the extent of membrane fusion would be even more severe under dehydration because of their high MGDG contents.

Our energetics analyses suggest that the preference of an elongated or rounded pattern by MGDG and DGDG is determined by the minimization of the system bending energy. Because of its conical shape, MGDG features a negative spontaneous curvature, which makes it more energetically favored to form elongated stalks with a negative mean curvature. In contrast, DGDG features a cylindrical shape and therefore a nearly zero spontaneous curvature, which makes it more energetically favored to form rounded stalks with a saddle-shaped surface (i.e., zero mean curvature). In addition, we have developed a mechanistic model to understand the effect of the hydration level on the membrane stalk formation. We assume that stalks would form when the thickness of the water layer in the inter-membrane region is lower than a crucial value d_c. As a result of stalk formation, the thickness of the water layer increases, and no additional stalks would form when it reaches the critical value. By fitting our model to simulation data, we have confirmed that the MGDG-containing system features a much larger d_c value than the DGDG-containing system. The model

estimates agree reasonably well with the observed water layer thicknesses in our simulations. This implies that a thicker inter-membrane water layer is required to separate the two membranes to prevent further stalk formation in MGDG-containing systems, indicating its higher stalk formation propensity due to its conical shape as compared to cylindrical-shaped DGDG.

We further confirm the mitigative effect of chloroplast membrane lipid remodeling on membrane stalk formation with more physiologically relevant membrane models, in which oligo-galactolipids and other minor lipids, including TGDG, TeDG, PG and SQDG, are included. Our simulations also demonstrate that the presence of minor lipids may alter the critical dehydration level that leads to stalk formation. As expected, no membrane stalk is observed in the variant with oligo-galacotolipids. The presence of oligo-galactolipids also increases the average thickness of the head-group region and thus increases the local density of hydroxyl groups which enhances the repulsive hydration force between opposed bilayers during dehydration (Wolfe and Bryant, 1999; Moellering et al., 2010). However, the presence of PG and SQDG tends to promote stalk formation. SQDG show more substantial colocalization with MGDG along the stalks, suggesting they are more conical than PG. In addition to the schemes identified by our simulations, galactolipid remodeling may also reduce membrane fusion by modulating the membrane tension. As multiple MGDGs are converted into one oligo-galactolipid, the membrane is expected to shrink, which may elevate the membrane tension and prevent the initiation of membrane stalk formation by suppressing membrane undulations (Markosyan et al., 1999).

The regulation of the abundance and the pattern of the inter-membrane stalks by the MGDG-to-DGDG conversion may protect the normal function of plant chloroplast membrane in multiple ways. First of all, both the reduced extent of membrane fusion and the smaller individual stalk structure facilitated by MGDG-to-DGDG remodeling allow the two membranes to be able to separate completely from each other upon rehydration, preventing irreversible damage to the chloroplast outer envelope membrane. In addition, compared with rounded stalks, formation of elongated stalks is accompanied by more severe structural disturbance to the membranes, causing them to bend and enclose cylindrical water channels. We hence speculate that the severe structural disturbance might affect the structure and normal function of transmembrane proteins in the chloroplast outer envelope membrane. Examples of transport proteins include the Tic/Toc supercomplex for protein translocation across the outer and inner chloroplast membranes into the stroma (Li and Chiu, 2010), the outer envelope porin (OEP) family of transporting channels that export amines and amino acids, carbohydrates and ATP synthesized in chloroplasts (Pottosin and Shabala, 2016), and trigalactosyl-diacylglycerol TGD4 protein that is involved in endoplasmic reticulum-to-chloroplast transport of phosphatidic acid (PA) through the outer envelope membrane (Wang et al., 2012). Any alteration of the membrane structure, such as an increase in membrane curvature as a result of the elongated stalk formation under dehydration, may affect the conformation and proper function of these important transmembrane proteins that may remain impaired upon rehydration. More importantly, the membrane bending caused by the stalk formation between the inner and outer chloroplast membranes may regulate the opening/closure of mechanosensitive ion channels and lead to leakage of ions from the stroma (Haswell and Meyerowitz, 2006; Clausen et al., 2017). Leakage of Mg2+ from the stroma during

freezing has been proposed to cause activation of SFR2 as a protective mechanism against dehydration (Barnes et al., 2016). Our study suggests that membrane fusion between sections of inner and outer chloroplast membranes might provide a pathway to realize this protective mechanism.

As a side effect of stalk formation, we have observed that stalk formation facilitates lipid exchange between lower and upper membranes (see Supplemental Note S2 for detailed discussion), which could function as an important lipid trafficking pathway in complementary to vesicular and other non-vesicular lipid transport pathways (Jackson et al., 2016). For instance, MGDG synthesized on the inner envelope membrane by MGDG synthase MGD1 needs to be transported to the outer envelope membrane as substrate for DGDG synthase DGD1 (Froehlich et al., 2001). The transfer of MGDG (and also DGDG) across the two membranes has been postulated to involve membrane fusion mediated by the binding of the (hydrophobic) N-terminal domain of DGD1 to PA in the membranes which lead to local aggregation of the non-lamellar phase PA lipid (Kelly et al., 2016). We speculate that local increase in MGDG density in the inner envelope membrane might also contribute to increasing the propensity for inter-membrane fusion when the inter-membrane gap gets closer during membrane undulations.

Conclusions

Remodeling of galactolipids in chloroplast membranes, via conversion of conical-shaped MGDG to cylindrical-shaped DGDG and higher order oligogalactolipids, was identified as a protective mechanism against cellular dehydration in plants. This study reveals the detailed molecular-level mechanisms through molecular-level simulations

of bi-lamellar membranes under various dehydration conditions. Our simulation results show that MGDG-to-DGDG remodeling regulates not only the severity of the membrane stalk formation, but also the pattern of the formed membrane stalks. More specifically, MGDG-containing membranes fuse more extensively with apposed phospholipid membranes at moderate dehydration levels via formation of elongated fusion stalks, whereas DGDG-containing membranes fuse less extensively with formation of small, rounded stalks. The reduced abundance along with its small, rounded stalks may facilitate easy membrane separation upon rehydration and avoid permanent damages to the chloroplast. Similarly, the higher MGDG content in mutant freeze-treated Arabidopsis chloroplast outer envelope membranes also produced more extensive, elongated membrane stalks during inter-membrane fusion. The formation of elongated stalks causes bending of the two apposed membranes, which may affect the structure and function of any transmembrane proteins in the outer chloroplast membrane such as Toc, a translocon, and possibly disrupts transport of material into the chloroplast even after rehydration. This suggests that through reducing MGDG content, galactolipid remodeling confers tolerance to dehydration via limiting the extent of inter-membrane fusion between chloroplast membranes and other extraplastidic membranes. As the gene encoding galactolipid remodeling enzyme has also been discovered in other land plants such as tomato, galactolipid remodeling is probably a common strategy employed by plants as a first line of defense against cellular dehydration brought about by environmental stresses in general. A clearer molecular-level understanding may thus facilitate further exploitation of this strategy to protect other crops of vital importance to humankind against environmental stresses.

Materials and Methods

Molecular dynamics simulations of bi-lamellar membrane systems

To study the effect of hydration on the fusion of phospholipid membrane with mixed galactolipid-phospholipid membrane, we constructed model bilayers and stacked them atop each other with a certain separation to create a bi-lamellar system. In order to simulate a sufficiently large system for microseconds, we adopted a coarse-grained lipid model based on the MARTINI force-field version 2.2 and used GROMACS version 2018.2 MD software for our simulations (Pronk et al., 2013; Abraham et al., 2015; Páll et al., 2015). The MARTINI parameters for MGDG and DGDG were taken from López *et al*. (López et al., 2013). In the MARTINI model, about four heavy atoms and associated hydrogens are grouped into one bead (Marrink et al., 2007), with the 18 carbon atoms along the unsaturated fatty acid tail mapping to four particles (Fig. 1B). The initial configurations and simulation set-up files for the lipid bilayers were generated using the Martini Bilayer Maker (Qi et al., 2015) within the CHARMM-GUI web-based platform (Jo et al., 2008). The simulation box was 28 nm in both length and width, with 2014 di-C18:3 PC lipids in the phospholipid bilayer. The lipid compositions of the galactolipid-phospholipid bilayer adopted in this study were listed in Table 1. The two bilayers were then placed atop each other with a certain separation gap and the remaining space in the simulation box was filled with water particles (Fig. 1C). The bi-lamellar systems with standard hydration levels (e.g. 24 waters/lipid for system with MGDG-PC bilayer) were simulated for 1 μs and the resultant configuration taken as the starting configuration for dehydration studies (except for system with MGDG-PC bilayer where the configuration at 0.5 μs before fusion occurs was taken instead). Various hydration levels were investigated by varying the waters/lipid in the

589 inter-membrane region. Each system with reduced inter-membrane hydration level
590 was simulated for between 1 to 4 μs.
591
592 For each bi-lamellar system, after energy minimization steps, MD simulations were
593 carried out with progressively reduced restraints on lipid head-group positions as the
594 simulation time-step was increased from 2 to 10 or 15 fs. For stability, the maximum
595 time-step for the MGDG-containing systems was set at 10 fs whereas that for the
596 DGDG-containing systems was set to 15 fs. Unrestrained simulations were then
597 carried out for 1-4 μs to obtain the steady-state configuration. Electrostatic interactions
598 were computed using Reaction-Field method with dielectric constant of 15 and cut-off
599 distance of 1.1 nm. Van der Waals interactions were computed using cut-off method
600 with the same distance of 1.1 nm. System temperature was maintained at 310 K with
601 the Velocity-rescale thermostat, whereas pressure was maintained at 1 bar using the
602 Parrinello-Rahman barostat with semi-isotropic coupling (X-Y plane coupled
603 separately from the Z or bilayer normal direction) with time-constant of 12 ps and
604 compressibility of 3×10^{-4} bar^{-1}.
605
606 **Quantification of the area and aspect ratio of membrane stalks**
607 We have computed the area and aspect ratio of the membrane stalks using Fiji Particle
608 Analyzer tool (Schindelin et al., 2012). Each color image of the stalks (for example,
609 from Fig. 3, A and B) was turned into an 8-bit binary image before running the tool. A
610 minimum area of 300 pixels-squared was used to avoid including very small features
611 due to noise. The area fraction of stalks was automatically computed together with the
612 areas of each feature detected. The aspect ratios for rounded features/stalks were
613 taken from directly from estimates by Particle Analyzer. For the elongated features,

we have estimated their aspect ratios by assuming that each elongated feature is composed of a long rectangular region capped by semi-circles with diameter taken from the average diameter (about 4 nm) of the smallest, rounded features in DGDG-containing system.

Supplemental Data

Supplemental Note S1: Derivation of the relationship between hydration level and fraction of membrane area occupied by membrane stalks.

Supplemental Note S2: Stalk formation facilitates the lipid exchange between the two bilayers.

Supplemental Figure S1. Molecular dynamics simulation of a bi-lamellar membrane system comprising a phosphatidylcholine (PC) upper bilayer membrane and a mixed monogalactosyl-diacylglycerol (MGDG)-PC (50:50) lower lipid bilayer.

Supplemental Figure S2. Estimation of the principal curvatures for monolayer in rounded stalks formed by monogalactosyl-diacylglycerol (MGDG) or digalactosyl-diacylglycerol (DGDG).

Supplemental Figure S3. Time evolution of the percentage area of inter-membrane region occupied by lipid stalks for monogalactosyl-diacylglycerol (MGDG)-containing systems at 13 and 15 waters/lipid (W/L).

Supplemental Figure S4. Effect of the minor lipid species on membrane stalk formation in systems containing Arabidopsis wild-type freeze-treated (WT-FT) chloroplast outer envelope membrane model and its variants.

Supplemental Figure S5. Time evolution of the fraction of (A) monogalactosyl-diacylglycerol (MGDG) and (B) digalactosyl-diacylglycerol (DGDG) lipids in the upper bilayer for MGDG- and DGDG-containing systems, respectively, at different hydration levels indicated by waters/lipid (W/L).

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Tables

Table 1. Lipid compositions (mol %) of chloroplast outer envelope membrane models. The total numbers of lipids for MDGG-PC, DGDG-PC, wild-type freeze-treated (WT-FT) and Mutant-FT membranes are 2196, 2004, 2134 and 2160, respectively.

Lipid type	MGDG-PC	DGDG-PC	WT-FT	Mutant-FT
di-C18:3 MGDG	50	-	33*	40*
di-C18:3 DGDG	-	50	25*	25*
di-C18:3 TGDG	-	-	1.5*	-
di-C18:3 TeDG	-	-	0.5*	-
C16:0/18:3 SQDG	-	-	5#	4#
di-C18:3 PC	50	50	27	24
C16:0/18:3 PG	-	-	4#	3.5#
C16:1/18:3 PG	-	-	4#	3.5#

*(Moellering et al., 2010); # (Block et al., 1983); MGDG: monogalactosyl-diacylglycerol; DGDG: digalactosyl-diacylglycerol; TGDG: tri-galactosyl-diacylglycerol; TeDG: tetra-galactosyl-diacylglycerol; SQDG: sulfoquinovosyl diacylglycerols; PC: phosphatidylcholine; PG: phosphatidylglycerols

Table 2. Estimated principal curvatures for the monolayer in rounded stalks formed by monogalactosyl-diacylglycerol (MGDG) or digalactosyl-diacylglycerol (DGDG).

Principal curvature	**MGDG**	**DGDG**
$K_{1,R} = K_1$ (nm^{-1})	−0.66	−0.75
$K_{2,R}$ (nm^{-1})	0.71	0.71

Table 3. Lipid compositions (mol %) of Arabidopsis wild-type freeze-treated (WT-FT) chloroplast outer envelope membrane model and variants. All systems consist of 2134 lipids in total.

Lipid type	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	WT-FT
di-C18:3 MGDG	33	33	33	33	33	33*
di-C18:3 DGDG	25	25	25	25	25	25*
di-C18:3 TGDG	-	1.5	-	-	-	1.5*
di-C18:3 TeDG	-	0.5	-	-	-	0.5*
C16:0/18:3 SQDG	-	-	5	-	5	5#
di-C18:3 PC	42	40	37	34	29	27
C16:0/18:3 PG	-	-	-	4	4	4#
C16:1/18:3 PG	-	-	-	4	4	4#
Fusion?	N	N	Y	Y	Y	Y

*(Moellering et al., 2010); # (Block et al., 1983); MGDG: monogalactosyl-diacylglycerol; DGDG: digalactosyl-diacylglycerol; TGDG: tri-galactosyl-diacylglycerol; TeDG: tetra-galactosyl-diacylglycerol; SQDG: sulfoquinovosyl diacylglycerols; PC: phosphatidylcholine; PG: phosphatidylglycerols

Figure legends

Figure 1. Molecular dynamics simulations of bi-lamellar membranes with one of the membranes containing monogalactosyl-diacylglycerol (MGDG) or digalactosyl-diacylglycerol (DGDG) glycolipids. A, Schematic illustration of cellular dehydration-induced interaction between chloroplast outer membrane and plasma membrane of the cell. B, Chemical structures and coarse-grained (CG) models of MGDG and DGDG lipids with two C18:3 fatty acid tails. CG particles are labelled and colored with the glycolipid head-groups as red, the glycerol groups as pink and the fatty acid tails as cyan. For the tails, "D" particles incorporate double-bonded carbon atoms whereas "C" particles incorporate only single-bond carbon atoms. Both MGDG and DGDG models share the same fatty acid tails. C and D**,** Cross-sectional views of the initial and equilibrated configurations of the bi-lamellar systems where a phosphatidylcholine (PC) bilayer is placed atop either a 50:50 MGDG-PC bilayer (C) or a 50:50 DGDG-PC bilayer (D) with a gap of about 2 nm. Water and PC head-group particles are colored as magenta and blue, respectively. The PC glycerol and fatty acid tail particles are colored the same as for MGDG or DGDG in (B).

Figure 2. Monogalactosyl-diacylglycerol (MGDG)- and digalactosyl-diacylglycerol (DGDG)-containing systems form distinct stalk patterns at steady state under dehydration. A, Simulation snapshots showing the progression of membrane stalk formation towards the steady-state pattern in the inter-membrane region (viewed from above the membranes; water particles colored purple) for the MGDG-containing system (PC: phosphatidylcholine) at an inter-membrane hydration level of 13 waters/lipid. Empty regions are where the two membranes are fused. The system is replicated along X and Y directions across periodic boundaries to better reveal the long-range patterns of the fused regions. Zoomed-in views within the dashed rectangles are shown on the right as insets, where the GL1 particles of MGDG lipids are shown in red. The black dashed ellipse in the zoomed-in view highlights the rounded end region of the elongated stalk. Scale bar: 10 nm. B, Schematics of the packing of conical MGDG lipids on the curved surface of elongated stalks (*Middle*) and rounded stalks (*Right*) with two principal curvatures K_1 and K_2, where the gray boundary outlines the effective tapered shape of MGDG (*Left*).

C, Simulation snapshots showing the progression of membrane stalk formation towards the steady-state pattern in the inter-membrane region for the DGDG-containing system at a hydration level of 12 waters/lipid. The same coloring scheme is used as in (A). Scale bar: 10 nm. D, Schematics of the packing of cylindrical DGDG lipids on the curved surface of elongated stalks (*Middle*) and rounded stalks (*Right*) with two principal curvatures K_1 and K_2, where the gray boundary outlines the effective cylindrical shape of DGDG (*Left*).

Figure 3. Membrane stalk formation in monogalactosyl-diacylglycerol (MGDG)- and digalactosyl-diacylglycerol (DGDG)-containing systems under various dehydration levels. A, Cross-sectional (*upper row*) and top (*lower row*) views of the bi-lamellar membranes with phosphatidylcholine (PC) lipids in the upper bilayer and mixed MGDG-PC (50:50) lipids in the lower one under different dehydration levels after equilibration (4 μs for 13 and 15 waters/lipid, and 3 μs for 17 and 24 waters/lipid). In the cross-sectional views, water particles above the upper bilayer and below the lower bilayer are omitted for clarity. In the top views, only the inter-membrane waters (purple) and the GL1 particles of MGDG (red) are shown, where empty regions (white) are where the two membranes are fused. The zoomed-in view within the dashed rectangle is shown on the right as an inset, where coarse-grained particles are colored with the head-groups as red for MGDG and blue for PC, the glycerol groups as pink and the fatty acid tails as cyan. The red dashed lines indicate the location where the cross-sectional views are taken. Each system is replicated along both X and Y directions to better reveal long-range patterns of the fused regions. Scale bar: 10 nm. B, Similar views for bi-lamellar membranes with PC phospholipids in the upper bilayer and mixed DGDG-PC (50:50) lipids in the lower one after equilibration (2 μs for 12, 14 and 17 waters/lipid, and 1 μs for 21 waters/lipid). GL1 particles of DGDG lipids are shown in red. Similar coloring scheme is used as (A) for the inset. Scale bar: 10 nm. C, The area fraction of the inter-membrane region occupied by membrane stalks as a function of the inter-membrane hydration level for MGDG- and DGDG-containing systems. Averages are taken over the last 300 ns of each simulation (error bars are standard errors: n = 6). D, The aspect ratio of the membrane stalks in the inter-membrane region as a function of the inter-membrane hydration level quantified from the images in (A) and (B). Error bars for DGDG data are standard errors and the sample sizes for hydration levels of 12, 13 and 14 are 12, 8 and 3, respectively.

Figure 4. Geometry-based consideration to rationalize the effect of the inter-membrane hydration level on the extent of stalk formation. A, Schematic illustration of the lipid membrane reorganization as stalks formed across the inter-membrane region, including the reduction of the lateral dimension of the bi-lamellar system and the increase in the thickness of the water gap in the inter-membrane region from d_0 to d. B, Variation of the water gap thickness with the hydration level for monogalactosyl-diacylglycerol (MGDG)- (red squares) and digalactosyl-diacylglycerol (DGDG)-containing (black circles) systems at steady state (error bars are standard errors; n = 30). Data points enclosed by the orange ellipse are from simulations without stalk formation. The theoretical curves according to Eqn. (3) are shown as red and black dashed lines for MGDG- and DGDG-containing systems, respectively. C, Variation of the membrane area with the hydration level for MGDG-containing (red squares) and DGDG-containing (black circles) systems (error bars are standard errors; n = 100). Data points enclosed by the orange ellipse are from simulations without stalk formation. D, Critical water layer thicknesses predicted by the model (light blue) and observed from the simulation (light gray) for MGDG-containing and DGDG-containing systems. Error bars are standard errors and n = 4 for both the model and simulation.

Figure 5. Molecular dynamics simulations of stalk formation in Arabidopsis wild-type (WT) vs mutant freeze-treated (FT) chloroplast outer envelope membrane under dehydration. A, Coarse-grained models of digalactosyl-diacylglycerol (DGDG), tri-galactosyl-diacylglycerol (TGDG) and tetra-galactosyl-diacylglycerol (TeDG) showing successive addition of sugar residues in the head-groups from DGDG to TGDG and then to TeDG. GC1-GC3 and GD1-GD3 groups in TGDG and TeDG are "translated" versions of GA1-GA3 group in DGDG in which same bond lengths and angles are adopted. Glycerol particles are colored pink and fatty acid tail particles are colored cyan. B, Models of the mutant-FT and WT-FT chloroplast outer envelope membranes with lipid head-groups colored as follows: phosphatidylcholine (PC) in gray, monogalactosyl-diacylglycerol (MGDG) in red, DGDG in blue, TGDG in orange, TeDG in green, sulfoquinovosyl diacylglycerols (SQDG) in yellow and phosphatidylglycerols (PG) in white. Glycerol groups and lipid tail particles are colored same as in (A). TGDG and TeDG only reside in the upper leaflet of the WT-FT membrane model. C-D, Cross-sectional (*top*) and top (*middle*) views of the equilibrated (after 1 μs) configurations of the bi-lamellar systems where a PC bilayer is placed atop the mutant-FT (C) or WT-FT (D) membrane with different inter-membrane hydration levels.

Water, lipid glycerol and lipid tail particles are colored as purple, pink, and cyan, respectively. In the top views, only the inter-membrane waters (purple) are shown, where empty regions (white) are where the two membranes are fused. The inter-membrane region is replicated along X and Y directions across periodic boundaries to better reveal the long-range patterns of the fused regions in white. Close-up views (*bottom*) of the top view of the inter-membrane region show the distribution of the lipids in the two leaflets facing the intermembrane region. Only one of the two glycerol particles (GL1) is shown for each lipid, with those of MGDG in red, DGDG in blue, TGDG in orange, TeDG in green, SQDG in yellow and PG in silver. PC lipids are omitted for clarity purpose.

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