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Should I stay or should I go: the functional importance and regulation of lipid diffusion in biological membranes

Running title: the importance and regulation of lipid diffusion in biological membranes

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Abstract

Biological membranes are highly dynamic notably due to the constant exchange of vesicles between the different compartments of the cell. In addition, the dynamic nature of membranes is also caused by their inherently fluid properties, with the diffusion of both proteins and lipids within their leaflets. Lipid diffusion is particularly difficult to study *in vivo* but recent advances in optical microscopy and lipid visualization now enable the characterization of lipid lateral motion. Here, we review the methods that enable the characterization of lipid lateral diffusion in plants. We will then discuss the parameters that impact lipid diffusion in membranes and explore their consequences on the formation of membrane domains at different scales. Finally, we will address how controlled lipid diffusion impact membrane functions during cell signaling, development and environmental interactions.

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Highlight:

This review aims to give insights on the contribution of lipids lateral diffusion to membrane domains formation and underlying impact on development.

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Keywords – six to ten, alphabetically

Barrier

Confinement

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Diffusion

Lipid

Membrane domain

Nanodomain

Particle tracking

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Introduction

Biological membranes are composed of different classes of lipids including sphingolipids, sterols, and glycerophospholipids (Boutté and Jaillais, 2020). The latter consists of a hydrophilic phosphate group linked via a glycerol molecule to two hydrophobic fatty acid tails. Abundant phospholipids, such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) are structural components of the membrane (Colin and Jaillais, 2020). By contrasts, phospholipids with an anionic headgroups (so called anionic phospholipids) are low abundant lipids that often play key regulatory functions in membranes. These include phosphatidic acid (PA), phosphorylated forms of phosphatidylinositol (i.e., phosphoinositides such phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)) and phosphatidylserine (PS).

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Membranes are both highly dynamics and interconnected (Kusumi *et al.*, 2012) (Holthuis and Menon, 2014; Gronnier *et al.*, 2018). They form a dense network that constantly exchange materials via vesicular trafficking and non-vesicular transfer at membrane contact sites. Within

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membranes, lipids themselves can be highly dynamic. They can move from one membrane leaflet to another, a type of movement called lipid flip-flop, which is often catalysed by enzymes (López-Marqués, 2021). Lipid can also diffuse in the plane of the membrane (within one membrane leaflet) a movement referred to as lipid lateral diffusion (Jaillais and Ott, 2020).
55 Lateral diffusion of lipid is mostly passive and follows a Brownian law. However, lipid diffusion can also be locally regulated, notably via lipid-lipid interactions and self-organization or via interaction with proteins. Such changes in lateral lipid diffusion can occur after biological, physical, hormonal or mechanical stimuli and may have profound impact on membrane organization. Notably, the local confinement of lipids ultimately contributes to the
60 lateral segregation of membranes into structural and functional domains.

Recent progresses in optical microscopy assays allow the study of lipid motion in complex biological systems. In this review, we first summarize some of the techniques enabling the study of lipid dynamics *in vivo*. Then, we review how confined lipid diffusion can impact on protein
65 localization, signaling activities and development, at the local, cellular and tissue scales.

Optical techniques to characterize molecular diffusion

Overall, there are three main techniques that are available to study molecular diffusion in living plants, that are based on photobleaching, fluorescence fluctuations and particle tracking of
70 protein probes or fluorescent lipids. Fluorescence fluctuations methods such as fluorescence correlation spectroscopy (FCS) have not been used to study lipid dynamics in plants and will not be described here but are reviewed in (Bayle *et al.*, 2021).

FRAP. In Fluorescent Recovery After Photobleaching (FRAP) a high-intensity laser beam is applied to a region of interest (ROI) (Box 1). This process will locally (i.e., within the ROI) photobleach the fluorescent molecules. This means that the fluorophores will be permanently
75 switched off. The mobility and diffusive behaviour of the ensemble of molecules is estimated from the kinetics of fluorescence recovery in the photobleached region. By using an appropriate model to fit the data, it is possible to extract numerical parameters such as the percentage of immobile molecule or their diffusion coefficient (Axelrod *et al.*, 1976). The advantage of FRAP experiments is that it can be done on most confocal microscopes and with virtually every kind of fluorophores. However, FRAP data should be interpreted with caution as the numerical data
80 obtained are as good as the underlying models used to calculate them (Bayle *et al.*, 2021). They are also a record of the dynamics of an ensemble of molecules, which may have individually very different diffusive behaviour. As such, FRAP data should be seen as the average dynamics
85 of a molecule population.

SPT. In single particle tracking (SPT), a fluorescent particle is tracked through time to record their individual trajectories. This can be used to track the dynamics of lipid-containing
90 structures such as nanodomains (Fratini *et al.*, 2021). From SPT data, it is possible to extract a host of numerical values including the diffusion coefficient, the mean square displacement (MSD), and the mode of motion of each tracked particle (Bayle *et al.*, 2021). Most SPT methods do not track the diffusability of the molecules *per se* but that of the structure it contains. Yet, SPT methods can be adapted to track single molecules, for example in sptPALM (single particle
95 tracking photoactivated localization microscopy, Box 1) experiments (Bayle *et al.*, 2021). The main advantage of sptPALM over FRAP, is that it is a single molecule not an ensemble imaging technique. It is therefore possible to uncover molecules within a population with very different diffusive behavior or with multiphasic or transitory behaviors. However, SPT techniques are
100 often more complex to implement.

How to label lipids in plant membranes?

105 All the techniques described above are based on fluorescence. To study lipid diffusion in vivo in membranes, it is thus required to label lipids with a fluorescent moiety. This is tricky to achieve experimentally and there is no perfect way to achieve this. Below, we describe the two main approaches to follow lipid in membranes via their direct and indirect labeling and we discuss their respective advantages and limitations.

110 **Fluorescent lipids.** Lipids can be coupled with fluorescent organic dyes. Fluorescent lipids are then supplied to the cells exogenously. These labeled lipids will then insert into the outer leaflet of the plasma membrane, where they can be flipped into the inner —cytosolic— membrane leaflets. These labeled lipids can follow bulk membrane trafficking or may be metabolized by endogenous enzymes. Such direct labeling strategies have been used in plants although not extensively (Poulsen *et al.*, 2015)(López-Marqués and Günther Pomorski, 2021). One of the obvious advantages of this strategy is the fact that the diffusion of the labeled-lipid is directly measured (Box1). In addition, the type of lipid molecular species is known. It is thus possible to analyze the impact of different fatty acid chain length and unsaturation degree on the diffusive behaviour of a phospholipid.

120 However, there are also several caveats that should be taken into account. First, the subcellular localization of an exogenously applied lipid may not reflect that of the endogenous lipid. It may also be addressed to the wrong membrane leaflet, depending on the localization and activity of the endogenous flippases (Box 1). Modifying lipids may affect their biological activity, their interaction with peripheral or integral proteins and their diffusion. There are two main ways to label lipids by attaching the fluorescent dye either to one of the fatty acid side chain or directly on the head group (Box 1). The fluorescent dye may be more or less hydrophobic, which will impact the stability of the labeled lipid within the membrane, its flip-flop between membrane leaflets and its non-vesicular lipid transfert (Kay *et al.*, 2012). Another parameter to take into consideration is the fact that a lipid may be rapidly transformed into other lipid species.

130 **Genetically-encoded lipid biosensors.** As an alternative to direct lipid labeling, lipids may be tracked through the use of genetically encoded fluorescently-tagged lipid probes (Colin *et al.*, 2022). Such probes can serve as in-vivo sensors and are based on lipid binding domains of proteins, (e.g. Pleckstrin Homology (PH) domain) that stereospecifically recognize a given lipid species. Lipid sensors have been instrumental in studying lipid localization, membrane lipid composition and lipid diffusion (Vermeer *et al.*, 2006; van Leeuwen *et al.*, 2007; Simon *et al.*, 2014). However, it is important to keep in mind that studying the diffusion of lipid sensors is not the same as studying the diffusion of its cognate lipid and any results coming from them should be interpreted accordingly. Indeed, lipid sensors are big compared to their cognate lipids and the interaction will thus slowdown the diffusion of such proteo-lipid complex. In addition, the type of fluorescent protein can also impact diffusion, notably if they are not truly monomeric. Second, although the membrane association of a lipid binding domain can be stereospecific for a given lipid, these domains often recognize additional membrane features such as the presence of specific chemico-physical properties or proteins (Platre and Jaillais, 2016; Heilmann, 2016). Lipid sensor mostly recognize the lipid headgroup, which can be seen as an advantage and drawback. It is an asset in the sense that it will probe the entire lipid class irrespective of their fatty acid chain composition. But this is also a limitation, as they cannot be used to test the impact of side chain composition on lipid diffusion. Finally, a limitation of lipid biosensors, is the fact that they interact ON and OFF with their cognate lipid and so they constantly cycle between a membrane-bound and cytosolic state (Box 1). This is important to

take this into account, notably when interpreting FRAP curve, as the dwell time of the sensor at the membrane will drastically impact the fluorescence recovery.

155 **Variations of lipid diffusion at the local scale in membrane-based signaling**

Formation of membrane domains with different lipid diffusion behaviour. Lateral lipid diffusion can drastically vary at the local scale within a membrane. These variations lead to local heterogeneities that ultimately translate into the formation of nanometer-sized membrane domains, also referred to as nanodomains. These membrane nanodomains are extremely diverse in lipid and protein composition (Jaillais and Ott, 2020). At any given time, many domains coexist within the same membrane and they can also vary through time according to changes in signaling activities or the cell environment.

160 *Membrane physico-chemical properties and lipid diffusion.* Membrane physico-chemical parameters can impact lipid diffusion within membranes, including for example electrostatics, curvature, thickness, tension, lipid packing or membrane order (Levental and Lyman, 2022) (Figure 1). These properties are strongly influenced by the lipid composition of the membrane. For example, variations in the fatty acid chain length and unsaturation degree of lipids tune lipid packing and membrane order. The type of lipid head group (e.g., charge, size) directly influence the electrostatic properties of the membrane or its curvature. Lipid-lipid interactions affects the lipid order of the membrane and hence the diffusion of lipids both within and between phase-separated membrane domains (Gronnier *et al.*, 2019; Jaillais and Ott, 2020; Levental and Lyman, 2022)(Figure 1). For example, sphingolipids and certain sterol species are known to interact, which favor a liquid-ordered state of the membrane (Mamode Cassim *et al.*, 2020). Finally, the two membrane leaflets may interact with each other in a process coined transbilayer coupling (Raghupathy *et al.*, 2015), in which lipids with long or very-long chains in one leaflet may impact the diffusion of a lipid on the other side of the membrane.

170 *Impact of protein-lipid interactions on lipid diffusion.* Protein-lipid complexes are much bigger than lipid alone, which affects their diffusion. Transmembrane proteins can act as physical obstacle to lipid diffusion by acting like pickets in the membrane. In addition, the cortical cytoskeleton may also prevent diffusion by acting like a fence (Figure 1). Similarly, the presence of membrane contact sites in between membranes organelles may also slow down lipid lateral diffusion. However, a recent study in animal cells, using PI(4,5)P₂ as a model lipid, suggest that cytoskeleton (with the exception of the septin cytoskeleton, see below) and membrane contact sites do not act as a strong physical barrier against lateral lipid diffusion within the cytosolic leaflet of the plasma membrane (Pacheco *et al.*, 2023).

180 *The cell wall as a potential modulator of lipid diffusion.* Certain lipid headgroups of the outer leaflet of the plasma membrane specifically interact with extracellular matrix components (Mamode Cassim *et al.*, 2019), which could impact the lipid lateral diffusion. Furthermore, lipid with large headgroup, such as the sphingolipid GIPCs (Glycosyl Inositol Phospho Ceramides), may be corralled by the presence of the cell wall, even in the absence of specific lipid-carbohydrate interaction (Figure 1).

190 Overall, very few of the parameters discussed above have been experimentally tested *in planta* and thus should be considered as theoretical possibilities rather than established facts. One should also consider that these different parameters likely act in a cooperative manner to control lipid diffusion.

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Functional impact of confined lipid diffusion at the nanoscale.

205 The functional impact of membrane nanodomains are more and more documented in plant signaling. Lipids are clearly involved in nanodomain formation in plants, notably sterols, sphingolipids and PI4P (Gronnier *et al.*, 2017, 2019; Legrand *et al.*, 2019; Ukawa *et al.*, 2022). However, there are few examples of studies addressing the importance of lipids on membrane diffusion, nanodomain formation and signalling activities. Two lipid species have been clearly shown to localize in nanodomains, phosphatidylserine (PS) and PI(4,5)P₂.

210 PS was shown to localize in plasma membrane nanodomains using genetically encoded biosensors and sptPALM super-resolution imaging (Platre *et al.*, 2019). Using this approach, about 50% of the PS sensor 2xPH^{EVECTIN2} was found to be immobile in the plasma membrane. Furthermore, clustering analysis revealed that the PS sensor was densely accumulating within
215 nanodomains of about 50–70 nm in size (Platre *et al.*, 2019). Genetic analyses suggested that PS is required to stabilize the Rho-related GTPase of Plant6 (ROP6) in these PS-containing nanodomains. In the absence of PS, ROP6 is not confined in plasma membrane nanodomains and not competent for signaling (Platre *et al.*, 2019).

220 Another elegant example of how lipid diffusion regulates cellular activity was shown by Fratini and co-authors (Fratini *et al.*, 2021). They uncovered multiple independent pools of PI(4,5)P₂ at the tip of growing tobacco pollen tube. Pollen tubes undergo polarized expansion with intensive trafficking and cytoskeletal dynamics at a narrow area of the subapical membrane at the apex of the tube. The PH^{PLC} PI(4,5)P₂ sensor shows an enrichment of this lipid at the
225 subapical plasma membrane region. Moreover, spinning disc microscopy analyses suggest that the lipid reporter is present in two distinct pools at the plasma membrane: a punctate signal suggest the possible local confinement of the lipid in nanodomains and a diffuse fluorescence showing that the lipid also laterally diffuses. The authors showed that these two pools of PI(4,5)P₂ are synthesized by different PI4P 5-kinases and reciprocal domain swapping
230 experiments demonstrated that they each have specific functions in pollen tube growth. Thus, lipids with different diffusible behavior may arise from their biosynthetic trajectories. It is possible that based on the localization of synthesis, PI(4,5)P₂ interacts with different lipids or proteins, thereby modifying its diffusion. It is also possible that different biosynthetic enzymes may favor the production of specific molecular species. For example, in animal cells it was
235 recently demonstrated that different pools of PI(4,5)P₂ exist with different fatty acid composition based on their metabolic routes (Kim *et al.*, 2022).

Diffusion barrier or how to isolate membrane domains.

240 In addition to the formation of distinct membrane nanodomains, lipid diffusion barriers also exist at the cellular scale. These barriers prevent exchanges of molecules between different polar domains within the cell.

245 For example, in animals, tight junctions act as a diffusion barrier for proteins and lipids between the apical and basolateral domain of epithelial cells (Ebnet and Gerke, 2022). They also act as a diffusion barrier for extracellular molecules (Figure 2A). Tight junctions essentially constitute a molecular belt at the frontier between the apical and basolateral poles of the cell, and this barrier is coordinated from cell-to-cell within the tissue. They consist of a macromolecular assembly that includes multiple transmembrane proteins (e.g., Claudin, Occludin, JAM). These proteins interact with each other from one cell to the other via their extracellular domains
250 (Figure 2A). Thus, lipid diffusion barrier can be built by transmembrane proteins.

Another interesting example of diffusion barrier concerns yeast cells and the physical separation of ER between mother and daughter cell during division. This diffusion barrier is created through the local accumulation of sphingolipids in a septin, bud1-GTPase dependent manner (Figure 2B) (Clay *et al.*, 2014). In this example, it is the confinement of sphingolipids in a restricted area of the ER membrane which acts as a diffusion barrier. The sphingolipids, but not Bud1, contributes to barrier formation in the outer membrane of the dividing nuclear membrane as well (Clay *et al.*, 2014). Note that the septin ring also plays a role as a diffusion barrier at the plasma membrane, supporting that polymerized peripheral proteins, not only transmembrane proteins, may act as a membrane diffusion barriers (Caudron and Barral, 2009; Saarikangas and Barral, 2011; Bridges and Gladfelter, 2015; Pacheco *et al.*, 2023).

In plants, the best characterized diffusion barrier at the cellular level is the casparian strip in the root endodermis (Figure 2C). The casparian strip is not only an apoplastic barrier but also blocks the diffusion of membrane components (Geldner, 2013). This has been clearly shown for transmembrane proteins but it is also likely true for lipids. Indeed, FM4-64, which is a lipidic membrane dye, does not diffuse through the casparian strip (Alassimone *et al.*, 2010). The strip is highly dense in proteins, which likely affect lipid and protein diffusion through this membrane domain (Roppolo *et al.*, 2011; Geldner, 2013; Barbosa *et al.*, 2022). In addition, it is tightly linked with an extremely differentiated extracellular matrix, which could also locally impact lipid diffusion. Whether the casparian strip membrane has a specific lipid composition is unknown. However, a PI(4,5)P₂ biosensor was shown to be enriched at the onset of the casparian strip formation (Kalmbach *et al.*, 2017). Another example where the differentiation of the cell wall may also play a role as a diffusion barrier is the trichome (Noack and Jaillais, 2020). Indeed, the trichome is made of two different polar domains with distinct lipid compositions separated by the so called Ortmannian ring, a callose- and silica-rich cell wall strip (Kulich *et al.*, 2018; Kubátová *et al.*, 2019). It is unknown whether the local modification of the cell wall directly or indirectly impact lipid diffusion between the two domains. It is also possible that the local deposition of a distinct cell wall at the border between the two domains is a consequence rather than the trigger of differential membrane properties.

Another likely barrier for lipid diffusion is established between the plasma membrane and the interfacial membrane built during plant-microbe interaction (Noack and Jaillais, 2020). Indeed, although they are continuous with the plasma membrane the interfacial membranes have a specific lipid signature. This is for example the case of the periarbuscular membrane established during arbuscular mycorrhizial symbiosis (Ivanov and Harrison, 2019), the infection thread established during rhizobial infection (Lace *et al.*, 2022), the extra-invasive hyphal membrane formed during *Colletotrichum* infection of root cells (Shimada *et al.*, 2019), or the extra-haustorial membrane induced during powdery mildews infection of leaf cells (Qin *et al.*, 2020). The neck region of these interfacial membranes likely acts as a diffusion barrier for both protein and lipid molecules. However, the exact nature of this barrier and whether it is lipid, protein and/or cell wall-based is currently unknown.

Plasmodesmata and cell-to-cell lipid diffusion in plants.

Plant cells are connected with membrane-lined channels embedded in the cell wall, which are called plasmodesmata. These structures are like pores which connect symplasmically the cell cytoplasm (Li *et al.*, 2021). However, plasmodesmata also connect the endoplasmic reticulum (ER) and the plasma membrane of neighbouring cells. As such, the plasma membrane and the ER of neighbouring cells is continuous, opening the possibility for lipids to diffuse from cell-to-cell in plants. This idea was substantiated by FRAP analyses of fluorescently-labeled lipids

added to soybean root cell culture (Grabski *et al.*, 1993). This analysis suggested that a fluorescent diacylglycerol (NBD-DAG) in the ER membrane could diffuse from a cell to its neighbour (Figure 3). By contrast, a fluorescent sphingolipid (NBD-sphingosine phosphocholine) and phosphatidylcholine (NBD-PC) at the plasma membrane could not diffuse from one cell to another. These data suggest that lipids can diffuse via the ER strand that runs through plasmodesmata, while the plasma membrane connection may act as a diffusion barrier. The plasmodesmata are enriched in sphingolipids and sterols (Grison *et al.*, 2015), which may explain the limited or no diffusion of lipid through the plasma membrane domain. However, whether or not sphingolipids and sterols are enriched in the plasma membrane, the ER membrane or both in plasmodesmata is unknown. Furthermore, plasmodesmata are area of close ER/PM contacts, which may be involved in restricting lipid movement within this structure. Overall, the functional implication of cell-to-cell lipid diffusion via plasmodesmata is currently unknown.

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Conclusions

In between staying or going the choice is not so difficult for lipids as they will undergo transitory lipid motion stages according to their membrane environment and stimuli. Interestingly, PI(4,5)P₂ for example was shown to lead to two different phenotypes in tobacco pollen tube tip regarding its diffused or confined behaviour and colocalization with metabolic kinases (Fratini *et al.*, 2021). Various diffusion barriers exist to limit or prevent lateral lipid diffusion such as interactions with transmembrane proteins, cytoskeleton, extracellular matrix components, electrostatics, or transbilayer coupling of lipids. However, as lipid in a certain context can also bypass these barriers (hop diffusion) the properties of a diffusion barrier has still to be defined. It is still unknown if some of these barriers will impact one or the two membrane leaflets for instance. Indeed, it will be very interesting to be able in the future to create artificial membrane diffusion barriers to restrict diffusion in a certain area of the cell. Having more comprehensive data on barriers of diffusion and being able to implement genetic engineering on them could be one of the challenges of the new decades.

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C.B. and Y.J. wrote the manuscript and prepared the figures.

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Conflict of interest statement:

The authors declare no conflict of interest.

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Figures legends:

475 **Box 1. Key developments in the characterization of lipid dynamics in plants.**

• **Fluorescent lipid uptake in living seedlings.** Poulsen et al., (2015) and Marques-Lopez et al., (2021) used NBD-conjugated lipids to understand their internalization in Arabidopsis seedling. The probe can be attached to the fatty acid chain or the polar head, which can impact their respective diffusion. NBD-PS and Topfluor-PS conjugates are given as examples.

480 • **Fluorescence recovery after photobleaching (FRAP) to understand the dynamics of lipid sensors.** Simon et al., (2016) used FRAP on genetically-encoded lipid sensors with various affinities to probe their dynamics in plant cells. Fluorescently-labelled lipids can be used to study lipid diffusion but it is difficult to control in which membrane leaflets they are inserted in. Note that FRAP on lipid sensors probes both their lateral diffusion as well as
485 dwell time in the membranes.

• **Single molecule imaging to probe lipid dynamics in the living plant plasma membrane.** Platre et al., (2019) and Bayle et al., (2021) used total internal reflection fluorescence microscopy (TIRFM) and single particle tracking photoactivated localization microscopy (sptPALM) to analyze the dynamics of phosphatidylserine in the plasma membrane of
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• **Spinning disk microscopy to study lipid nanodomains in pollen tubes.** Fratini et al., (2021) used spinning disk microscopy to identify PI(4,5)P₂- and PIP₅K₂-containing nanodomains in pollen tube. These localizations impact on actin filament occupancy at the tip of growing pollen tubes.
495

Figure 1 Examples of factors that can, in theory, impact lipid diffusion at the local scale

Diverse types of lipid lateral diffusion constraints are depicted in membrane bilayer. Labels mention the different types of lipids and proteins.

500 **Figure 2. Examples of membranes diffusion barriers at the cell level**

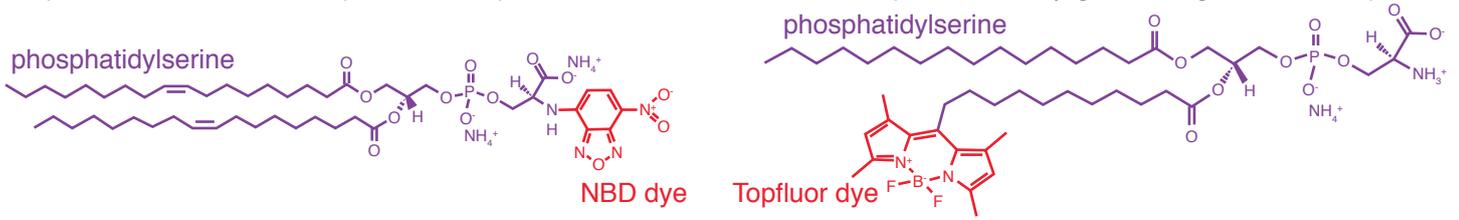
(A) Tight junction around two epithelial cells shown in 3D. The tight junction zone in between two contiguous membrane cells is enlarged and example of transmembrane proteins involved in tight junction are shown. These proteins are preventing diffusion in membranes and membrane interspace. (B) Schematic representation of a dividing baker yeast cell. The bud neck is magnified at the bottom. A sphingolipid belt prevents the diffusion of ER membrane
505 misfolded proteins from stressed mother cell to daughter cell in a septin dependent manner. (C) Schematic representation of two endodermal cells in 3D with their casparian strip highlighted. The membrane junction zone where the casparian strip is present is enlarged. CASP cluster as well as modified cell wall prevent lipid/protein diffusion.

510 **Figure 3. Model for cell-to-cell diffusion across plasmodesmata**

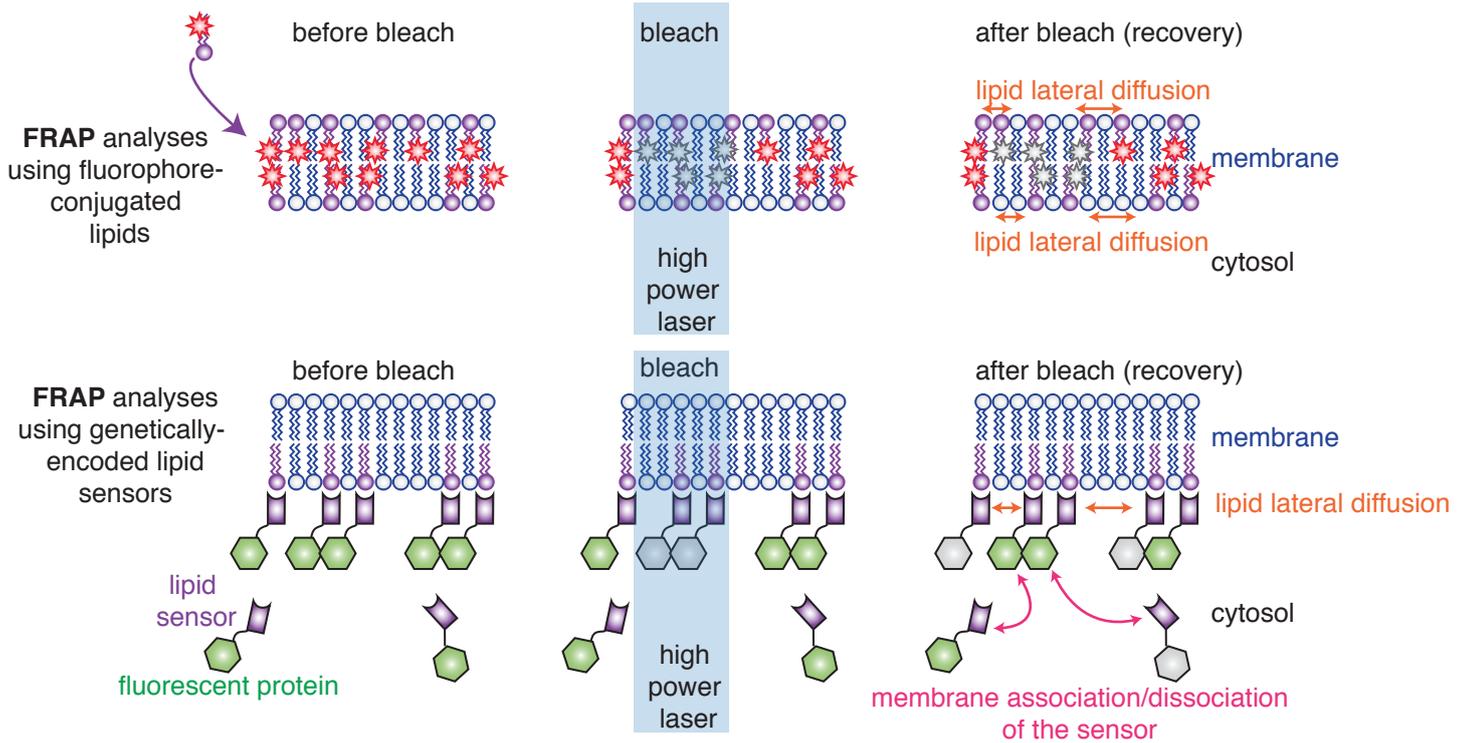
Plasma membrane/ER membrane continuity at the plasmodesmata junction in between two adjacent plant cells is shown. A putative sphingolipid and sterol-rich domain present in the PM can presumably limit lipid lateral diffusion whereas in the ER membrane lipid diffusion may
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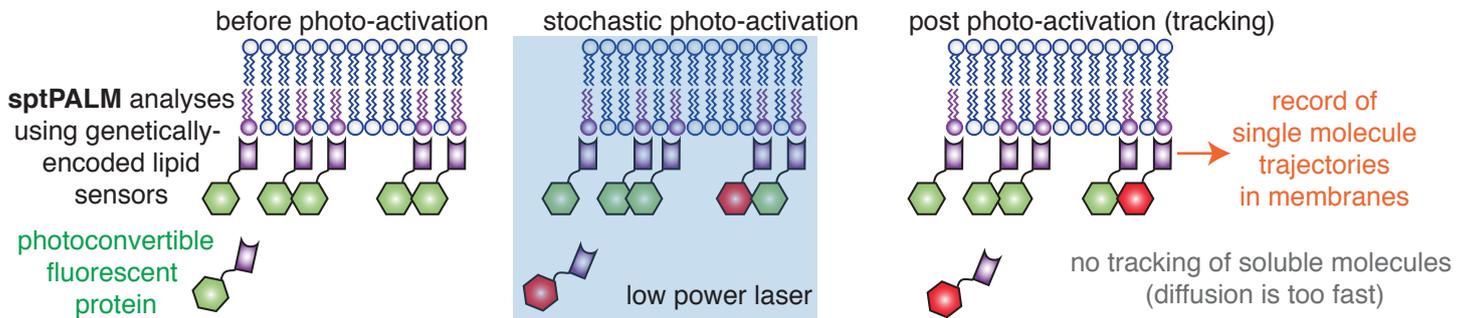
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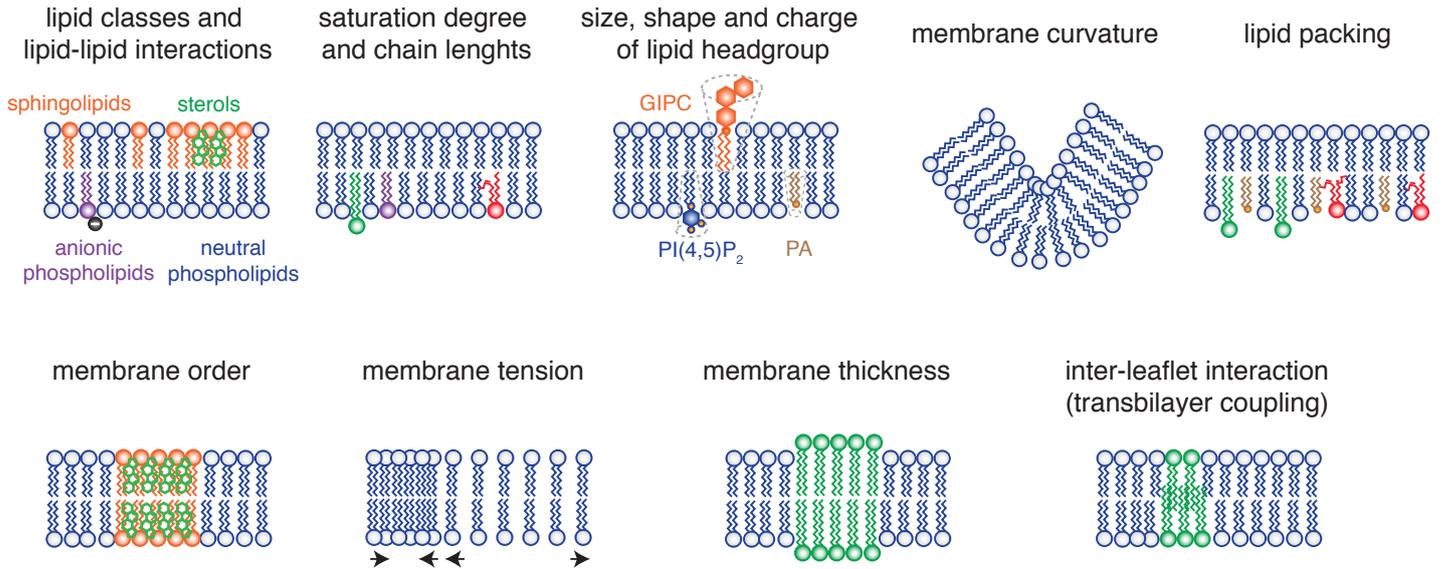


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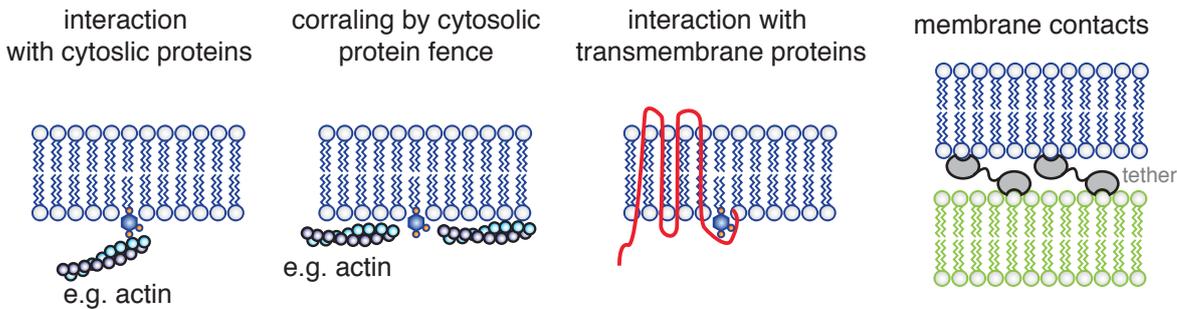


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Membrane physico-chemical properties and lipid species



Protein-lipid interactions



Cell wall-lipid interactions

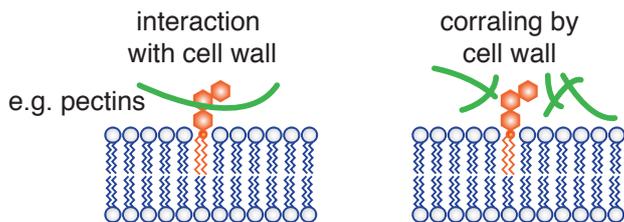


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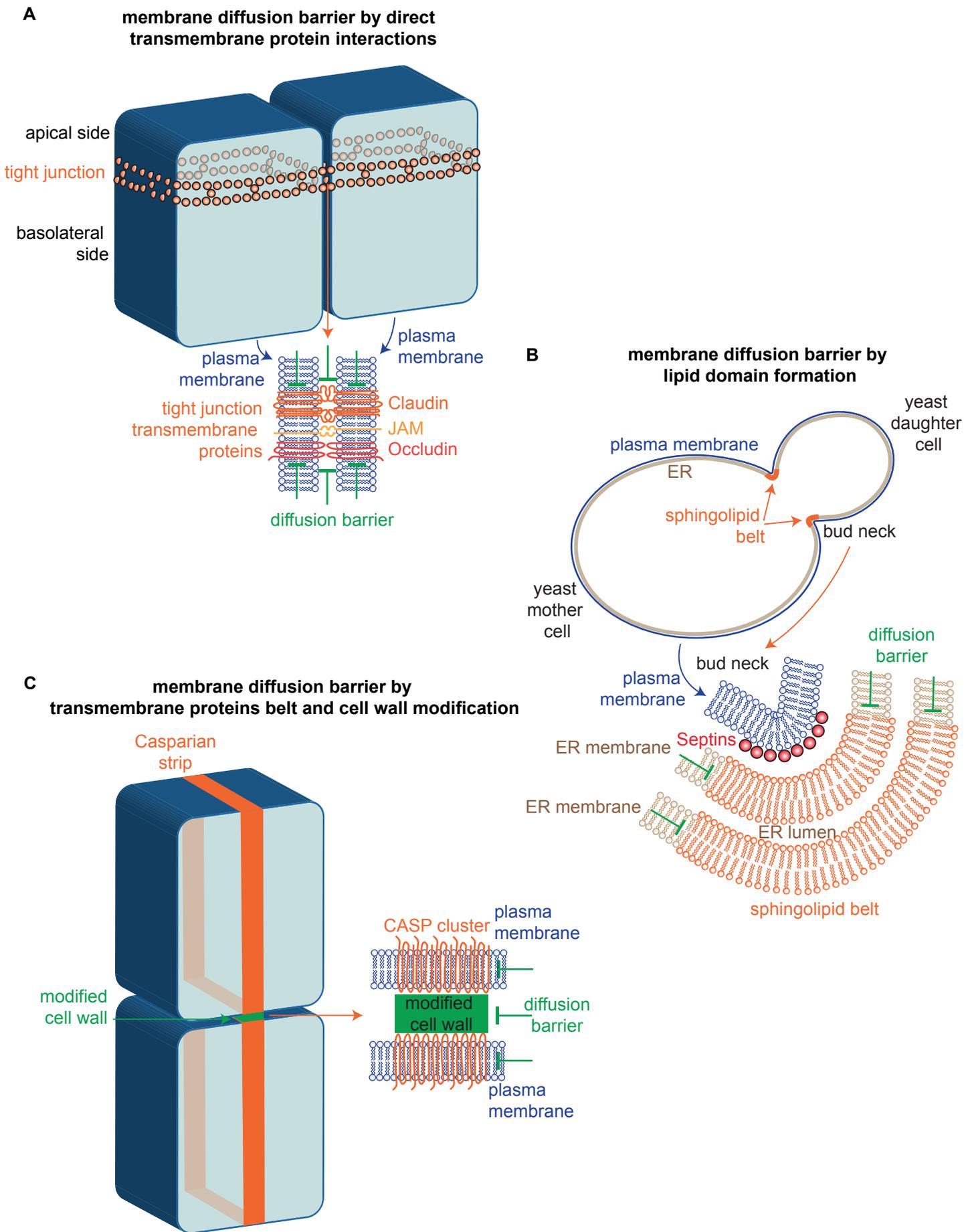


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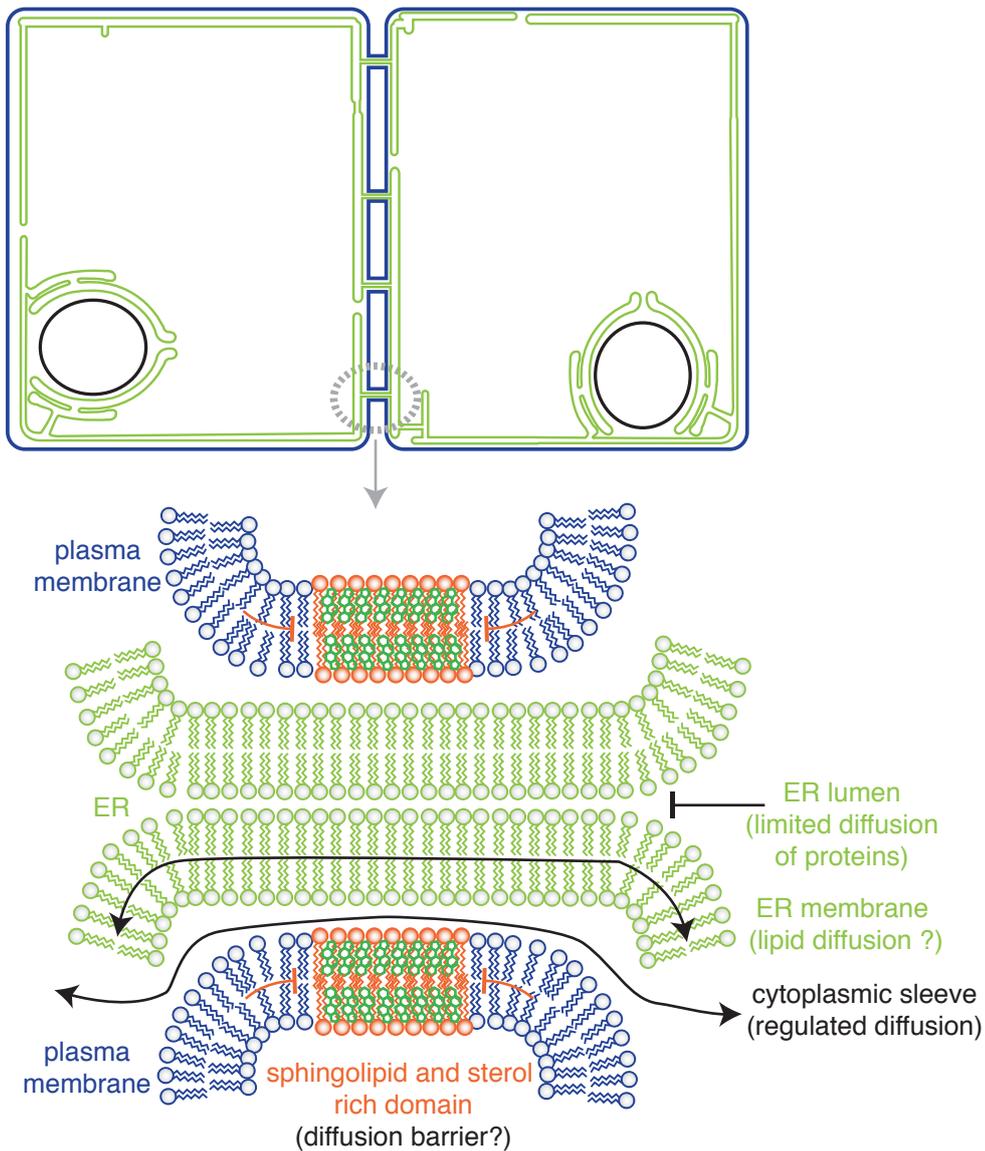


Figure 3. Model for cell-to-cell diffusion across plasmodesmata. Plasma membrane/ER membrane continuity at the plasmodesmata junction in between two adjacent plant cells is shown. A putative sphingolipid and sterol-rich domain present in the PM can presumably limit lipid lateral diffusion whereas in the ER membrane lipid diffusion may be possible from a cell to another.